Investigation of Quantitative and Qualitative MtDNA Alteration in Breast Cancer

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Xiucheng Fan

aus Shenyang, China

Basel, 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Michael N. Hall
Prof. Raija LP Lindberg
Prof. Xiao Yan Zhong,

Basel, den 23 June 2009

Prof. Dr. Eberhard Parlow
Dekan

Table of Content

ABSTRACT

Mitochondrial DNA (mtDNA) alterations including copy number variations and sequence variations are suspected to be associated with carcinogenesis. We established a multiplex quantitative real-time PCR to examine the quantities of mtDNA and nuclear DNA (nDNA) for analysing relative mtDNA content in blood and tissue samples of patients with breast cancer. We also developed a novel matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based MicroARRAY multiplex assay to identify mtDNA sequence variants at 22 nucleotide positions (np) in a single reaction.

For the quantitative analysis, mtDNA content was significant decreased in cancerous breast tissues (51 cases) compared with the paired normal breast tissues (p = 0.000). The down-regulation of mtDNA was observed in 82% of the cancerous samples. The similar down-regulation has been also found in whole blood and plasma samples from patients with breast cancer. Using the MALDI-TOF MS, we analysed the 22 mtDNA mutations related to breast cancer in the 51 paired breast tissues (cancerous and normal). 154 mtDNA mutations were found in total, 49.35% in cancerous tissues and in 50.65% in paired normal samples. Forty one tissue samples contain more than 2 mutations each. All these sequence variants were distributed at 5 np in a hotspot region around the displacement loop (D-loop). We investigated the relationship between the quantitative and qualitative mtDNA alterations in breast tissues, as well as the correlation between the alterations of mtDNA and some clinical/pathological parameters, such as patient age, tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors in breast cancer. No associations were found between the quantitative and qualitative changes, as well as between the mtDNA changes and clinical/pathological parameters.

Our data suggest that mtDNA alterations are indeed involved in breast cancer. Investigating mtDNA alterations in cancer might be helpful for developing biomarkers in the management of cancer patients. The methods used in this study for the investigation can be introduced as simple, accurate and cost-efficient tools.

Acknowledgements

Much invisible strength has been put into this thesis, and many unforgettable thanks ought to be announced here.

I am deeply indebted to Prof. Dr. Xiao Yan Zhong for giving me such an opportunity to carry out my PhD study in her laboratory. As the direct supervisor, bearing scientific and practical thought, she has actively kept encouraging me to work creatively, intellectually and efficiently. The timely and helpful instructions to my thesis work from her let me surpass the hurdles on the way to the PhD thesis. Moreover, she has been concerned about the life besides work of the international student. Certainly, these supports led me to steady and successful academic performance and a happy life in this country.

I am very grateful to Prof. Dr. Wolfgang Holzgreve for accepting me as research member of his well-known team under his extraordinary guidance in the first two years of my study.

I would like to thank Prof. Michael N. Hall and Prof. Raija Lindberg seriously but scientifically conducting their responsibility for my PhD thesis. I cordially thank Prof. Michael N. Hall, who accepted to be my “Doctor Vater” and the chairman of my PhD committee. It is a great honour on me to be so close to a great scientist.

I would like to thank Ms. Vivian Kiefer for her sufficient laboratory support and kindly help on managing my personal daily life issues. I would like to thank Nicole Chiodetti for her abundant

arrangement of experiment consumables. I would like to thank Dr.Ying Li at the first time giving me the introduction of MALDI-TOF MassARRAY technology in this lab.

I would like to thank Prof. Sinuhe Hahn, Dr. Corinne Rusterholz**,** Dr. Dorothy Huang, Dr. Simon Grill, Marianne Messerli, Prassad Vara Kolla for their constractive suggestions, discussions and kindly help and support.

I would like to thank Rebecca Zachariah, Dr. Shereen El Tarhouny, Martin Seefeld, and Dr. Xia Peng for their great work for me. I would like to thank Corina Cohler and Ramin Radpour for their suggestive opinions.

Undoubtedly, without the help from all the professors, doctors and colleagues, these three years' PhD study would never be so colourful, so cheerful and so meaningful in my life.

Abbreviation

ATP	Adenosine Triphosphate
ALL	Acute Lymphoblastic Leukemia
ANT	Adenosine Nucleotide Translocase
BC	Breast Cancer
BP	Base Pair
Ccf	Circulating Cell Free
CEA	Carcinoembryonic Antigen
CIN	Chromosomal Instability
Cyt	Cytochrome
CoQ	Coenzyme Q
COX	Cytochrome C Oxidase
CRS	Cambridge Reference Sequence
DCIS	Delocalized Lipophilic Cations
D-loop	Displacement loop
ER	Estrogen receptor
FAD	Flavin Adenine Dinucleotide
FADP	Flavin Adenine Dinucleotide Phosphate
GE	Genome Equivalent
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine Triphosphate
HCCs	Hepatocellular Carcinomas
HER2/neu (ErbB-2)	Human Epidermal growth factor Receptor 2
HPA	Human Platelet Antigen
IBC	Inflammatory Breast Cancer
IDC	Invasive Ductal Carcinoma
LCIS	Lobular Carcinoma In Situ
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
MDS	Myelodysplastic Syndromes
MS	Mass Spectrometry
Mt	Mitochondrial
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
ND	NADH Dehydrogenase
nDNA	Nuclear DNA
O_H	H-strand Origin
O_L	L-strand Origin
Oxphos	Oxidative Phosphorylation
Nox	NADPH Oxidase
PLTs	Platelets
PCT	Photochemotherapy
POLRMT	MtRNA Polymerase
PR	Progesterone Receptor
TFAM	Mitochondrial Transcription Factor
TFB1M	Mitochondrial Transcription Factor B1
PC	Prostate Cancer
Ref 1	Redox Factor 1

ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
rRNA	Ribosome RNA
SNP	Single Nucleotide Polymorphism
TCA	Tricarboxylic Acid
tRNA	Transfer RNA
VEGF	Vascular endothelial growth factor

Part I Background of mtDNA

1. General information of mtDNA

1.1 Mitochondrial structure and function

Mitochondria (singular mitochondrion, Fig 1) are membrane-bound organelles like the nucleus have a double membrane found in the cytoplasm of most eukaryotic cells. These organelles show the incredible diversity on both the size (0.5 to10 μm in diameter) and the copy number (1 to over 1000) per cell. They are about the size of *Escherichia coli* with different shapes according to the cell types. However, the structures of mitochondria are pretty similar regardless of their size, number per cell, plant or animal origin. Generally, a mitochondrion has an inner membrane and an outer membrane as well. There is a space between the inner and outer membranes called the intermembrane space. The outer membrane is fairly smooth, whereas the inner membrane is greatly convoluted, forming folds or invaginations called cristae. The cristae largely expand the inner membrane surface area. The space enclosed by the inner membrane is so called matrix. It contains a highly-concentrated mixture of hundreds of enzymes, mitochondrial ribosomes, tRNA, and several copies of the mitochondrial genome (Bruce et al. 2002).

Mitochondria are involved in a series of cellular processes including cellular differentiation and proliferation, cell signaling, programmed cell death, the control of the cell cycle and cell growth (McBride et al. 2006). Besides these, mitochondria are so called cellular power plants for generating the most of the cellular chemical energy, adenosine triphosphate (ATP) for cell use (Campel et al. 2006). Mitochondria play the dominant role in oxidative phosphorylation (OXPHOS), combining the electron-transferring respiratory chain complexes I–IV and the ATP synthase (complex V).

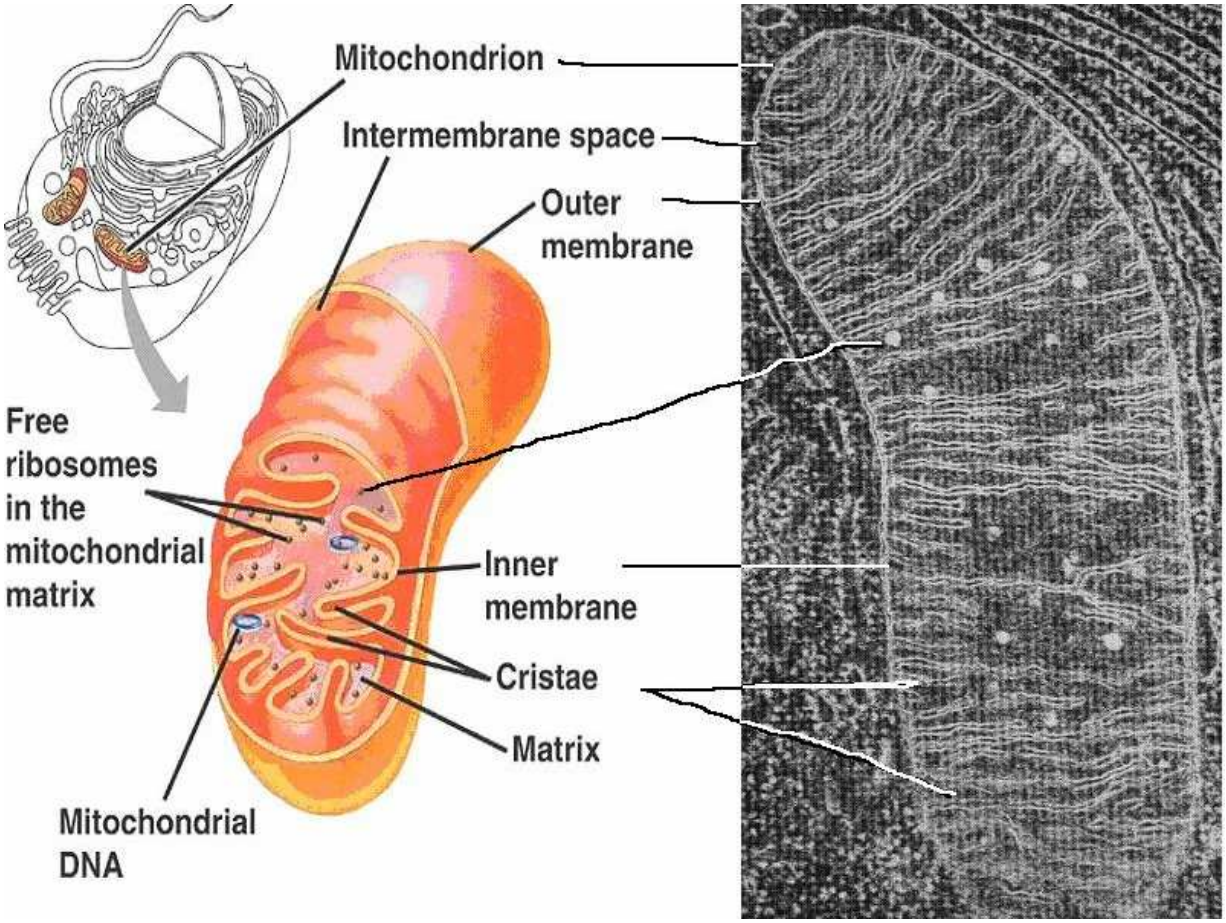


Figure 1. The strcture of mitochondrion (right part: electron micrograph).

In the catabolism of carbohydrates, glucose is broken down into pyruvate from glycolysis in cytoplasm, and then pyruvate was transported from the cytoplasm into the mitochondria. The process of converting one molecule of glucose into two molecules of pyruvate generates 2 net nicotinamide adenine dinucleotides (NADH) finally. In mitochondrion one molecule of pyruvate undergoes the subsequent oxidation and decarboxylation to 2 molecule of acetyl coenzyme A by a cluster of three major protein complexes of pyruvate dehydrogenase, which is located in mitochondrial matrix. During the oxidative decarboxylation of pyruvate, one molecule of NADH is formed per pyruvate oxidized. Acetyl coenzyme A is oxidized through a cycle involving eight

catalytic steps, which is called citric acid cycle, and also known as the tricarboxylic acid cycle (TCA cycle), or the Krebs cycle.

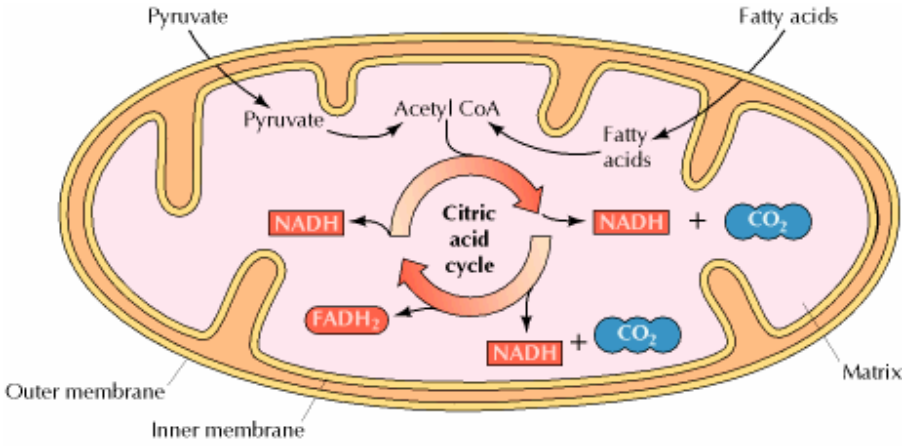


Figure 2. Metabolism in the matrix of mitochondria Pyruvate and fatty acids are imported from the cytosol and converted to acetyl CoA in the mitochondrial matrix. Acetyl CoA is then oxidized to CO_2 via the citric acid cycle, the central pathway of oxidative metabolism (Cooper et al, 2000).

Each round of the TCA cycle results in the production of two molecules of CO2, 3 molecules of NADH one molecule of reduced flavin adenine dinucleotide (FADH2), and one molecule of GTP (the energetic equivalent as ATP). In the next stage of the aerobic metabolism of oxidative phosphorylation, the respiratory substrates of NADH and FADH2 generated through the TCA cycle are oxidized in a process coupled to ATP synthesis. Substrate oxidation involved in a series of respiratory enzyme complex is located within the mitochondrial membrane and the ability to accept any free electrons in a particular sequence based on the relative redox potential and substrate specificity. Complex I (NADH coenzyme Q reductase) accepts electrons from the TCA cycle electron carrier NADH, and passes them to coenzyme Q (ubiquinone; CoQ), which also receives electrons from complex II (succinate - ubiquinone reductase). Complex II consists of

four protein subunits; one is the FADH2-linked TCA cycle enzyme succinate dehydrogenase, transferring NADH from succinate to CoQ. CoQ passes electrons to complex III (Cytochrome c reductase/Cytochrome b complex), an 11 - subunits of respiratory enzyme complex involved in the transfer of electron from membrane-bound CoQ to oxidised cytochrome C (Cyt C)within the outer surface of the mitochondrial membrane. Cyt C passes electrons to Complex IV. Complex IV (cytochrome C oxidase, COX) is the terminal mobile electron acceptor composed of 13 kinds of different protein subunits, which uses the electrons to reduce molecular oxygen to water. Three of the electron carriers (complexes I, III and IV) are proton pumps and function as the reception sites for the translocation of protons from the matrix side to the external side of the inner mitochondrial membrane. The resulting transmembrane proton gradient is used to make ATP via ATP synthase (complex V). Thus, each molecule of NADH leads to 3 molecule of ATP and each molecule of $FADH_2$ leads to 2 molecules of ATP. Thereby each molecule of pyruvate enters the TCA cycle generating12 molecules of ATP. Totally one

The ATP produced in the mitochondrion which is not utilized by mitochondrion need to exit to the cytosol via the enzyme adenine nucleotide translocase (ANT) for an exchange of cytosolic ADP. This exchange is the principal control for the rate of oxidative phosphorylation, which is the major supply of the cellular energy under aerobic conditions and is required to sustain cell viability and normal cell functions.

Fatty acid oxidation is another important source of energy for many organisms, which metabolic catabolism also occurs in mitochondria. Fatty acid is catalyzed and transported from cytoplasm to inner mitochondrial space into fatty-CoA ready for beta oxidation machinery. β-oxidation splits the long chain fatty acid into acetyl CoAs, which can enter the TCA cycle to generate NADH and $FADH_2$. β-oxidation enzymes are separated to two functional groups, the inner membrane-bound

complex responsible for long-chain fatty acid oxidation and the soluble matrix responsible for the degradation of medium- and short-chain fatty acids (Liang et al. 2001), which carry on the 4-step repeat cycle. In each round of the cycle one molecule of acetyl CoA is decarboxylated to one acetyl-CoA and one acyl-CoA molecule with two carbon atoms shorten, which can re-enter the β -oxidation cycle until completely degraded to acetyl-CoA. The resulting acetyl-CoA molecules enter the TCA cycle for further oxidation. However, under some certain physiological conditions for instance the long-term fasting and hungriness, or under some pathological conditions such as diabetes, the oxidation of fatty acids results into ketone bodies, β - hydroxybutyrate, acetoacetate and acetone, which is called ketogenesis catalyzed by the enzymes also located in the mitochondrial matrix. In these cases, the ketone bodies are used as an alternative energy source of energy in the skeletal muscles, heart and brain (Voet et al, 2006; McBride et al. 2006).

In addition to oxidative metabolism, mitochondria are also involved in other metabolic tasks, for example, some enzymes functioning in gluconeogenesis (Sobll. 1995) and the urea cycle (Nakagawa et al. 2009) and are located in mitochondrial matrix. Mitochondria of the cells involved in regeneration of NAD, and steady-state in the cells of the inorganic ions such as calcium Calcium signaling (Hajnóczky et al, 2006), steroid synthesis (Rossier 2006).

1.2 Mitochondrial Genome

In addition to nuclear genomes, eukaryote cells also have cytoplasmic genomes which are compartmentalized in the mitochondria. Human mitochondrial DNA is extremely small molecule only about 16,569 base pairs (bp) in length located within the mitochondrial matrix and present in thousands of copies per cell., like most bacterial and prokaryote DNA, organized in a closed

circle like a donut, unlike human nuclear DNA, which is about 3.0 billion bp in length and is arranged in a long spiraled and coiled thread like structure and present only one pair of copy per cell. Unlike human nuclear DNA has 46 chromosomes (23 pairs) and about 30000 genes, human mitochondrial DNA genome only encodes 37 genes (Table 1). According to the nucleotide content, mitochondrial genome is differentiated into two strands. The guanine rich strand is referred to as the heavy strand and the cytosine rich strand is referred to as the light strand. The heavy strand encodes 28 genes, and the light strand encodes 9 genes for a total of 37 genes. The heavy strand encodes 12 of the 13 polypeptide-encoding genes, 14 of the 22 tRNA-encoding genes and both rRNA-encoding genes. Of the 37 genes, 13 are essential polypeptides of the OXPHOS system; 22 are for transfer RNA (tRNA) and two are for the small subunit and large subunit of ribosomal RNA (rRNA), which construct the necessary RNA machinery for their translation within the organelle (Fig. 3). The remaining protein subunits that make up the respiratory-chain complexes, together with those required for mtDNA maintenance, are nuclear-encoded, synthesized on cytoplasmic ribosomes, and are specifically targeted and sorted to their correct location within the organelle. Therefore mitochondria are under the dual genetic control of both nuclear DNA and the mitochondrial genome (Taylor et al. 2005).

Human mtDNA has no introns but extremely high proportion of contiguous coding sequences (Anderson et al. 1981, Wallace et al. 1992, Zeviani et al. 1998). The only non-coding segment of mtDNA is the displacement loop (D-loop), a region of 1121 bp that contains the origin of replication of the H-strand (O_H) and the promoters for L and H-strand transcription. The mtDNA is replicated from two origins. DNA replication is initiated at O_H using an RNA primer generated from the L-strand transcript. H-strand synthesis proceeds two-thirds of the way around the mtDNA, displacing the parental H-strand until it reaches the L-strand origin (O_L), situated in a

cluster of five tRNA genes. Once exposed on the displaced H-strand, O_L folds a stem-loop structure and L-strand synthesis is initiated and proceeds back along the H-strand template. Consequently, mtDNA replication is bidirectional but asynchronous (Clayton 1982). MtDNA transcription is initiated from two promoters in the D-loop, P_L and P_H. Transcription from both promoters creates a polycistronic precursor RNA that is then processed to produce individual tRNA and mRNA molecules (Clayton et al. 1991, Ojala et al. 1981). To initiate transcription, the dedicated mitochondrial RNA polymerase (POLRMT) requires mitochondrial transcription factor A (TFAM,) and either mitochondrial transcription factor B1 (TFB1M) or B2 (TFB2M) (Falkenberg et al. 2002, Fernandez et al. 2003). Recent evidence shows that TFAM induces a structural change of the light-strand promoter that is required for POLRMT-dependent promoter recognition (Gaspari et al. 2004). The importance of mitochondrial transcription to cellular dysfunction as a result of pathogenic mtDNA mutations is a neglected area of research that might give important insights into some of the tissue-specific or mutation-specific effects.

Furthermore, the genetic code in human mitochondria has come to differ from that used in the nucleus, and thus mtDNA genes are no longer intelligible to the nucleocytosolic system (Wallace 1982). UGA is read as tryptophan rather than ‘stop’, AGA and AGG as ‘stop’ rather than arginine, AUA as methionine rather than isoleucine, and AUA or AUU is sometimes used as an initiation codon instead of AUG (Anderson et al. 1981, Montoya et al. 1981).

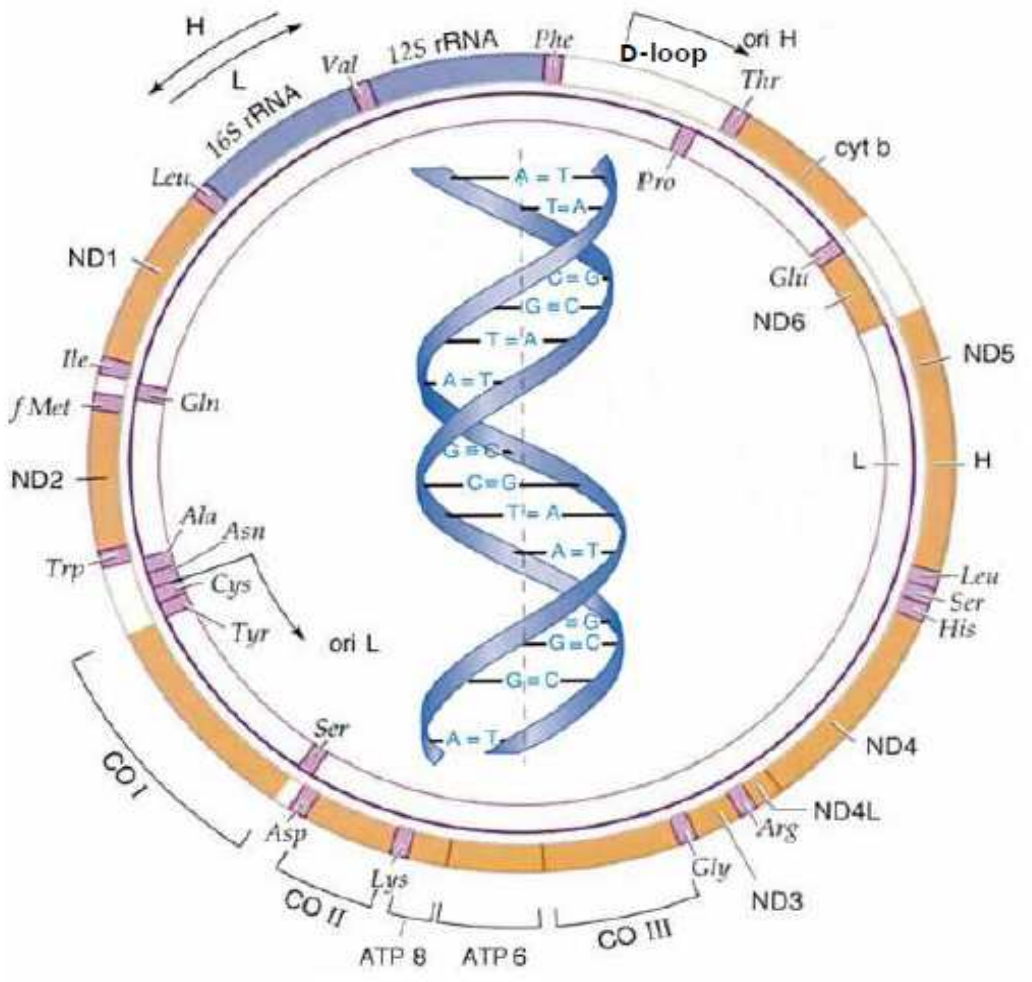


Figure 3. The human mitochondrial genome encodes 13 subunits of respiratory chain complexes: seven subunits (ND 1–6 and 4L) of complex I, cytochrome b (Cyt b) of complex III, the COX I–III subunits of cytochrome oxidase or complex IV, and the ATPase 6 and 8 subunits of F_OF_1 ATP synthase. MtDNA also encodes 12S and 16S rRNA genes and 22 tRNA genes. The abbreviated amino acid names indicate the corresponding amino acid tRNA genes. The outer strand is heavy-chain DNA and the inner one light-chain DNA. O_H and O_L are the replication origins of the light and heavy chain, respectively, while P_H and P_L indicate the transcription sites.

(Modified from http://ipvgen.unipv.it/docs/projects/torroni_eng.html)

Product Category	Symbol	Gene Type	nucleotide position
12S RNA	MT-RNR1	rRNA	648..1601
16S RNA	MT-RNR2	rRNA	1671..3229
Cytochrome C Oxidase (complex IV)	COX1	protein coding	5904..7445
	COX2	protein coding	7586..8269
	COX3	protein coding	9207..9990
ATP synthase (complex V)	ATP8	protein coding	8366..8572
	ATP6	protein coding	8527..9207
NADH dehydrogenase (complex I)	ND1	protein coding	3307..4262
	ND2	protein coding	4470..5511
	ND4L	protein coding	10470..10766
	ND5	protein coding	12337..14148
	ND4	protein coding	10760..12137
	ND6	protein coding	14149..14673
	ND3	protein coding	10059..10404
Coenzyme Q - cytochrome c reductase /Cytochrome b (complex III)	CYTB	protein coding	14747..15887
No	D-loop	Non-coding	16024..16569; 1..576
Phenylalanine	MT-TF	tRNA	577..647
Valine	MT-TV	tRNA	1602..1670
Leucine	MT-TL1	tRNA	3230..3304
Isoleucine	MT-TL2	tRNA	12266..12336
	MT-TI	tRNA	4263..4331
Glutamine	MT-TQ	tRNA	4329..4400
Methionine	MT-TM	tRNA	4402..4469
Tryptophan	MT-TW	tRNA	5512..5579
Alanine	MT-TA	tRNA	5587..5655
Asparagine	MT-TN	tRNA	5657..5729
Cysteine	MT-TC	tRNA	5761..5826
Tyrosine	MT-TY	tRNA	5826..5891
Serine	MT-TS1	tRNA	7446..7514
	MT-TS2	tRNA	12207..12265
Aspartic acid	MT-TD	tRNA	7518..7585
Lysine	MT-TK	tRNA	8295..8364
Glycine	MT-TG	tRNA	9991..10058
Arginine	MT-TR	tRNA	10405..10469
Histidine	MT-TH	tRNA	12138..12206
Glutamic acid	MT-TE	tRNA	14674..14742
Threonine	MT-TT	tRNA	15888..15953
Proline	MT-TP	tRNA	15956..16023

Table 1. MtDNA regions, encoding genes, and nucleotide positions.

Characteristic	Nuclear genome	Mitochondrial genome
Size	~3.3 x 10^9 bp	16,569 bp
Number of DNA molecules per cell	23 in haploid cells; 46 in diploid cells	Several thousand copies per cell (polyploidy)
Number of genes encoded	~20,000–30,000	37 (13 polypeptides, 22 tRNAs and 2 rRNAs)
Gene density	~1 per 40,000 bp	1 per 450 bp
Introns	Frequently found in most genes	Absent
Percentage of coding DNA	~3%	~93%
Codon usage	The universal genetic code	AUA codes for methionine; TGA codes for tryptophan; AGA and AGG specify stop codons
Associated proteins	Nucleosome-associated histone proteins and non-histone proteins	No histones; but associated with several proteins(for example, TFAM) that form nucleoids
Mode of inheritance	Mendelian inheritance for autosomes and the X chromosome; paternal inheritance for the Y chromosome	Exclusively maternal
Replication	Strand-coupled mechanism that uses DNA polymerases α and δ	Strand-coupled and strand-displacement models; only uses DNA polymerase γ
Transcription	Most genes are transcribed individually	All genes on both strands are transcribed as large polycistrons
Recombination	Each pair of homologues recombines during the prophase of meiosis	There is evidence that recombination occurs at a cellular level but little evidence that it occurs at a population level

Table 2. Comparision between the human nuclear and mitochondrial genomes. *Table modified from (Taylor et al, 2005). TFAM, mitochondrial transcription factor A; rRNA, ribosomal RNA.

Except the difference at the codon usage, the copy numbers, mechanism of replication, the control of replication, mitochondrial genetics is also different from Mendelian genetics on its uniparental inheritance (Taylor et al, 2005) (Table 1). Human mtDNA is normally inherited exclusively from the mother, known as maternal inheritance. The mammalian egg contains 100,000 to 1,000,000 mtDNA molecules, whereas a sperm contains only 100 to 1000 mtDNA molecules (Chen et al. 1995b, Manfredi et al. 1997). When the sperm fertilizes the egg, the sperm detaches the tail and except the nucleus of the sperm is used to fertilise, all the paternal mitochondria including mtDNA are lost early in embryogenesis, soon after fertilization, between the two-cell and four-cell stages. This could be due either to destruction of sperm mitochondria or to impaired replication of sperm mtDNA in the cells (Manfredi et al. 1997). However, this inheritance could be altered by cloned embryos or subsequent rejection of the paternal mitochondria. The paternal mtDNA was reported presenting at the blastocyst stage in some abnormal (polyploidy) human embryos produced by in vitro fertilization and intracytoplasmic sperm injection techniques (St John et al. 2000). A case study showed 2-bp pathogenic deletion in the mtDNA NADH dehydrogenase subunit-2 (ND2) gene in the muscle of a patient with mitochondrial myopathy was paternal in origin and accounted for 90 percent of the patient's muscle mtDNA (Schwartz et al. 2002). Although no any evidence of paternal transmission have been shown on the other patients with the same disease in the subsequent studies (Taylor et al. 2003, Filosto et al. 2003, Schwarz 2004).

Mitochondria are descendants of α-proteobacteria that formed an endosymbiotic relationship with ancestral eukaryotic organisms. In 1963 it was discovered that DNA was contained within the mitochondrion, and not only could they translate mRNA into protein, but also that the very genes for these proteins are present in the organelles. During its evolution into the present-day ‘powerhouse’ of the eukaryotic cell the mitochondrion transferred many of its genes to the

nucleus. Whilst the mitochondrion is largely dependent on nuclear-encoded factors some functional independence remains. By definition, mitochondria in all organisms are able to carry out two functions: the expression of an integral genome and the generation of ATP coupled to electron transport (Futuyma 2005).

In mitochondria, the cellular energy (ATP or the equivalent GTP) is produced through a process so called oxidative phosphorylation (OXPHOS), in which hydrogen is oxidized to generate water and ATP. MtDNA is located in the mitochondrial matrix close to the internal mitochondrial membrane. Due to the close proximity to ATP production site, mtDNA is highly exposed to strongly mutagenic reactive oxygen species (ROS) generated as by-products of OXPHOS. Moreover mitochondria seem to lack protective proteins such as histones and lack an efficient DNA repair system (Richter et al. 1988, Bogenhagen et al. 1999). Therefore mtDNA is vulnerable to oxidative damage and accumulate sequence mutations. Furthermore, it seems deviant mitochondrial metabolism might accelerate the rate of mtDNA mutation (Lightowlers et al. 1997). These unique features probably cause the mutation rate of mtDNA is 10 times higher than that in nuclear DNA (Cavalli et al. 1998).

Sometimes mutations arising in mtDNA generate an intracellular mixture with both mutant and normal mtDNAs, which is termed as heteroplasmy. If only the wild-type or all mutant mtDNA is found in cells, which condition is described as homoplasmy. During the cell division, the mitochondria and their genomes undergo a process so called replicative segregation, in which mitochondrial genomes random replicate and partition into daughter cells. Hence only a small number of mtDNA molecules in the mother are passed on to the next generation, which results to the mitochondrial genetic bottleneck. It could also explain that although a high copy number of

mtDNA present in mature oocytes versus a relatively small number of cell divisions in the female germline, mtDNA sequences could variate remarkably between generations (Poulton et al. 1998).

2 MtDNA and human cancer

2.1 MtDNA changes

To date, various types of mtDNA alterations including point mutations, instability of mono- or dinucleotide repeats, mono- or dinucleotide insertions, deletions, or quantitative alterations have been identified virtually in solid tumors, such as colon, stomach, live, kidney, bladder, prostate, skin and lung cancer (Chatterjee et al, 2006; Brandon et al. 2006), and hematologic malignancies, such as leukaemia and lymphoma (Fontenay et al. 2006).

In a study, by the entire mitochondrial genome sequencing analysis of human colorectal cancer cell lines, 70% (7/10) were found to carry mutations in protein coding genes or rRNA qenes, which also revealed that most of the mtDNA mutations were homoplasmic (Polyak et al. 1998). It has been reported that 64% (9/14) of bladder cancers, 46% (6/13) of head and neck cancers, and 43% (6/14) of lung cancers harboring point mutations of mtDNA. It was as well addressed that the majority of these somatic mutations of mtDNA were homoplasmic (Fliss et al. 2000). Other than point mutations, a 40 bp insertion within the COX I gene has been reported to be associated with renal cell oncocytoma (Welter et al. 1989), while it has been found a deletion happened to NADH dehydrogenase subunit III, which lead to the loss of mtDNA, is specifically linked to renal carcinoma (Selvanayagam et al. 1996). Two types of frame-shift mutations of 3571_3572 ins C and 11038 del A have also been detected in thyroid oncocytomas as well as in renal oncocytoma tissues (Mayr et al. 2008). It has been reported that the frequency of missense

mutations on COX I in prostate cancer patients was significantly higher compared to the non-cancer controls in a population based study (Petros et al. 2005).

In addition to mutations in the coding region of mtDNA, a high frequency of somatic mutation was located in the non-coding displacement loop (D-loop) region of mtDNA. The D-loop region has been described as the most frequent host for mtDNA mutations in variety of human cancers. Several studies of somatic mutation in the D-loop region of mtDNA has revealed that insertions or deletions at nucleotide position (np) 303-309, a polycytidine stretch (C-tract) termed D310, are the most common mutations of mtDNA in human cancers including colorectal cancer (Lievre et al. 2005), gastric cancer (Wu et al. 2005), hepatocellular carcinoma (Tamori et al. 2004), melanoma (Takeuchi et al. 2004), ovarian cancer (Liu et al. 2001), uterine serous carcinoma (Pejovic et al. 2004). The D-loop is a triple stranded non-coding region with regulatory elements required for replication and transcription of the mtDNA. Hence mtDNA mutations in this region might responsible for the changes on copy number and gene expression of the mitochondrial genome.

Based on the published data, Carew and his colleagues addressed four common features of mtDNA mutations in all tumor types including that the base substitutions are the most common mutations; mutations occur in all protein coding mitochondrial genes; the D-loop region is the hot spot of somatic mutations among most of tumor types; and the presence of homoplasmic mutant mtDNA in tumors suggests that they may play an important role in the development of tumors (Carew et al.2002).

Large-scale deletions of mtDNA have been detected in various types of cancers (Carew et al. 2002). For example, it was reported that a 4,977 bp deletion was largely accumulated in sun-

exposed skin tissues, the squamous cell carcinomas and precancerous skin tissues (Pang et al. 1994). The 4,977 bp deletion of mtDNA was later detected in oral cancers and paired non-malignant oral tissues of patients with betel quid chewing history (Lee et al. 2001). Moreover, an increase of mtDNA large-scale deletions was reported in radiation-associated thyroid tumors (Rogounovitch et al. 2002). However, even the 4,977 bp deletion of mtDNA has been frequently detected in various types of cancers; the incidence and amount of the 4,977 bp-deleted mtDNA are significantly lower in the malignant tissues as compared with the paired normal tissues of cancer patients. It has been suggested that during cancer progression the mtDNA with a deletion is decreased (diluted) as a result of clonal expansion of cell lineages that contain less or no mtDNA deletion. The study with micro-dissected tumor tissues further confirmed the lower incidence of 4977 bp mtDNA deletion in most tumors (Dani et al. 2004).

Alterations in the copy number have also been found in human cancers. The copy number of mtDNA was found to be increased in papillary thyroid carcinomas (Mambo et al 2005) and during endometrial cancer development (Wang et al. 2005). While the elevated mtDNA content has been detected in saliva from patients with primary head and neck squamous cell carcinoma, which was significantly higher than that of controls, and it was found that the increase of mtDNA content was associated with advanced tumor stage (Jiang et al. 2005). In addition, it was observed in head and neck cancers that mtDNA content was increased with histopathologic grade from normal, moderate, dysplasia, severe dysplasia to invasive tumors, which demonstrated the rising incidence with histopathologic grade (Kim et al. 2004). The increase in mtDNA content was thought to be a feedback mechanism that compensates for a decline in respiratory function.

In contrast, it has been reported that the copy numbers of mtDNA were frequently reduced in hepatocellular carcinomas (HCCs). And, this reduction of mtDNA copy number of was more

frequently observed in female patients with HCCs as compared with male patients with HCCs. This finding suggests that the differential alterations in the mtDNA copy number of cancer tissues of male and female patients may contribute to the differences in clinical manifestation, progression, and mortality rate between male and female HCC patients (Yin et al. 2004). It has also been reported that mtDNA content was reduced in HCCs compared with the corresponding non-cancerous liver tissues, and that low mtDNA content of HCCs was significantly correlated with large tumor size and liver cirrhosis (Yamada et al. 2006). In gastric cancers, the association between the clinicopathological features and the mtDNA content has been addressed and it was found that a decrease of mtDNA content is significantly associated with ulcerated and infiltrating type (Borrmann's type III) and diffusely thick type (Borrmann's type IV) of gastric carcinomas (Wu et al. 2004). These findings suggest that a decrease in the mtDNA content is associated with the progression of ovarian cancer.

However, both increases and decreases in mtDNA content in contrast to non-malignant controls were observed in each cancer type in a comprehensive investigating on mtDNA copy number in study with 54 hepatocellular carcinomas (HCCs), 31 gastric, 31 lung, and 25 colorectal cancers (Lee et al. 2005). The mtDNA content in ovarian carcinomas was found to be significantly higher than that in normal ovaries (Wang et al. 2006). Whereas, it was shown that the mtDNA content in the pathologically high-grade (poorly differentiated) ovarian cancer was lower than that of the low-grade (well differentiated) ovarian cancer (Wang et al. 2006). Recently, a study of 153 colorectal cancer patients revealed that mtDNA content in colorectal cancers was higher than that in the corresponding non-cancerous colon tissues. However, the mtDNA content was decreased in colorectal cancers with higher TNM stages and poorer differentiation (Lin et al. 2008).

These findings suggest that a change in the content of mtDNA might not be associated with a certain type of cancers, and the actual copy number of mtDNA in certain cancers might depend upon the specific sites of mtDNA mutations attached to that cancer. On one hand, it was suggest that somatic mutations in the D-loop of mtDNA and impairment in mitochondrial biogenesis may contribute to the decrease of mtDNA copy number in human cancers (Lee et al. 2005). On the other hand, mtDNA mutations located within genes encoding oxidative phosphorylation proteins might be expected to result in an increase in mtDNA copy number. It has been hypothesized that this might occur as a compensatory response to the decline in respiratory chain function (Kim et al. 2004).

2.2 MtDNA and Carcinogenesis

Uncontrolled cell growth and altered energy metabolism are two essential properties of tumour. Mitochondria play a fundamental important role in energy metabolism, and programmed cell death, suggesting mitochondria might serve as the key switch for carcinogenesis (Cavalli et al. 1998). In 1920s Otto Warburg observed in tumour cells the most cellular energy was produced by glycolysis even under aerobic condition, which is termed as aerobic glycolysis. Since aerobic glycolysis is in contrast to 'Pasteur effect' in normal cells, Warburg hypothesized cancer was caused by the irrversible injury to the mitochondrial respiratory machinery (Warburg et al . 1924, Warburg 1956).

Warburg’s observation extremely inspired investigation on mitochondrial function in tumors. In 1998 it was reported by Vogelstein and colleagues that mtDNA mutations were present in 7 out of 10 colorectal cancer cell lines in their landmark study (Polyak et al. 1998). This is the first paper to describe the presence of somatic mtDNA mutations in solid human tumours, in this case

colon cancer. In many cases, the mtDNA mutations had accumulated to homoplasmic levels and were not evident in the matched normal tissue from the same patient. A causal relationship between mtDNA mutations and tumorigenesis is yet to be established. Since then, the presence of somatic mtDNA mutations has been reported in both solid tumours and leukaemias (Robert et al. 2005). It was suggested that the high rates of mtDNA mutations observed in cancer cells may lead to mitochondrial dysfunction and reduce the cellular ability to generate ATP through OXPHOS (Carew et al. 2002; Singh 2004; Brandon et al. 2006). Moreover, malfunction of mitochondrial respiratory chain could also enhance electron leakage, leading to increased ROS production. This speculation led Carew and colleagues to use primary human leukemia cells isolated from patients to examine mtDNA mutations and their correlation with alteration in cellular ROS and mitochondrial mass. It was found that mtDNA mutations in leukemia cells were closely associated with increased ROS (Carew et al. 2004).

ROS are well known for its damage effect and take a role in decreasing mitochondrial ATP production, as well as in both the initiation and promotion of tomor (Shigenaga et al. 1994; Gille et. al 1992; Zhang et al. 1990). It has been shown HeLA cells with DNA-depleted mitochondria generate high levels of ROS, in part due to electron leakage generated by the presence of nuclear DNA-encoded Complex II system and ubiquinone (Miranda et al. 1999). A research group reported that mutants completely devoid of mtDNA in yeast show 3 to 6 fold increases in spontaneous nuclear mutation rates (Flury et al. 1976). Model systems expressing altered levels of adenine nucleotide translocators, Mn-superoxide dismutase, ubiquinone or nitric oxide synthase could be used to study the predicted association between mitochondrial ROS production and nuclear mutation frequency. Recent data indicate that Mn-SOD knockout mice show increased oxidative DNA damage (Melov et al. 1999). Oxidative damage induced by ROS is

probably a major source of mitochondrial genomic instability leading to respiratory dysfunction resulting in cancer growth.

Fragments of mtDNA are sometimes found in nuclear genes, for example, sequences representing subunits ND4 (Complex I) and subunits cytochrome *c* oxidases I, II and III (Complex IV) are present in the nuclear DNA of various tissues (Corral et al. 1989). And the insertion of mtDNA in nuclear genes has been suggested as a mechanism by which oncogenes are activated (Corral et al. 1989; Reid et al. 1983).

Recently some studies suggest the functional significance of mtDNA mutations and depletions in tumorigenesis and/or tumor progression. It has been reported some somatic mtDNA mutations and mtDNA depletion in gastric cancer might be involved in carcinogenesis of breast and gastric carcinoma (Boddapati et al. 2005), while it has been shown mtDNA mutations also appear to play a role in development digestive tract cancer (Kose et al. 2005). Shidara and colleagues have shown that specific point mutations in mtDNA accelerated tumor growth and reduced apoptosis (Shidara et al. 2005). These point mutations are in the mitochondrial ATP synthase subunit 6 gene (MTATP6) and are associated with maternally inherited Leigh syndrome but have also been detected in a variety of tumors (Maximo et al. 2002, Yeh et al. 2000, Tan et al. 2002). These data support the notion that point mutations occurring in tumors within mtDNA can have functional advantages as they promote tumor growth. In another comprehensively study of 25 colorectal cancers, 31 gastric cancers, 54 hepatocellular carcinomas and 31 lung cancers , it has been reported the incidence of somatic D-loop mutations is higher in later stage cancers than that of early stage cancers (Lee et al. 2005). These findings suggest that instability in the D-loop region of mtDNA may be involved in carcinogenesis of human cancers.

In prostate cancer both germline and somatic mtDNA COI missense and nonsense mutations have been found to be associated with prostate cancer. Moreover, when the mtDNAs of a prostate cancer cell (PC3) were substitution with a patient mtDNA harboring the pathogenic np T8993G ATP6 mutation the resulting PC3 (mtDNA T8993G) cell lines generated much more rapidly growing tumors in nude mice than did the PC3 prostate cancer cell lines in which the resident mtDNA was replaced with a mtDNA from the same heteroplasmic patient but harboring the normal base, PC3 (mtDNA T8993T). This increased tumorigenicity was associated with increased ROS production, indicating that mtDNA mutations that increase ROS production may bean important factor in tumorigenicity (Petros et al., 2005).

It has also been suggested by the trans-mitochondrial hybrid (cybrid) studies that mtDNA plays an important role in establishing and/or maintaining the tumorigenic phenotype. For example, the evidence of increased tumorigenic phenotype had been shown in a rho0 derivative of human osteosarcoma cells, which showed increased anchorage independent growth compared to the parental cells. In turn, the parental phenotype was restored by transfer of wild type mitochondrial DNA to rho0 cells displaying reduced anchorage independent growth (Singh et al. 2005). These studies suggest that inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis and that retrograde signaling from mitochondria to nucleus may be an important factor in restoration of the non-tumorigenic phenotype. In the further studies by Singh's group it is found that retrograde mitochondria-to-nucleus signaling has important role in regulation of NADPH oxidase (Nox1), and that over-expression of Nox1 in most of breast and ovarian tumors (Desouki et al. 2005). The cluster of Nox enzymes consist of seven structurally related homologues, Nox 1-5 and dual oxidase 1 and 2 (Desouki et al. 2005; Lambeth et al). 2004), and Nox 1 encoded by nuclear DNA is a major source of endogenous ROS in the cell

(Desouki et al. 2005). With the same technique the functional significance of mtDNA mutations is demonstrated in another studys. Cybrids harboring the ATP6 T8993G mtDNA mutation in prostate cancer (PC3) cells were found to generate tumors that were 7 times larger than wild type cybrids, which barely grew in mice (Petros et al. 2005). In addition, cybrids constructed using a common HeLa nucleus and mitochondria containing a point mutation at nucleotide position 8993 or 9176 in ATPsynthase subunit 6 were present a growth advantage in early tumor stages after transplantation into nude mice. This growth advantage might possibly occur *via* prevention of apoptosis (Shidara et al. 2005). These studies indicate that mtDNA mutations might directly promote tumor growth *in vivo*.

Moreover, it is also reported that mitochondrial dysfunction leads to chromosomal instability (CIN), a hallmark of cancer cells, present in a variety of primary human tumors, which suggests mitochondria-led nuclear mutations may be a causative factor in tumorigenesis. In addition, the redox factor 1 (Ref1, also known as Ape1 and Hap1) was found to plays a key role in genomic instability. Ref1 expression was altered in a variety of tumors. Together, these studies suggest that mitochondria-to-nucleus retrograde redox regulation due to mitochondrial dysfunction may also contribute to tumorigenesis (Singh et al. 2005)

2.3 MtDNA and cancer diagnosis

In the last twenty years various approaches have been developed and investigated on detecting specific molecular markers in clinical samples to improve the outcomes of conventional cancer screening (Sidransky, 2002). Though the changes of nuclear genetic and epigenetic have been regarded as the cornerstone of such studies, mitochondrial cellular content and mutations are also emerging as new molecular markers for clinical application. The feature of sheer abundance and

homoplasmic tendency make mtDNA an attractive biomarker for cancer (Sing et al. 1998 and 1999, Polyak et al. 1998). In patients with lung cancer, bronchoalveolar lavage samples were found to harbour almost 200-fold more mitochondrial mutations than nuclear TP53 (Fliss et al. 2000). In addition to bronchoalveolar lavage samples in lung cancer, mtDNA mutations have been readily detected in urine and blood from patients with bladder and head and neck cancers (Fliss et al. 2000); serum of hepatocellular carcinoma patients (Okochi et al. 2002); nipple aspirate fluid from patients with breast cancer (Zhu et al. 2005). Mitochondrial DNA can serve as a reliable and sensitive biomarker of cumulative UV radiation exposure in skin (Harbottle et al. 2006). MtDNA mutations within the D-loop control region have been used as clonal markers in hepatocellular carcinoma (Nomoto et al. 2002) and breast cancer (Parrella et al. 2001). Moreover, mtDNA sequence variants have been detected with a rapid and high throughput sequencing method in patient tumor tissue and blood samples (Jakupciak et al. 2005).

However, the mitochondrial genome is highly variable and the ease of whole-genome sequencing does not resolve some diagnostic dilemmas because the interpretation of a novel sequence change can be difficult in relation to potential pathogenicity (McFarland et al. 2004). It has been also mentioned that direct sequencing of tumor mtDNA, common performed in many studies, is a poor screening technique, since it misses levels of heteroplasmy below approximately 20%; whereas a better method is denaturing high-performance liquid chromatography followed by confirmatory polymerase chain reaction/restriction fragment length polymorphism analysis (Zanssen et al, 2005).

In despite of extensive study reports on the identification of mitochondrial DNA mutations in a wide range of human cancers, the exact role of mitochondrial DNA mutations in tumor

development and progression has not been established. Thus, new technologies need to be further investigated to detect mitochondrial genetic and somatic alterations so as to provide an opportunity for large-scale analysis of mitochondrial mutations in human cancers. Therefore the link of functional mtDNA alteration to cancers could be applied on routine clinical diagnosis including screening.

The alterations of mtDNA including mutations, insertions, deletions and instability are emerging as new biomarkers for detecting many cancers in tissue samples and body fluids which can be probably implemented in population screening trials (Verma et al, 2007). By using MitoChip for rapid sequencing of the entire mitochondrial genome, somatic mtDNA alterations were observed in preneoplastic lesions of the gastrointestinal tract, even in the absence of histopathological evidence of dysplasia (Sui et al. 2006). Undoubtedly single clinical application with MitoChip could be to augment diagnosis, but by the validation of mtDNA detection in body fluids harbouring shed tumor derivatives this application could be significantly advanced (Folkman 2001). The development of the high-throughput mtDNA resequencing microarray is a milestone in mutation and polymorphism detection techniques, which is applicable to improve cancer diagnosis. These findings support the rationale for exploring the mitochondrial genome as a biomarker for the early diagnosis of cancer.

2.4 MtDNA and cancer treatment

MtDNA not only represents a signature of personal identity, but also serves as a log book accumulating mutations unique to each person. Thus, the mitochondrial genomic information has already impacted genetic counselling procedures and provided insights into novel avenues for treatment (Jakupciak et. 2006). Since mitochondria play a critical role of in apoptosis, it is

conceivable that mutations in mtDNA in cancer cells could significantly affect the cellular apoptotic response to anticancer agents. Experiments with rho^0 cells without mtDNA evaluate the role of respiration in drug sensitivity resulting in various results, which might reflect complex interactions between rho^0 cells and anticancer agents with disparate function mechanisms. To evaluate the changes in drug sensitivity in cancer cells bearing mtDNA mutations could be even more complicated, since different types of mtDNA mutations are likely to have diverse effects on the apoptotic response. Nevertheless, the clonal selection/expansion hypothesis could predict that the mutations recovered from cancer cells which survive chemotherapy are likely to be associated with resistance to the particular anticancer drugs being used in previous therapy. Furthermore, some particular mtDNA mutants in cancer cells are likely to arouse the respiratory chain dysfunctions and increase ROS generation. This biochemical change offers a unique opportunity to selectively kill this population of cancer cells by using agents that inhibit free radical elimination and cause further ROS accumulation, leading to lethal damage in the cancer cells (Huang et al. 2000). Taken together, it is evident that mtDNA mutations are clinically relevant and have potential therapeutic implications.

The cancer cells with defective mitochondria and mtDNA mutants also produce larger amounts of ROS and are thus exposed to higher oxidative stress. The mitochondria with higher oxidative stress might utilize the retrograde signaling pathways to modulate the expression of nuclear genes involved in glycolysis and mitochondrial respiration and OXPHOS. This phenomenon, so called Warburg effect, might explain the observed increase in glucose utilization and higher lactate production in the formation and progression of cancers (Lee et al). The distinct differences in mtDNA structure and function between cancer cells and normal cells provide the potential for clinical use of mitochondria and mtDNA as targets for novel and site-specific anticancer agents

(Weissig et al. 2001, Modica-Napolitano et al. 2002). Therefore, development of drugs that target to mitochondria or mtDNA may improve treatment of some types of human cancers in the future.

One chemotherapeutic strategy it to employ delocalized lipophilic cations (DLCs) which selectively accumulate in carcinoma cells in response to elevated mitochondrial membrane potential. Several of these compounds have exhibited some degree of efficacy in carcinoma cell killing *in vitro* and *in vivo* (Sun et al. 1994, Koya et al. 1996, Weisberg et al. 1996). Efforts have also been made to enhance the selective tumor cell killing of DLCs by combination with other anti-cancer agents, including AZT (Modica-Napolitano et al. 2004). Some DLCs have been applied in photochemotherapy (PCT), an investigational cancer treatment involving light activation of a photoreactive drug, or photosensitizer, that is selectively taken up or retained by malignant cells (Modica-Napolitano et al. 2003, Lo et al. 2005). It has been considerably interested in PCT as a form of treatment for neoplasms of the brain, breast, bladder, lung, skin or any other tissue accessible to light transmitted either through the body surface or internally *via* fiber optic endoscopes. Cationic photosensitizers are particularly promising as potential PCT agents. Similar as other DLCs, these compounds are converged by cells into mitochondria in response to transmembrane potentials, and are thus particularly accumulated in the mitochondria of carcinoma cells. The photosensitizer can be converted to a more reactive and highly toxic species in response to localized photoirradiation, so as to strengthen the selective toxicity to carcinoma cells and offer a means of highly specific tumor cell killing without injury to normal cells (Chatterjee et al. 2008).

One alternative strategy is to employ mitochondrial membrane protein-import machinery to deliver macromolecules into mitochondria. Using the similar machinery, a mitochondrial signal sequence has been used to direct green fluorescent protein to mitochondria, which promises the

visualization of mitochondria within living cells (Rube et al. 2004). Some peptides containing two functional domains, one homing motif for targeting particular cell types and the other pro-apoptotic sequence, readily infiltrate via the mitochondrial membrane and turn into toxic when internalized into the targeted cells by disruping mitochondrial membranes (Modica-Napolitano et al. 2004**).** Another chemotherapeutic strategy is to target specific mitochondrial membrane proteins to alter membrane permeabilization and ultimately induce apoptosis (Cullen et al. 2007). Attempts have been made also to develop mitochondriotropic drug and mtDNA delivery systems. One study demonstrates that conventional liposomes can be conferedd mitochondria-specific by attaching to the known mitochondriotropic residues to the liposomal surface (Liguori et al. 2008)

Furthermore, DQAsomes made from derivatives of the self-assembling mitochondriotropic bola-amphiphile dequalinium chloride, have been exhibited the capacity to bind and transport oligonucleotides as well as plasmid DNA conjugated to a mitochondrial leader sequence (MLS) to mitochondria in living mammalian cells and release DNA on contact with mitochondrial membranes (Dsouza et al. 2005). The long-term therapeutic goal of this type of research is to produce mitochondria-specific vehicles which could effectively deliver drugs or mtDNA into the organelle to destroy malfunctioned mitochondria or restore mitochondria with healthy copies of the genome.

Due to the important role of mitochondria in ATP metabolism, in generation of free radicals, and in regulation of apoptosis, it has been indicated mtDNA mutations are likely to affect cellular energy capacities, increase oxidative stress, cause ROS-mediated damage to DNA, and alter the cellular response to apoptosis induction by anticancer agents (Penta et al. 2001, Copeland et al. 2001). However, apoptosis was found to occur less frequently in the mutant cybrids in cultures as

compared with wild-type cybrids, which suggests that the pathogenic mtDNA mutations might promote the growth of tumors by preventing apoptosis (Shidara et al. 2005). The mutant mtDNA in cybrids also exhibited resistance to cisplatin-induced apoptosis (Shidara et al. 2005). These results suggest that pathogenic mtDNA mutations might contribute to the progression of cancers and tolerance against anticancer drugs. The presence of somatic D-loop mutations might be a factor of resistance to fluorouracil based adjuvant chemotherapy in stage III cancers (Lievre et al. 2005). It has been also found that mtDNA mutations in leukemia cells were closely associated with altered sensitivity to drug treatment (Carew et al. 2003). Moreover, mtDNA has also been shown to determine the hormone dependence in breast cancer cell lines. Naito and colleagues stablished hydroxytamoxifen-resistant breast cancer cells by growing human breast cancer cells MCF-7 in the presence of hydroxytamoxifen. They found that the mtDNA content was significantly reduced in the hydroxytamoxifen-resistant breast cancer cells. They further demonstrated that depletion of mtDNA induced by hormone therapy or other independent insults could trigger a shift to acquired resistance to hormone therapy in breast cancers (Naito et al. 2008).

2.5 MtDNA and cancer prognosis

Numerous biomarkers have been evaluated to predict morbidity and mortality in patients with cancer, although few have proved entirely useful. In a small scale study with 19 cases of cervical cancer, mtDNA D-loop mutations are found to be possibly caused by HPV infection, and are not associated with the histopathological grade and tumor staging (Sharma et al. 2005). Similarly a rarely mtDNA D-loop 16519 somatic mutations found in pancreatic cancer, which cannot be considered causative events for this tumor type and probably are epiphenomena, but probably worsens pancreatic cancer prognosis (Navaglia et al. 2006). In a mutation analysis of eight

sample pairs of papillary thyroid carcinomas and six of follicular thyroid carcinomas tissue with the corresponding normal thyroid tissue, it has not been found mtDNA mutations to be correlated with statistically validated clinical prognosticators for recurrence or survival (Witte et al. 2007). A study of 109 patients with head and neck cancers revealed that the presence of D-loop mutations of mtDNA was not associated with the prognosis or the response of patients to neoadjuvant chemotherapy (Lièvre et al. 2006). Moreover, no significant association was found between somatic mtDNA mutations and clinicopathological characteristics in esophageal cancer (Hibi et al. 2001), gastric cancer(Wu et al. 2005), lung cancer (Jin et al. 2007), and ovarian cancer (Bragoszewski et al. 2008) respectively.

However, in other studies mtDNA exhibits the potential to be a molecular biomarker to monitor cancer prognosis. In a 10 years retrospective study on 41 patients with invasive carcinoma of the uterine cervix, the results suggest that multiple mtDNA mutations are an independent marker of poor prognosis (Allalunis-Turner et al. 2006). It has been suggested by a study with analysis on somatic mutations in the D-loop region, the common 4,977-bp deletion, and the copy number of mtDNA in breast cancer and paired nontumorous breast tissues from 60 patients that somatic mtDNA mutations in D-loop region could be used as a molecular prognostic biomarker in breast cancer (Tseng et al. 2006). It has been also report in a study with 59 cases of invasive breast tumors and paired non-tumorous tissues indicated that patients with reduced mtDNA content had significantly poorer disease-free survival and overall survival rate, which suggested that reduced copy number of mtDNA may be involved in breast neoplastic transformation or progression and mtDNA content might be potentially used as a tool to predict prognosis (Man et al, 2007).

Moreover, it has also been found in 202 patients with non-small cell lung cancer, the average mutation rate in the D-loop of mtDNA of patients at stage IIIB or stage IV was significantly higher than that of patients at lower clinical stages. And the stage IIIB or stage IV cancer patients carrying point mutations in the D-loop of mtDNA exhibited poorer prognosis compared with those free of the mtDNA mutations (Matsuyama et al. 2003).

Additionally, a population-based study on 365 patients with colorectal cancer recorded with 3 years follow-up, the presence of tumor D-loop mutation appears to be a factor of poor prognosis in colorectal patients (Lievre et al. 2005). Another study of 153 colorectal cancer patients revealed that mtDNA content in colorectal cancers was higher than that in the corresponding non-cancerous colon tissues. Whereas the mtDNA content decreased in colorectal cancers was associated with higher TNM stages and poorer differentiation. The decrease in mtDNA content was correlated with a lower expression level of mitochondrial transcription factor A (mtTFA) or β subunit of the mitochondrial ATP synthase (β-F_1-ATPase). It was suggested that mitochondrial dysfunction is associated with poor prognosis of colorectal cancer (Lin et al. 2008). It has been reported that patients with lower mtDNA content in HCCs tended to show poorer 5-year survival compared with the patients with higher mtDNA content in HCCs, which suggest that decrease in the mtDNA content may be associated with malignancy of HCCs (Yamada et al. 2006). Similarly, most patients with types III and IV gastric cancers, respectively, were found to have poor prognosis and lower 5-year survival rate after gastric resection. These results suggest that the reduction in the content of mtDNA may contribute to the malignancy and progression of gastric cancers (Wu et al. 2004).

The correlations between clinicopathological parameters and somatic mtDNA alterations in certain cancers indicate mtDNA alterations might potentially be used as a molecular prognostic

indicator of cancers. Their correlations with poorer prognosis suggest that somatic mtDNA alterations in cancers may contribute to tumor recurrence and drug resistance in the process of cancer progression. In contrast, these correlations are absent in other cancers such as esophageal cancer, head and neck cancer, which suggest the function of mtDNA alteration might be site or tissue specific.

3. MtDNA in breast cancer

3.1 Alterations of mtDNA in breast cancer

Several studies have examined the presence of mtDNA mutations in breast cancer. In one of the most comprehensive studies 19 sets of paired normal and tumor tissues from the same patients with breast cancer has been analyzed by using a combination of temporal temperature gel electrophoresis and direct DNA sequencing of the complete mitochondrial genome. Somatic mutations were identified in 74% of patients. The bulk of the mutations (81.5%) were restricted to the D-loop region, while other mutations were detected in the 16*S* rRNA, ND2, and ATPase 6 genes. Of these mutations, five (42%) were deletions or insertions in a homopolymeric C-stretch between nucleotides 303–315 (D310) within the D-loop. The remaining seven mutations (58%) were single-base substitutions in the coding or non-coding regions (D-loop) of the mitochondrial genome (Tan et al., 2002).

In another study, somatic mutations were detected in 61% (11/18) of the fine needle aspirates from primary breast tumors harbored mtDNA mutations that were not detected in matched lymphocytes from the same patient or in age-matched normal breast tissue and most of the

mutations identified were in the D-loop region. While 42% of the mutations were present in the homopolymeric C stretch D310 region encompassed within the control region (D-loop). In 39% (7 of 18) somatic mutations in ND and cytochrome b genes were present. Again, these mutations were all homoplasmic suggesting a high clonal stability (Parrella et al. 2001). Zhu and colleagues could detect mutations in as much as 93% (14 of 15) of the examined breast tumor cells. Many of these tumors had multiple mtDNA mutations and the relative mutation frequency in D-Loop mutations was seven fold higher compared to that in gene coding areas (Zhu et al. 2005).

As observed in the aforementioned study, it has been found in a study with paired tumorous and nontumorous breast tissues from 60 patients 30% breast cancers displayed somatic mutations in mtDNA D-loop region. The occurrence of D-loop mutations was associated with an older onset age (≥50 years old), and tumors that lacked expressions of estrogen receptor and progesterone receptor and significantly poorer disease-free survival (Tseng et al. 2006). It was indicated a D-loop mutation is a significant marker independent of other clinical variables. A study on somatic mutation in the D-loop region of mtDNA has revealed that insertions or deletions at nucleotide position (np) 303-309, a polycytidine stretch (C-tract) termed D310, are the most common mutations of mtDNA in human cancers including breast cancer (Tan et al. 2002). In addition, it has been also reported that breast cancers harbouring mutations in D-loop region, particularly at the polycytidine stretch or close to the replication origins of the heavy-strand, had a significantly lower copy number of mtDNA than the ones without D-loop alterations (Man et al. 2007).

Although the most common mtDNA mutations detected in breast cancer have been largely single base substitutions or insertions, a large deletion of 4977 bp has been detected in both the malignant and paired normal breast tissues of patients with breast cancer (Sharp et al. 1992,

Bianchi et al. 1995). The incidence of the 4,977-bp deletion in nontumorous breast tissues (47%) was much higher than that in breast cancers (5%) (Tseng et al. 2006).

All together, these observations suggest that somatic mutation in the D-loop of mtDNA can be considered as a new prognostic marker for some types of cancers, and that mtDNA mutations may play a role in cancer progression and in response to anticancer drug treatment.

In addition to alterations on the sequence of mitochondrial genome, a decrease in mtDNA copy number was found to associate with an older onset age (≥ 50 years old) and a higher histological grade of breast cancer. In addition, patients with reduced mtDNA content had significantly poorer disease-free survival and overall survival rate (Yu et al. 2007). In breast cancer it was reported that mtDNA content is reduced in 80% cases relative to normal controls (Mambo et al 2005). These results suggest that reduction in the content of mtDNA may be involved in neoplastic transformation or progression of breast cancers. However, no similar association was found in other studies of breast cancer patients (Tseng et al. 2006, Mambo et al. 2005).

3.2 MtDNA as a potential biomarker for breast cancer

Earlier diagnosis and treatment of breast cancer play an important role in reducing mortalities (Pantel et al. 2003). Many researchers attempted to establish molecular biological and immunological methods for detection of individual metastatic breast cancer cells in peripheral blood and bone marrow (Zhong et al. 1999a, Diel et al. 2000). However, a human eukaryotic cell containing only one or two copies of each gene limited the sensitivity of using genomic alterations as markers on the nuclear DNA level to identify single tumour cells in circulation.

Lack of cancer specific markers limited the specificity of using mRNA and proteins for gene expression analysis to distinguish normal and malignant cells (Zhong et al 1999b). Therefore, there is no reliable screening test for early diagnosis of breast cancer which measures less than 2mm, and there are no well-established measures to screen for micrometastases.

It has been shown that early diagnosis and accurate identification of haematogenic metastasic tumor cells in breast cancer can improve the success of treatment and patients' survival time. The ideal tumor biomarkers in the peripheral circulation could provide a better solution on the management of cancer. The ideal biomarkers for cancer can be sensitive detection markers for screening and earlier diagnosis, classification markers for treatment selection, and clinical response markers and risk assessment markers for monitoring and follow up of cancer patients (Fig 3).

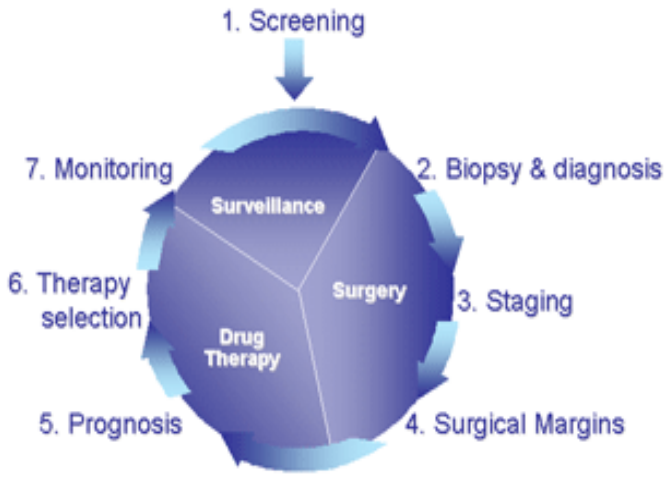


Figure 4. Biomarkers as potential tools for clinical applications.

More biomarkers are still needed to apply to the clinical implementation of cancer. Estrogen receptor and HER-2/neu status in breast tumours are currently routinely used as a guide for adjuvant therapy. As more new anticancer agents are being developed, more new biomarkers need to be found to aid in the detection of micrometastasis and to guide treatment using the new agents. However, there are still many obstacles to developing clinically useful biomarker tests for routine clinical practice. A lack of tumour marker specificity and lack of sensitivity of testing systems limit their clinical use.

The instabilities and alterations of mtDNA in tumorigenesis may serve as earlier markers for cancer development and may have the potential for tracking tumour progression and tumour metastasis. A human eukaryotic cell contains hundreds or thousands of mitochondria and each mitochondrion contains 1-10 copies of mtDNA. In addition, three facilities of mtDNA make it have an application value for. The first, the high copy number in comparison with the nuclear DNA enables detection of rare target cells, even at low levels. The second, mutant mtDNA has been reported to be 10-200 times more abundant than mutated nuclear DNA in cancer cells (Jackson et al. 2002; Hood et al. 2003; Petros et al. 2005), suggesting that they may be of promising clinical utility. The third, it has been showed that elevated mtDNA level is present in plasma of prostate cancer patients with a poor survival. Amplification of mitochondrial nucleic acids shows increased sensitivity and specificity over genomic DNA as diagnostic and prognostic marker in prostate cancer patients (Mehra et al 2007). Altogether, the properties of mtDNA, such as high copy numbers, high prevalence of mutations and quantitative and qualitative alterations in cancer, encourage us to investigate the clinical relevance of mtDNA alterations in cancers. In

addition, the simple structure and short length of mtDNA makes the genome-wide screening of mtDNA in life science easier and more cost-effective than using nuclear DNA.

In the last decades, a number of studies have been carried out on the investigation of mtDNA as a potential biomarker for cancer. Furthermore, the high frequency of mtDNA alterations in cancer and their presence in the early stages of disease could possibly be exploited as clinical markers for early cancer detection (Modica-Napolitano et al. 2002).

To measure the plasma mtDNA, or cell-free nucleic acid, has been entertained as a prognostic marker. Plasma mtDNA could be defined as fragments of mtDNA that were detectable in the extracellular fluid. Recent studies demonstrated that circulating mtDNA mutations can be detected in melanoma, prostate cancer, colon cancer, hepatocarcinoma, and pancreatic carcinoma. To assess whether such mtDNA mutations could be detected mutations in women with breast cancer, and the possibility to aid in the diagnosis of breast cancer, a study has been performed with 27 paired samples (14 patients with breast cancer and 13 healthy controls) of white blood cells and serum. The mtDNA D-loop region was amplified and sequenced. Polymorphisms were detected in all specimens, but no mtDNA mutations were found in any of the study groups (Losanoff et al. 2008). It has been suggested that the method used in this study are extremely sensitive in polymorphism detection, but could not detect mtDNA mutations in the blood of women with breast cancer.

Indeed, tumor-derived circulating nucleic acids in the plasma and serum of cancer patients have been sought as a noninvasive tool for cancer detection over one decade. Though the test criteria, sensitivity and specificity, compare favorably with conventional diagnostic measures, to date the methodical tediousness of circulating nucleic acids analysis prevented it from becoming a clinical

routine application. But, with speeding development of state-to-art technology towards automated high-throughput platforms, it would not be surprised in the nearby future to see analyses of circulating nucleic acids in plasma and serum becoming routing methods for diagnosis and follow-up monitoring of cancer patients. The dream is that the application of circulating nucleic acids in plasma and serum as a cancer biomarker and potential profiling tool will finally translate into a longer survival and better quality of life for cancer patients.

However, the role of mtDNA mutations in cancer development, genetic instability and disease progression, and the development of drug resistance remains ambiguous, which warrants a comprehensive investigation in blood and tissue samples of patients with breast cancer as well as in healthy people.

Part II

Summary of Publications and Manuscripts

1. Study aim and experimental design

Aim

In order to establish a test system for facilitating the early detection and monitoring of breast cancer and its metastasis, we will investigate:

i. Whether any quantitative and qualitative alterations of mitochondrial DNA (mtDNA) exist in breast cancer.

ii. Whether tumour cell or/and tumour-derived mtDNA shedding from tumour tissues into peripheral blood could serve as sensitive and specific markers for clinical application in screening, monitoring and follow-up of breast cancer.

Experimental design

We first intend to investigate quantitative alteration and qualitative alteration of mtDNA in breast cancer tissue. Tumour-specific mtDNA alterations will be used as markers to identify tumour-derived cell free and cellular DNA in the blood samples of patients with breast cancer. The relative proportion of tumour derived and non-tumour derived mtDNA will be detected by MALDI-TOF mass spectrometry assay. The levels of cell-free tumour derived and non-tumour derived mtDNA in three study groups, namely, breast cancer, breast benign lesion and healthy control, will be compared. The association between levels of tumour-derived mtDNA in peripheral blood and traditional clinical parameters, such as tumour size, lymph node involvement, and extent of metastasis, histological grade, receptor status and HER's-2/neu status will be analyzed.

2. Summary of background

2.1 Instability of mitochondrial genome

Eukaryotic cells have a nuclear genome and additional cytoplasmic genomes that are compartmentalized in the mitochondria. The human mitochondrial genome is a circular double stranded DNA of 16.6 kb, including the coding regions for 13 respiratory chain protein subunits and the hypervariable non-coding D-loop regions (Anderson et al 1981; Fernandez-Silva et al. 2003). The mitochondria produce energy to support cellular activities and also generate reactive oxygen species (ROS).

In comparison to nuclear genomic DNA,

- MtDNA molecules are markedly exposed to ROS (**ROS enhanced aggression)**.
- Due to the lack of protective histone proteins, mtDNA is highly sensitive to oxidative DNA damage by ROS (**high sensitivity to damage)**.
- The replication and repair of mtDNA depend on nuclear genes (**deficient repair of damage**).
- The limited DNA repair mechanism allows mtDNA mutations to accumulate (**high rate of mutations)**.

Thus, the properties of mtDNA suggest their potential importance in aging, apoptosis and especially carcinogenesis (Augenlicht et al 2001; Bartnik et al. 2001, Bianchi et al. 2001).

2.2 MtDNA and human cancers

MtDNA aberrations, which include point mutations, instability of mono- or dinucleotide repeats, mono- or dinucleotide insertions, deletions or quantitative alterations, have been found in

- solid tumours, such as colon, stomach, liver, kidney, bladder, prostate, skin and lung cancer (Chatterjee et al, 2006; Brandon et al 2006),
- hematologic malignancies, such as leukaemia and lymphoma (Fontenay M et al. 2006).

The instability of mtDNA may play an important role in tumor development. A high rate of mutation, the presence of most of the mutations in coding sequences, their subsequent accumulation because of limited repair mechanisms, and insertion of mutations into nuclear DNA have all been noted in mtDNA.

For instance:

- MtDNA mutations in coding sequences have been found in pre-malignant histological benign-appearing glands of the prostate, implying that the mtDNA alterations might be involved in the **early events** of prostate cancer development (Jeronimo et al 2001).
- A high prevalence of mtDNA mutations in colorectal cancer tissues, with lower numbers in the pre-cancerous lesions and no mutations in the surrounding normal tissues have been observed, suggesting that, while the histology appears pre-malignant, the genotype is moving **towards the tumour state** (Akhionbare et al. 2004).
- Ultraviolet radiation in sunlight is an important factor in the development of skin cancer and has been shown to induce mtDNA damage in human skin, which could **not be repaired** in mitochondrial genomes (Pascucci et al. 1997; Croteau et al. 1997).

2.3 MtDNA and breast cancer

Breast cancer is the most common malignant disease in women of Western industrial countries. There have been a variety of mtDNA alterations found in breast tumour tissues:

- Tan et al. (2002) performed an analysis of all known mtDNA genome mutations. They could identify 27 mtDNA mutations in 74% of patients with breast cancer. The mutations were located in coding regions, and mostly in the hypervariable D-loop regions.
- Zhu et al. (2005) found **mutated mtDNA** in 93% of breast cancer tissues, with the frequency of mutations higher in the coding regions and D-loop regions than in other loci tested.
- Breast cancer-specific **deletions** of mtDNA have been observed in 77% of breast cancer tissues by Zhu et al. (2004) and in 46% of breast cancer tissues by Dani et al. (2004).
- In the fine-needle aspirates of patients with breast cancer, Parrella et al. (2001) found mtDNA mutations in 61%, deletions or **insertions** in 42% and **single base substitutions** in 58%, which were localized in the coding and D-loop regions.

3 Quantitative analysis of mtDNA in breast cancer

3.1 Method setup for quantitative analysis

Eukaryotic cells have nuclear DNA (nDNA) and additional cytoplasmic mitochondrial DNA (mtDNA). It has been demonstrated that cell-free nucleic acids, *i.e.*, cell-free (ccf) nuclear DNA (cf-nDNA) and ccf mtDNA exist in circulation (Sozzi et al., 2003). Quantification of circulating nucleic acids in plasma and serum could be used as a non-invasive diagnostic tool for monitoring a wide variety of diseases and conditions. We describe here a rapid, simple and accurate multiplex real-time PCR method for direct synchronized analysis of circulating cell-free (ccf) mitochondrial (mtDNA) and nuclear (nDNA) DNA in plasma and serum samples. The method is based on one-step multiplex real-time PCR using a FAM-labeled MGB probe and primers to amplify the mtDNA sequence of the ATP 8 gene, and a VIC-labeled MGB probe and primers to amplify the nDNA sequence of the glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) gene, in plasma and serum samples simultaneously. The efficiencies of the multiplex assays were measured in serial dilutions. Based on the simulation of the PCR reaction kinetics, the relative quantities of ccf mtDNA were calculated using a very simple equation. Using our optimised real-time PCR conditions, close to 100% efficiency was obtained from the two assays. The two assays performed in the dilution series showed very good and reproducible correlation to each other. This optimised multiplex real-time PCR protocol can be widely used for synchronized quantification of mtDNA and nDNA in different samples, with a very high rate of efficiency.

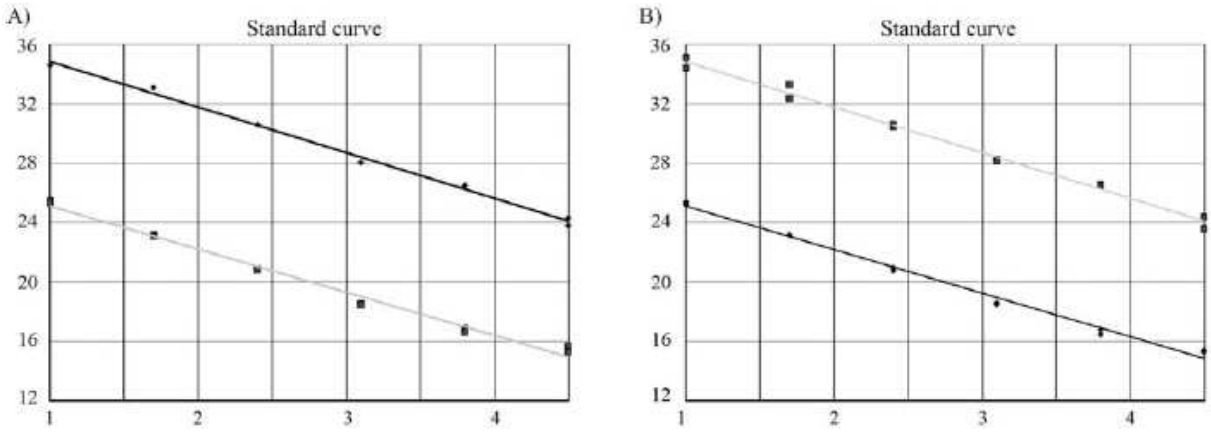


Figure 5. Simulation of real-time PCR kinetics for amplifying mtDNA and nDNA on a serials of dilutions. The figure shows reproducible standard dilution curves for identification of the mtDNA and nDNA. The upper lines are nDNA standard dilution curves and the lower lines are mtDNA standard dilution curves. The numbers on the y axis represent the values of cycle threshold (Ct) and numbers on the x axis represent the dilution points.

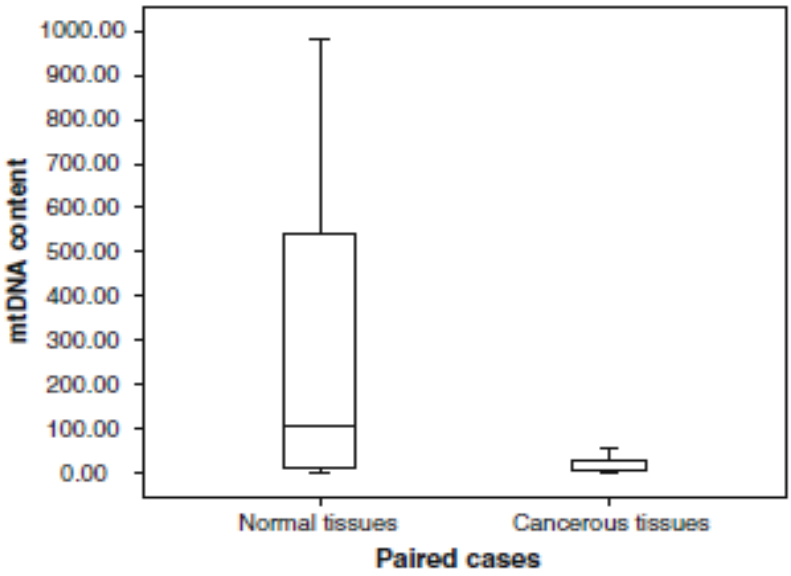


Figure 6. MtDNA content in paired adjacent normal and cancerous breast tissue. The content of mtDNA in cancerous tissues is significantly lower than that in normal tissues (Mann–Whitney *U* test $P < 0.001$)

3.3 MtDNA quantification in cancer tissues of patients with breast cacner

Human cells contain a nuclear genome and additional cytoplasmic genomes that are compartmentalised in the mitochondria. In contrast to nuclear DNA, mitochondrial DNA (mtDNA) reveals high mutation rates caused by constant exposure to mutagenic oxygen radicals and lacks the protective mechanisms of DNA repair. These properties of mtDNA suggest their potential importance in ageing, apoptosis and especially carcinogenesis (Zhang and Qi 2008; Zhang et al. 2008). Quantitative aberrations of mtDNA have been observed in various sample types from patients with cancers (Mambo et al. 2005). While increased mtDNA content has been found in prostate (Mizumachi et al. 2008a), head and neck (Kim et al. 2004), endometrial adenocarcinoma (Wang et al. 2005), etc., reduced mtDNA content in renal (Meierhofer et al. 2004) and liver cancers (Yin et al. 2004) has been reported. Because of these mutations and the quantitative aberrations involved in the development of human cancers, mtDNA may have promising clinical applications for cancers (Jiang et al. 2005; Jain et al. 2007). Using the established multiplexed assay, we found down-regulated mtDNA in breast cancerous tissues compared to the paired breast normal tissues from 51 patients with breast cancer. The amounts of nDNA and mtDNA in 102 tissue samples were quantified for both glyceraldehype-3-phosphodehydrogenase (GAPDH) gene and mtDNA encoded ATPase (MTATP) 8 gene. The average threshold cycle (Ct) number values of the nDNA and mtDNA were used to calculate relative mtDNA content in breast tissues. The median delta Ct (ΔCt) and the median mtDNA content for normal and cancerous breast tissues were 6.73 and 2.54, as well as 106.50 and 5.80 (P = 0.000 respectively). MtDNA content was decreased in 82% of cancerous breast tissues compared with the normal ones. The changes were associated with hormone receptor status. Our

finding suggests that decreased mtDNA content in breast cancer may have diagnostic and prognostic value for the disease.

3.3 MtDNA quantification in whole blood of patients with breast cancer

Alterations of mtDNA have been implicated in carcinogenesis. MtDNA alterations were also detected in bodily fluids, suggesting that mtDNA changes might serve as sensitive early biomarker for non-invasive detection of several types of solid cancer including breast cancer (Fliss et al. 2000). We sought to investigate whether mtDNA content in the peripheral blood of breast cancer patients is associated with clinical and pathological parameters. Using an accurate multiplex quantitative real-time PCR for synchronized determination of mtDNA and nuclear DNA, we found down-regulated mtDNA in the whole blood samples from in stage I breast cancer patients, compared to normal controls and advanced breast cancer patients ($P = 0.023$). Reduced mtDNA was found often in post menopausal cancer group ($P = 0.024$). No difference in mtDNA content, in regards to age ($p = 0.564$), lymph node involvement ($p = 0.673$), ER ($p = 0.877$), PR ($p = 0.763$), and Her-2/neu expression ($p = 0.335$), was observed. In the present study, decreased mtDNA content in the peripheral blood of patients with breast cancer was strongly associated with stage I. The use of mtDNA may have value for earlier diagnosis of breast cancer. Due to the unclear mechanism of compensated mtDNA quantities in advanced stage breast cancer, further studies are required to clarify the biology of the phenomena (Xia et al 2009, revision submitted).

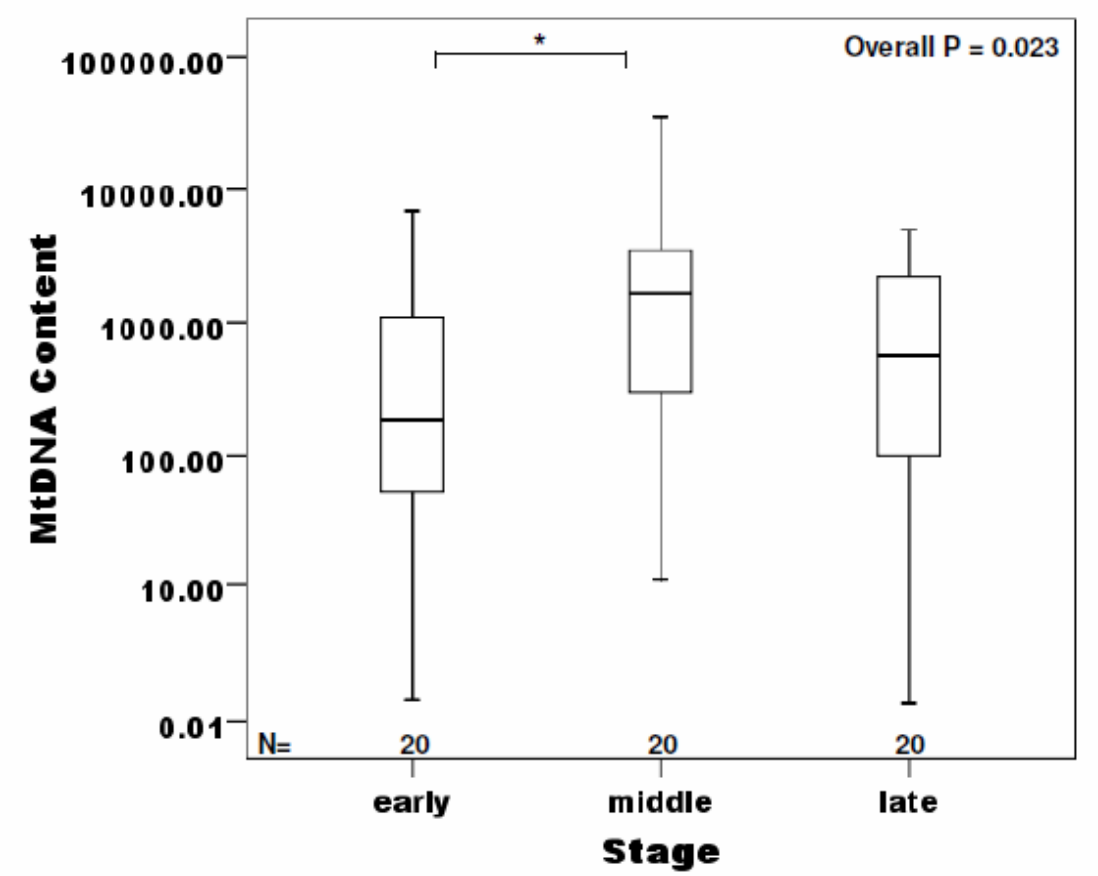


Figure 7. Box plot analysis illustrating levels of mtDNA in peripheral blood of normal group and breast cancer groups according the stage of carcinomas. The quantitative mtDNA content (as described in the text) is shown on the Y axis. The mtDNA content in peripheral blood of stage I breast cancers is significant lower than in the other stages as well as in normal control group according the One-way ANOVA on the ranks (*P = 0.018). Horizontal lines: group medians; boxes: boxes: 25–75% quartiles; boxes: 25–75% quartiles, range, peak and minimum.

3.4 MtDNA quantification in plasma of patients with breast cancer

The discovery of circulating cell-free (ccf) DNA in circulation has opened up the possibilities of non-invasive diagnosis and monitoring of a wide variety of malignant diseases. With the aim to simplify cancer management, cancer research lately dedicated itself more and more to discover and develop non-invasive biomarkers. In this connection, circulating cell-free (ccf) DNA seems to be a promising candidate. Altered levels of ccf nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have been found in several cancer types and might have a diagnostic value. Using multiplex real-time PCR we investigated the levels of ccf nDNA and mtDNA in plasma samples from patients with malignant and benign breast tumors, and from healthy controls. While the levels of ccf nDNA in the malignant disease group were significantly higher in comparison with the benign group and the healthy control group, lower level of ccf mtDNA was found to be elevated in the two tumour-groups. A cut-off value selected by ROC curve could allow distinguishing between the breast cancer cases and the healthy controls using ccf nDNA as marker and between the breast tumour group and the healthy controls using ccf mtDNA as marker. Our data suggest that both species might have a potential as biomarkers in breast tumor management. However, ccf nDNA seems to be the stronger biomarker regarding sensitivity and specificity (Kohler et al. 2009, submitted).

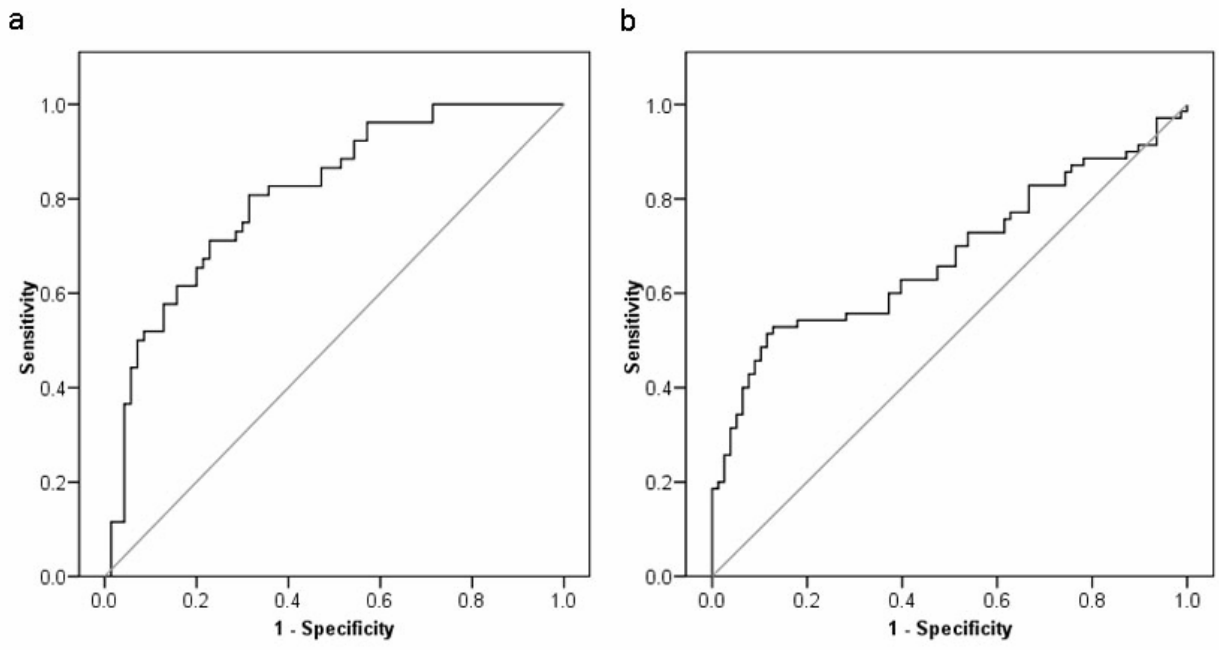


Figure 8. ROC curves using ccf nDNA and mtDNA for discriminating between the study-groups. A) ROC curve of ccf nDNA for discriminating between the cancer group and the healthy control group (sensitivity = 81 %; specificity = 69 %). B) ROC curve of ccf mtDNA for distinguishing between the tumor group and the healthy control group (sensitivity = 53 %; specificity = 87 %).

4. Qualitative analysis of mtDNA in breast cancer

4.1 Method setup for qualitative analysis

MtDNA is a circular cytoplasmic double stranded DNA of 16.6 kb, including the coding regions and the hypervariable non-coding D-loop region (Anderson et al. 1981, Fernandez-Silva et al. 2003). The features of mtDNA, such as matrilineal inheritance, high copy numbers per cell and lack of recombination make it a powerful tool in life science. Single nucleotide polymorphisms

(SNPs) of mitochondrial DNA (mtDNA) are involved in physiological and pathological conditions. Because of the importance of mtDNA SNPs in life science, a rapid, accurate, highly sensitive and high-throughput approach with low cost to identify physiologic and pathogenic mtDNA variants is needed for the analysis of large-scale samples, multiple SNPs or rare mtDNA. Hence we developed uniplex and multiplex assays for mtDNA SNP detections by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The detection limit achieved with the assays corresponded to the identification of five-genome equivalence of mtDNA per reaction after first round PCR amplification. The testing system enabled the discrimination of as little as 5% of mtDNA polymorphism in the predominating background of mtDNA not containing the SNP. No false positive and false negative results were obtained using the uniplex and multiplex MALDI-TOF MS assays for the analysis of the 18 SNPs compared with those obtained by sequencing analysis (Fan et al. 2007). Based on the successful uniplex assay, we developed a multiplex assay allowing analysis of 22 mtDNA SNPs in a single reaction, which as well demonstrated very high efficiency as expected (Fan et al, MS in preparation). This novel application enables the rapid, sensitive and accurate identification of single or multiple mtDNA SNP variants in a single reaction, which could serve as a powerful and sensitive tool for the studies including forensic medicine, tracing of matrilineage, transplantation immunology, transfusion medicine, the diagnosis of mtDNA mutation related disorders, and the research regarding aging, apoptosis and carcinogenesis based on physiologic and pathogenic alterations of mtDNA for the analysis of large-scale samples, multiple SNPs or rare mtDNA.

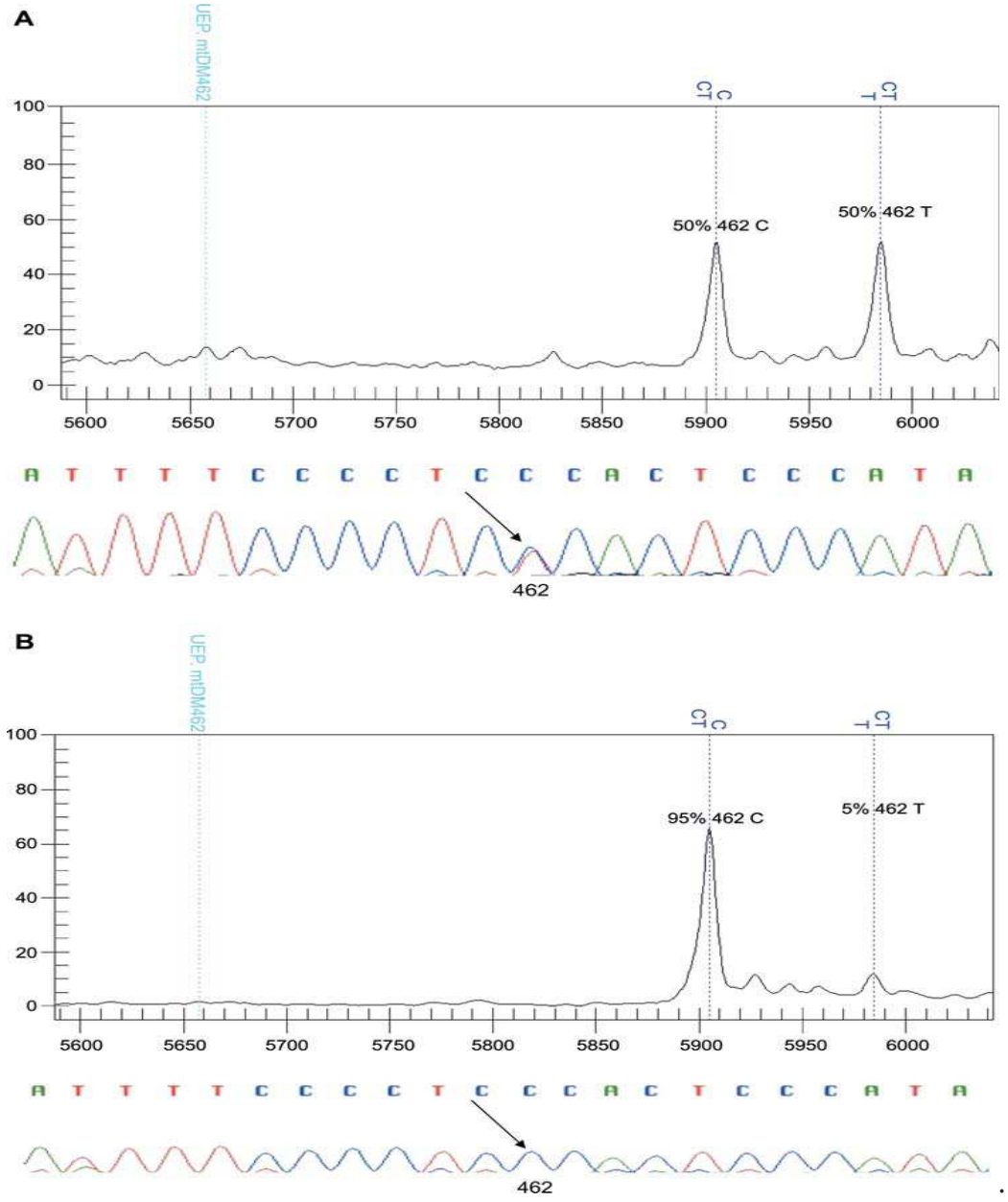


Figure 9. Comparison of MALDI-TOF and sequencing analysis for the sensitivity test. MtDNA sample containing the SNP of ‘‘T’’ at the position of 462 was mixed into the mtDNA sample containing a ‘‘C’’ at the same position. Panel (A) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 50%–50%. Panel (B) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 5%–95%.

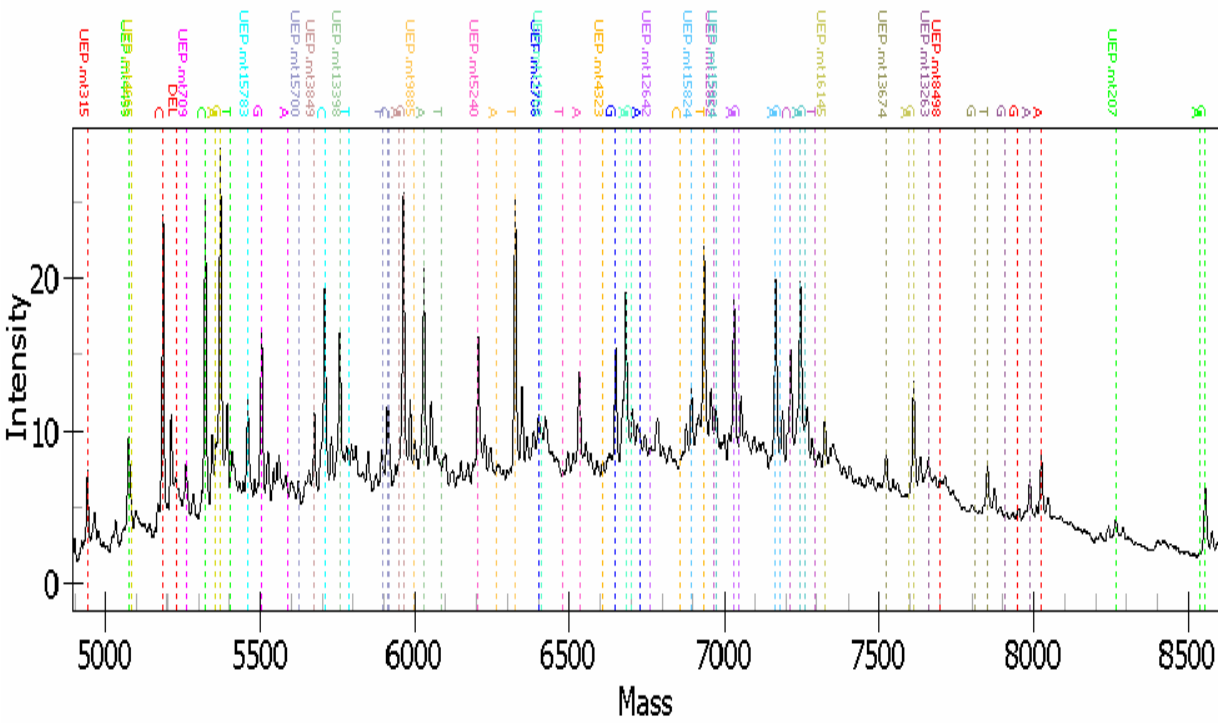


Figure 10. Mass spectra of mtDNA 22plex assay. Mass peaks of unextended extension primers and different alleles were labelled in different colours.

4.2 Method validation with HPA study by sequencing

Genotyping of single nucleotide polymorphisms (SNPs) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging technique, where finally tools for end users have become available to design primers and analyze SNPs of their own interest. This study investigated the potential of this technique in platelet (PLT) genotyping and developed a validated method for genotyping of clinical relevant human PLT antigens (HPAs). A multiplex assay using MALDI-TOF MS to analyze six HPA loci (HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15) simultaneously in a single reaction was applied for the genotyping of 100 DNA samples from a cohort of plateletpheresis donors and a patient population (n = 20) enriched for rare alleles. The genotyping results using MALDI-TOF MS

were validated by the comparison with the results from typing by polymerase chain reaction with sequence-specific primers and conventional DNA sequencing. Both homozygous and heterozygous genotypes of HPA-1 to -5 and -15 of the 120 individuals were easily identified by a six-plexed assay on MALDI-TOF MS. The three approaches achieved a 100 percent concordance for the genotyping results of the six HPA loci.Compared to conventional methods, the MALDI-TOF MS showed several advantages, such as a high velocity, the ability to perform multiplexed assays in a single reaction, and automated high-throughput analysis of samples. This enables cost-efficient large-scale PLT genotyping for clinical applications.

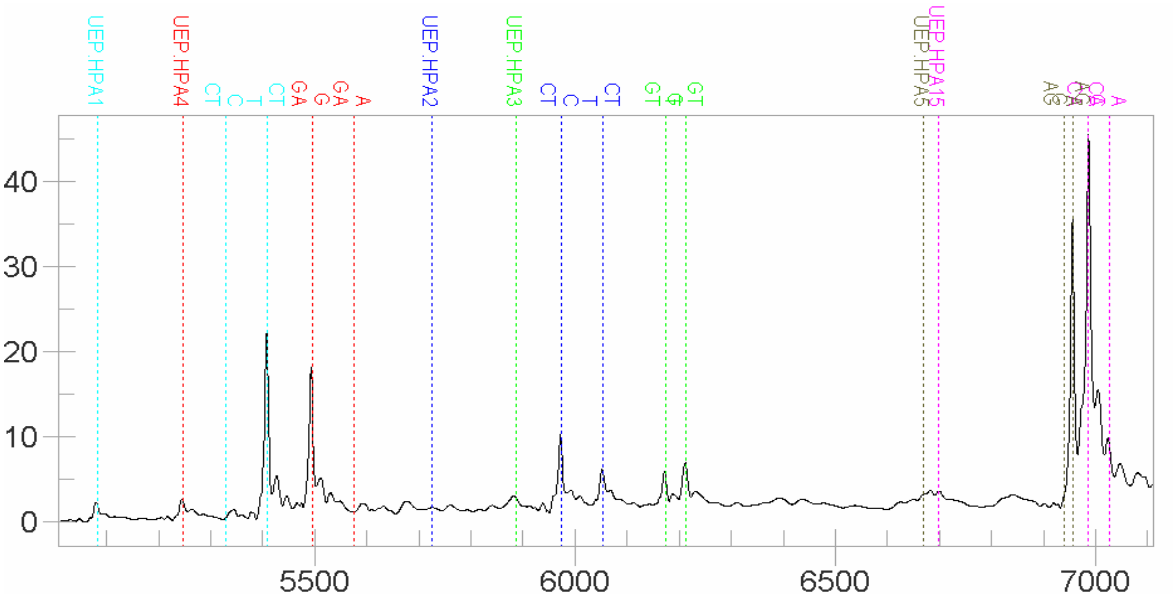


Figure 11. By using MALDI-TOF MS based MicroARRAY multi-plex assay, 100% concordance has been achieved for the genotyping of the 6 HPAs by the comparison with the results from typing by polymerase chain reaction with sequence-specific primers and conventional DNA sequencing.

4.3 MtDNA mutations in breast cancer

Mutations and up-/down-regulations of mitochondrial DNA (mtDNA) have been found in various cancers (Singh et al. 2007). The mtDNA alterations are suspected to be associated with carcinogenesis. However, the clinical relevance of the finding remains unknown and multiplexed assays for simultaneously detecting multiple mutations of mtDNA in a single reaction are currently not available. By using the microarray chip based multiplex assay using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), we detected 22 mtDNA single nucleotide variants simultaneously in 6 breast cancer cell lines and in the 102 cancerous and adjacent breast tissue samples from 51 patients with breast cancer. Totally, 164 mutations were identified in 66.67% of breast cancer cell lines and in 49.12% patients' tissue samples, in both the cancerous and adjacent parts. Most of the detected mtDNA variants in breast cancer samples were distributed in the regions of D-loop, 12S ribosomal RNA, 16S ribosomal RNA and tRNA. We also investigated the relationship between the quantitative and qualitative alterations of mtDNA in breast cancer. No correlation was found between the down-regulation and the mutations of mtDNA in breast cancer tissues. Similarly there was no association was found between the mutations and clinic-pathological parameters including tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors. Our study suggests that down-regulation and mutations of mtDNA in breast cancer tissues may be resulted by different mechanism. Both the qualitative and quantitative alterations of mtDNA in breast cancer may serve as biomarkers independent of clinic-pathological parameters for breast cancer on early screening, diagnosis, monitoring treatment effects, prognosis, and follow up. MALDI-TOF MS based MicroARRAY multiplex

assay is a high-throughput, and cost-efficient tool for large scale allelotyping of mtDNA mutations in life science.

Sample counting	Sample ID	characteristics	Nucleotide position (np)	Adjacent	Cancer (Cell line)
1	MDA-MB 231		709		A
2	MCF-7		2706		A
3	BT 549		2706		A
4	SKBR 3		2706		A
1	802	IDC	709	A	A
2	803	IDC	2706	A	A
3	804	ILC	16145	A	A
4	807	IDC	16145	AG	A
5	812	IDC	16145	AG	
	812	IDC	709		A
6	815	AMC	2706	A	A
7	817	IDC	16145	A	A
8	819	IDC	709	A	A
9	821	IDC	16145	A	A
10	822	IDC	311-315	C ins/-	C ins/-
11	823	IDC	709	A	A
	823	IDC	2706	A	A
12	824	ILC	709	A	
13	825	IDC	16145	A	A
14	827	IDC	15924	G	G
15	828	IDC	16145	AG	A
16	829	IDC	709	A	
17	830	IDC	15924		G
18	838	IDC	15924	G	G
19	840	IDC	709	A	A
20	841	IDC	311-315	C ins/-	C ins/-
21	843	IDC	2706	A	A
22	844	IDC	16145	AG	
	844	IDC	15924		G
23	846	IDC	16145	A	
24	851	IDC	15924	G	G
25	853	IDC	709	A	A

Table 4. mtDNA sequence variants were found in tissue samples. *Besides 822n/p and 841n/p paired samples only have one of C ins/- heteroplasmic mutations, each of the rest samples and cell lines have one mutation of C ins. (IDC = invasive ductal carcinoma, ILC=invasive lobular carcinoma, AMC=atypical medullary carcinoma).

5. Correlation Studies

5.1 Correlation study between quantitative and qualitative changes of mtDNA in tissues

Mitochondrial DNA alterations were long suspected to associate with carcinogenesis. Both alterations of mtDNA content and the mtDNA sequence have been detected in breast cancer, while it is clear yet whether the two alterations are correlated to each other. Using the MALDI-TOF MS based and multiplex MicroARRAY assay, 22 somatic mtDNA mutations were simultaneously detected in a single reaction. Six breast cancer cell lines and 102 cancerous and adjacent breast tissue samples from 51 patients with breast cancer were studied. Most of the detected mtDNA variants in breast cancer samples were distributed in the regions of D-loop, 12S ribosomal RNA, 16S ribosomal RNA and tRNA. In our previous study, we found down-regulation of mtDNA in the breast cancerous tissues compared to the adjacent tissues. In this study, we investigated the relationship between the quantitative and qualitative alterations of mtDNA in breast cancer. No correlation was found between the down-regulation and the mutations of mtDNA in breast cancer tissues. Also there was no association was found between the mutations and clinic-pathological parameters including tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors.

Group	Case (percentage)	mtDNA content	P value
709-	89 (87.25)	15.35 (1.17-69272.73)	0.098*
709+	13 (12.75)	56.69 (1.56-2730.60)	
2706-	94 (92.16)	15.19 (1.17-69272.73)	0.700*
2706+	8 (7.84)	39.98 (1.49-2730.60)	
15924-	94 (92.16)	15.19 (1.17-69272.73)	0.429*
15924+	8 (7.84)	63.60 (2.17-13587.57)	
16145-	86 (84.31)	21.05 (1.17-69272.73)	0.800*
16145+	16 (14.71)	13.27 (1.35-749.61)	
Var -	60 (58.82)	9.73 (1.17-69272.73)	0.039*
Var+	42 (41.18)	37.42 (1.35-13587.57)	
[1]N & Var -	28 (27.45)	60.38 (3.05-9877.98)	1vs2: 0.130*; 3vs4: 0.070*
[2]N & Var+	23 (22.55)	216.02 (1.35-3888.51)	1vs3: 0.000*; 2vs4: 0.013*
[3]C & Var -	32 (31.37)	3.89 (1.17-69272.73)	0.421**
[4]C & Var+	19 (18.63)	15.03 (1.49-13587.57)	

Table 5. Sample profile of mtDNA content and variant groups (excluding 311-315). N: normal; C: cancerous; Var: Sequence Variant; "-": without; "+": with. * Mann-Whitney U test in normal tissue; **Chi-square test was applied to analyze the association between tissue type and mutation.

5.2 Correlation study between mtDNA content in paired blood and in tissue samples

Using multiplex quantitative Real-Time PCR, we investigated the levels of nDNA and mtDNA in 10 paired plasma and tissue samples from patients with breast carcinomas. The median delta Ct (ΔCt) and median mtDNA content for plasma and cancerous tissues were 5.79 and 5.03, as well as 374280.44 and 13918579.50 (P = 0.005). While the mtDNA contents in the tissue are correlated with those in plasma (Spearman's rank correlation coefficient $\rho = 0.648$, $P = 0.043$)

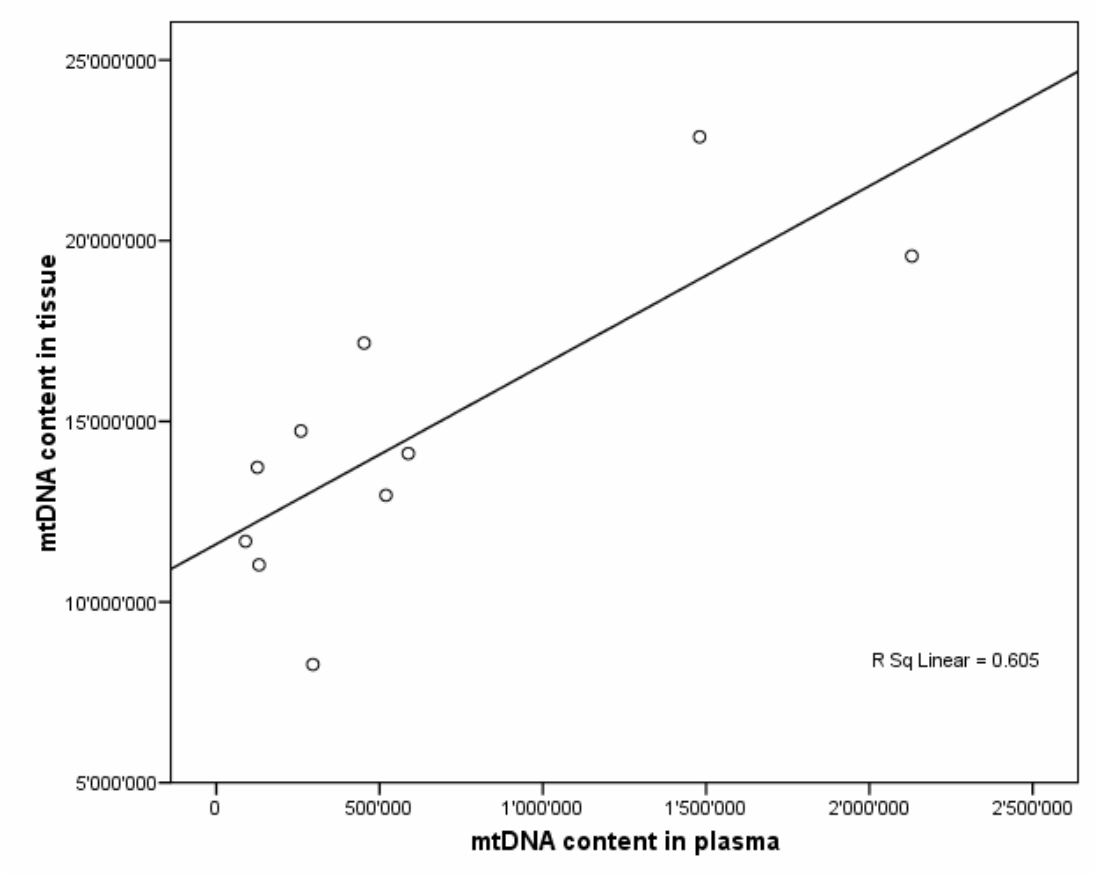


Figure 12. MtDNA contents in tissue are correlated with those in plasma (ρ=0.648, P=0.043).

5.3 Correlation study between mtDNA content in paired blood and in tissue samples

Most mtDNA polymorphisms have been identified in the displacement loop (D-loop) region harbouring the major promoters responsible for replication and transcription. Likewise in one previous study we found mtDNA polymorphisms at the region around D-loop in paired breast cancer tissue samples. It is not know yet whether different mtDNA variants found in the breast cancer tissue can also be found in the correspondent plasma. This study is to be targeted on the identification of mitochondrial DNA (mtDNA) polymorphisms in three hyperviable region in

breast cancer patients and the investigation on the role of mtDNA mutation in tumorigenesis. Paired plasma and tissue samples from 10 unrelated patients with breast cancer were analyzed for mtDNA nucleotide variations in three hyper variable regions of mtDNA, by the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method using 4 pairs of primers followed by direct DNA sequencing. 221 homoplasmic variants but no any heteroplasmic ones were detected at 65 nucleotide positions (np) in 10 paired samples. Each of these 20 samples displayed at least 6 mtDNA allelic variants. Of all the variants, 70 variants were detected in plasma samples but not in paired tissue samples. Reversely, 49 variants were identified in tissues samples but not in paired plasma samples (Table 6).

It has been proposed the plasma mtDNA, or cell-free mtDNA, could be use in tracking the tumor derived mtDNA shedding from tumour tissues into peripheral blood. The divergence between mtDNA mutations present in plasma and those in tissue could be caused by tumor, and might serve as a specific biomarker for tumor diagnosis, prognosis and follow-up. All the mutations detected in this study are homoplasmic, and no any heteroplasmic was present, which indicated no tumor metastasis occurred. The heteroplasmic condition was not detected in plasma might be due to the mutant allele present in a very low frequency compared to the wild type allele. Our method is not sensitive enough to detect lower than 2% mutant DNA from wild type background. The development of novel techniques, for example digital PCR and BEAMing-up method, might conquer this difficulty, but it is either too expensive, or too complicated, or both to expand it to routine application.

Sample ID	Nucleotide position	Reference sequence	Plasma	Tissue
701	16290	C	T	C
	16295	C	C	T
	16319	G	A	G
	146	T	T	C
	235	A	G	A
	303-309	6C	6C	7C
	308	C	T	C
	310	T	DEL	T
	489	T	T	C
	514-523	5CA	6CA	4CA
702	16051	A	G	A
	16129	G	G	A
	16189	T	C	T
	16257	C	C	T
	16261	C	C	T
	16362	T	C	T
	152	T	C	T
	235	A	G	A
	456	C	T	C
	489	T	C	C
703	16126	T	T	C
	16234	C	C	T
	16290	C	C	T
	16304	T	C	T
	16319	G	G	A
	189	A	A	G
	198	C	T	C
	235	A	A	G
	249	A	DEL	A
	303-309	7C	8C	9C
704	16126	T	C	T
	16163	A	G	A
	16186	C	T	C
	16189	T	C	T
	16924	C	T	C
	16304	T	T	C
	152	T	C	T
	195	T	C	T
	207	G	G	A
	248	A	A	DEL
	303-309	6C	7C	6C
705	16051	A	A	G
	16092	T	T	C

	16129	G	A	G
	16140	T	C	T
	16145	G	G	A
	16166	A	G	A
	16189	T	C	T
	16217	T	C	T
	16245	C	C	T
	16257	C	C	T
	16274	G	A	G
	16261	C	C	T
	16293	A	G	A
	16335	A	G	A
	195	T	C	T
706	16189	T	T	C
	16234	C	T	C
	16274	G	G	A
	146	T	C	T
	195	T	C	T
	217	T	C	T
707	16129	G	G	A
	16145	G	G	A
	16162	A	A	G
	16172	T	T	C
	16189	T	C	T
	16223	C	T	C
	16266	C	T	C
	16304	T	T	C
	16362	T	C	T
	146	T	C	T
	150	C	T	T
	248	A	DEL	A
	489	T	C	T
	548	C	C	T
708	16093	T	C	T
	16129	G	G	A
	16183	A	DEL	A
	16189	T	DEL	T
	16217	T	C	T
	16218	C	T	C
	16223	C	C	T
	16325	T	T	C
	16362	T	T	C
	56	A	DEL	A
	58	T	A	T
	72 INS	-	G	-
	94	G	A	G

	303-309	7C	9C	7C
709	16140	T	T	C
	16182	A	DEL	A
	16183	A	DEL	A
	16189	T	C	T
	16217	T	C	T
	16223	C	C	T
	16261	C	T	C
	16299	A	G	A
	16311	T	C	T
	16362	T	T	C
	193	A	G	A
	298	C	C	T
	303-309	7C	9C	7C
	489	T	T	C
	514-523	5CA	4CA	5CA
710	16129	G	A	G
	16140	T	T	C
	16162	A	G	A
	16172	T	C	T
	16182	A	A	DEL
	16183	A	A	DEL
	16189	T	T	C
	16217	T	T	C
	16274	G	G	A
	146	T	T	C
	150	C	C	T
	249	A	DEL	A
	514-523	5CA	4CA	5CA
	548	C	T	C

Table 6. MtDNA mutations found in 10 paired plasma and tissue samples.

6. Prospect

For the future plan regarding the research interest on mtDNA, the following issues need to be clarified:

- The path-physiology and biology of mtDNA changes in cancer through detecting more clinical cases, investigating cell lines and/or establishing animal models
- Whether the mtDNA changes in blood can be used for developing blood based test in clinical application in the aim of screening, earlier diagnosis, monitoring of patients with cancer
- Whether the mtDNA changes in tissues can be used as classification markers for treatment selection
- Whether the mtDNA changes in both blood and tissues can be used as clinical response markers and predictive markers for monitoring and following up of cancer patients.
- The predictive and prognostic value of tumour derived mtDNA in peripheral blood should be evaluated. The dynamic process of the tumour derived molecules in circulation need to be investigated before, during and after clinical interventions such as chemotherapy.

7. Publication and manuscript list

1) **Fan AX**, Garritsen HSP, Tarhouny S, Morris M, Hahn S, Holzgreve W, Zhong XY. A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med. 2008; 46(3):299-305.
2) Seefeld M, Tarhouny S, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Parallel assessment of circulatory cell-free DNA and nucleosomes in patients with benign and malignant breast tumours. Int J Biol Markers. 2008 Apr-Jun;23(2):69-73.
3) Zanetti-Dällenbach RA, Wight E, **Fan AX**, Lapaire O, Holzgreve H, Zhong XY. Positive Correlation of cell-free DNA in plasma/serum in patients with malignant and benign breast disease. Anticancer Res. 2008 Mar-Apr;28(2A):921-5.
4) Tarhouny S, Seefeld M, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Comparison of Serum VEGF and its Soluble Receptor sVEGFR1 with Serum Cell Free DNA in Patient with Breast Tumour. 2008 Cytokine.
5) Xia P, Radpour R, Zachariah R, **Fan AX**, Kohler C, Hahn S, Holzgreve W, ZHong XY. Simultaneous Quantitative Assessment of Circulating Cell-Free Mitochondrial and Nuclear DNA by Multiplex Real-Time PCR. Genetics and Molecular Biology, 2009, 32, 1, 20-24.
6) Radpour R, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Current Understanding of Mitochondrial DNA in Breast Cancer. The Breast Journal, review (accepted).
7) **Fan AX**, Garritsen HSP, Hannig H, Holzgreve W, Zhong XY. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for Genotyping Human Platelet Specific Antigens (HPAs) Transfusion. 2008 Oct 29. [Epub ahead of print]

8) Zachariah R., Schmid S, Nicole Buerki, Radpour R, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Is Circulating Cell Free DNA a Potential Biomarker for Developing Non-invasive Diagnostic Test in Minimal and Mild Endometriosis? Reprod Biomed Online. 2009 Mar;18(3):407-11.
9) Radpour R, Haghighi MM, **Fan AX**, Torbati PM, Hahn S, Holzgreve W, Zhong XY. High-throughput hacking the methylation patterns in breast cancer by in vitro transcription and thymidine-specific cleavage mass array on MALDI-TOF silico-chip. Mol Cancer Res. 2008 Nov; 6(11):1702-9.
10) Xia P, Radpour R, Kohler C, Dang CX, **Fan AX**, Holzgreve W, Zhong XY. A selected pre-amplification strategy for genetic analysis using limited DNA targets. Clin Chem Lab Med. 2009 Feb 4. [Epub ahead of print]
11) Radpour R, Haghighi MM, **Fan AX**, Kohler C, Holzgreve W, Zhong XY. Methylation profile in breast cancer by MALDI-TOF Mass Array (revised version submmited).
12) **Fan AX**, Radpour R, Hahn S, Holzgreve W, Zhong XY. Mitochondrial DNA Content in Paired Adjacent Normal and Cancerous Breast Tissue Samples from Patients with Breast Cancer. J Cancer Res Clin Oncol. 2009 Jan 6. [Epub ahead of print]

Search PubMed for “Fan AX OR Alex Xiu-cheng Fan”.

Part III References

Aleisa, M., Zeitouni, A.G. and Cullen, K.E. (2007) Vestibular compensation after unilateral labyrinthectomy: normal versus cerebellar dysfunctional mice. J Otolaryngol, 36, 315-21.

Allalunis-Turner, J., Ma, I., Hanson, J. and Pearcey, R.G. (2006) mtDNA mutations in invasive cervix tumors: a retrospective analysis. Cancer letters, 243, 193-201.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J., Staden, R. and Young, I.G. (1981) Sequence and organization of the human mitochondrial genome. Nature, 290, 457-65.

Andreyev, A.Y., Kushnareva, Y.E. and Starkov, A.A. (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry Biokhimii a, 70, 200-14.

Armstrong, J.S. (2007) Mitochondrial medicine: pharmacological targeting of mitochondria in disease. British journal of pharmacology, 151, 1154-65.

Bacolla, A., Collins, J.R., Gold, B., Chuzhanova, N., Yi, M., Stephens, R.M., Stefanov, S., Olsh, A., Jakupciak, J.P., Dean, M., Lempicki, R.A., Cooper, D.N. and Wells, R.D. (2006) Long homopurine*homopyrimidine sequences are characteristic of genes expressed in brain and the pseudoautosomal region. Nucleic acids research, 34, 2663-75.

Baranowski, T., Missaghian, M., Watson, K., Broadfoot, A., Cullen, K., Nicklas, T., Fisher, J. and O'Donnell, S. (2008) Home fruit, juice, and vegetable pantry management and availability scales: a validation. Appetite, 50, 266-77.

Baranowski, T., Watson, K., Missaghian, M., Broadfoot, A., Baranowski, J., Cullen, K., Nicklas, T., Fisher, J. and O'Donnell, S. (2007) Parent outcome expectancies for purchasing fruit and vegetables: a validation. Public Health Nutr, 10, 280-91.

Bass, B.P., Cullen, K. and McCall, K. (2007) The axon guidance gene lola is required for programmed cell death in the Drosophila ovary. Dev Biol, 304, 771-85.

Beraneck, M. and Cullen, K.E. (2007) Activity of vestibular nuclei neurons during vestibular and optokinetic stimulation in the alert mouse. J Neurophysiol, 98, 1549-65.

Bianchi, M.S., Bianchi, N.O. and Bailliet, G. (1995) Mitochondrial DNA mutations in normal and tumor tissues from breast cancer patients. Cytogenetics and cell genetics, 71, 99-103.

Boddapati, S.V., Tongcharoensirikul, P., Hanson, R.N., D'Souza, G.G.M., Torchilin, V.P. and Weissig, V. (2005) Mitochondriotropic liposomes. Journal of liposome research, 15, 49-58.

Boultwood, J., Fidler, C., Mills, K.I., Frodsham, P.M., Kusec, R., Gaiger, A., Gale, R.E., Linch, D.C., Littlewood, T.J., Moss, P.A. and Wainscoat, J.S. (1996) Amplification of mitochondrial DNA in acute myeloid leukaemia. British journal of haematology, 95, 426-31.

Bragoszewski, P., Kupryjanczyk, J., Bartnik, E., Rachinger, A. and Ostrowski, J. (2008) Limited clinical relevance of mitochondrial DNA mutation and gene expression analyses in ovarian cancer. BMC cancer, 8, 292.

Brandon, M., Baldi, P. and Wallace, D.C. (2006) Mitochondrial mutations in cancer. Oncogene, 25, 4647-62.

Campbell, N.A., Williamson B, Heyden B.J. (2006). Biology: Exploring Life. Boston, Massachusetts: Pearson Prentice Hall.

Canter, J.A., Kallianpur, A.R., Parl, F.F. and Millikan, R.C. (2005) Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. Cancer research, 65, 8028-33.

Capuano, F., Varone, D., D'Eri, N., Russo, E., Tommasi, S., Montemurro, S., Prete, F. and Papa, S. (1996) Oxidative phosphorylation and F(O)F(1) ATP synthase activity of human hepatocellular carcinoma. Biochem Mol Biol Int, 38, 1013-22.

Carew, J.S. and Huang, P. (2002) Mitochondrial defects in cancer. Mol Cancer, 1, 9-20.

Carew, J.S., Nawrocki, S.T., Xu, R.H., Dunner, K., McConkey, D.J., Wierda, W.G., Keating, M.J. and Huang, P. (2004) Increased mitochondrial biogenesis in primary leukemia cells: the role of endogenous nitric oxide and impact on sensitivity to fludarabine. Leukemia, 18, 1934-40.

Casellas, P., Galiegue, S. and Basile, A.S. (2002) Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int, 40, 475-86.

Cavalli, L.R. and Liang, B.C. (1998) Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? Mutat Res, 398, 19-26.

Chatterjee, D.K., Fong, L.S. and Zhang, Y. (2008) Nanoparticles in photodynamic therapy: an emerging paradigm. Adv Drug Deliv Rev, 60, 1627-37.

Chelli, B., Lena, A., Vanacore, R., Da Pozzo, E., Costa, B., Rossi, L., Salvetti, A., Scatena, F., Ceruti, S., Abbracchio, M.P., Gremigni, V. and Martini, C. (2004) Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. Biochem Pharmacol, 68, 125-34.

Chelli, B., Rossi, L., Da Pozzo, E., Costa, B., Spinetti, F., Rechichi, M., Salvetti, A., Lena, A., Simorini, F., Vanacore, R., Scatena, F., Da Settimo, F., Gremigni, V. and Martini, C. (2005) PIGA (N,N-Di-n-butyl-5-chloro-2-(4-chlorophenyl)indol-3-ylglyoxylamide), a new mitochondrial benzodiazepine-receptor ligand, induces apoptosis in C6 glioma cells. Chembiochem, 6, 1082-8.

Clayton, D.A. (1991) Replication and transcription of vertebrate mitochondrial DNA. Annu Rev Cell Biol, 7, 453-78.

Copeland, W.C., Wachsman, J.T., Johnson, F.M. and Penta, J.S. (2002) Mitochondrial DNA alterations in cancer. Cancer Invest, 20, 557-69.

Corrado, G., Palmisano, P., Cavaliere, M., Capuano, M., Frandina, G. and Antonelli, M. (1995) Cystic fibrosis: genetics and clinical applications. Rivista europea per le scienze mediche e farmacologiche = European review for medical and pharmacological sciences = Revue europeenne pour les sciences medicales et pharmacologiques, 17, 67-76.

Corral, M., Baffet, G., Kitzis, A., Paris, B., Tichonicky, L., Kruh, J., Guguen-Guillouzo, C. and Defer, N. (1989) DNA sequences homologous to mitochondrial genes in nuclei from normal rat tissues and from rat hepatoma cells. Biochem Biophys Res Commun, 162, 258-64.

Cullen, K.J., Yang, Z., Schumaker, L. and Guo, Z. (2007) Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer. J Bioenerg Biomembr, 39, 43-50.

Cuomo, R., Pumpo, R., Sarnelli, G., Capuano, G. and Budillon, G. (1995) Nicotinamide methylation and hepatic energy reserve: a study by liver perfusion in vitro. Journal of hepatology, 23, 465-70.

Daley, E., Wilkie, D., Loesch, A., Hargreaves, I.P., Kendall, D.A., Pilkington, G.J. and Bates, T.E. (2005) Chlorimipramine: a novel anticancer agent with a mitochondrial target. Biochem Biophys Res Commun, 328, 623-32.

Dani, M.A.; Dani, S.U.; Lima, S.P.; Martinez, A.; Rossi, B.M.; Soares, F.; Zago, M.A.; Simpson, A.J. (2004) Less Delta mtDNA 4977 than normal in various types of tumors suggests that cancer cells are essentially free of this mutation. Genet. Mol. Res., 3, 395-409.

Dastidar, S.G. and Sharma, S.K. (1989) Activities of glycolytic enzymes in rapidly proliferating and differentiated C6 glioma cells. Experimental cell biology, 57, 159-64.

Decaudin, D. (2004) Peripheral benzodiazepine receptor and its clinical targeting. Anti-cancer drugs, 15, 737-45.

Desouki, M.M., Kulawiec, M., Bansal, S., Das, G.M. and Singh, K.K. (2005) Cross Talk Between Mitochondria and Superoxide Generating NADPH Oxidase in Breast and Ovarian Tumors. Cancer Biol Ther, 4, 1367-73.

DiMauro, S. and Schon, E.A. (2001) Mitochondrial DNA mutations in human disease. American journal of medical genetics, 106, 18-26.

D'Souza, G.G.M., Boddapati, S.V. and Weissig, V. (2005) Mitochondrial leader sequence--plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. Mitochondrion, 5, 352-8.

Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G. and Gustafsson, C.M. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat Genet, 31, 289-94.

Fernandez-Silva, P., Enriquez, J.A. and Montoya, J. (2003) Replication and transcription of mammalian mitochondrial DNA. Exp Physiol, 88, 41-56.

Filosto, M., Mancuso, M., Vives-Bauza, C., Vila, M.R., Shanske, S., Hirano, M., Andreu, A.L. and DiMauro, S. (2003) Lack of paternal inheritance of muscle mitochondrial DNA in sporadic mitochondrial myopathies. Ann Neurol, 54, 524-6.

Fliss, M.S., Usadel, H., Caballero, O.L., Wu, L., Buta, M.R., Eleff, S.M., Jen, J. and Sidransky, D. (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science (New York, N Y), 287, 2017-9.

Flury, F., von Borstel, R.C. and Williamson, D.H. (1976) Mutator activity of petite strains of Saccharomyces cerevisiae. Genetics, 83, 645-53.

Folkman, J. (2001) Can mosaic tumor vessels facilitate molecular diagnosis of cancer? Proceedings of the National Academy of Sciences of the United States of America, 98, 398-400.
Futuyma, D.J. (2005). On Darwin's Shoulders. Natural History 114 (9): 64–68.

Galiegue, S., Tinel, N. and Casellas, P. (2003) The peripheral benzodiazepine receptor: a promising therapeutic drug target. Current medicinal chemistry, 10, 1563-72.

Gaspari, M., Falkenberg, M., Larsson, N.G. and Gustafsson, C.M. (2004) The mitochondrial RNA polymerase contributes critically to promoter specificity in mammalian cells. EMBO J, 23, 4606-14.

Gauldi, G.F., Ceroni, A.M., Burrai, L., Capuano, R. and Polettini, E. (1995) [Radiologic evaluation of parietal infiltration of bladder cancer (integrated imaging: US, TC, RM) and comparison with transurethral resection (TUR)]. La Clinica terapeutica, 146, 691-711.

Gille, J.J. and Joenje, H. (1992) Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. Mutation research, 275, 405-14.
Glaichenhaus, N., Leopold, P. and Cuzin, F. (1986) Increased levels of mitochondrial gene expression in rat fibroblast cells immortalized or transformed by viral and cellular oncogenes. The EMBO journal, 5, 1261-5.

Hajnoczky, G., Csordas, G., Das, S., Garcia-Perez, C., Saotome, M., Sinha Roy, S. and Yi, M. (2006) Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium, 40, 553-60.

Harbottle, A. and Birch-Machin, M.A. (2006) Real-time PCR analysis of a 3895 bp mitochondrial DNA deletion in nonmelanoma skin cancer and its use as a quantitative marker for sunlight exposure in human skin. British journal of cancer, 94, 1887-93.

Heath, H., Odelberg, S., Jackson, C.E., Teh, B.T., Hayward, N., Larsson, C., Buist, N.R., Krapcho, K.J., Hung, B.C., Capuano, I.V., Garrett, J.E. and Leppert, M.F. (1996) Clustered inactivating mutations and benign polymorphisms of the calcium receptor gene in familial benign hypocalciuric hypercalcemia suggest receptor functional domains. The Journal of clinical endocrinology and metabolism, 81, 1312-7.

Heddi, A., Faure-Vigny, H., Wallace, D.C. and Stepien, G. (1996) Coordinate expression of nuclear and mitochondrial genes involved in energy production in carcinoma and oncocytoma. Biochimica et biophysica acta, 1316, 203-9.

Hibi, K., Nakayama, H., Yamazaki, T., Takase, T., Taguchi, M., Kasai, Y., Ito, K., Akiyama, S. and Nakao, A. (2001) Mitochondrial DNA alteration in esophageal cancer. International journal of cancer Journal international du cancer, 92, 319-21.

Huang, P., Feng, L., Oldham, E.A., Keating, M.J. and Plunkett, W. (2000) Superoxide dismutase as a target for the selective killing of cancer cells. Nature, 407, 390-5.

Jakupciak, J.P., Dakubo, G.D., Maragh, S. and Parr, R.L. (2006) Analysis of potential cancer biomarkers in mitochondrial DNA. Current opinion in molecular therapeutics, 8, 500-6.

Jakupciak, J.P., Wang, W., Markowitz, M.E., Ally, D., Coble, M., Srivastava, S., Maitra, A., Barker, P.E., Sidransky, D. and O'Connell, C.D. (2005) Mitochondrial DNA as a cancer biomarker. The Journal of molecular diagnostics : JMD, 7, 258-67.

Jiang, W.-W., Masayesva, B., Zahurak, M., Carvalho, A.L., Rosenbaum, E., Mambo, E., Zhou, S., Minhas, K., Benoit, N., Westra, W.H., Alberg, A., Sidransky, D., Koch, W. and Califano, J. (2005) Increased mitochondrial DNA content in saliva associated with head and neck cancer. Clinical cancer research : an official journal of the American Association for Cancer Research, 11, 2486-91.

Jin, X., Zhang, J., Gao, Y., Ding, K., Wang, N., Zhou, D., Jen, J. and Cheng, S. (2007) Relationship between mitochondrial DNA mutations and clinical characteristics in human lung cancer. Mitochondrion, 7, 347-53.

Kim, M.M., Clinger, J.D., Masayesva, B.G., Ha, P.K., Zahurak, M.L., Westra, W.H. and Califano, J.A. (2004) Mitochondrial DNA quantity increases with histopathologic grade in premalignant and malignant head and neck lesions. Clinical cancer research : an official journal of the American Association for Cancer Research, 10, 8512-5.

Kose, K., Hiyama, T., Tanaka, S., Yoshihara, M., Yasui, W. and Chayama, K. (2005) Somatic mutations of mitochondrial DNA in digestive tract cancers. Journal of gastroenterology and hepatology, 20, 1679-84.

Koya, K., Li, Y., Wang, H., Ukai, T., Tatsuta, N., Kawakami, M., Shishido and Chen, L.B. (1996) MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer research, 56, 538-43.

Kulawiec, M., Arnouk, H., Desouki, M.M., Kazim, L., Still, I. and Singh, K.K. (2006) Proteomic analysis of mitochondria-to-nucleus retrograde response in human cancer. Cancer biology & therapy, 5, 967-75.

Kulawiec, M., Ayyasamy, V. and Singh, K.K. (2009) p53 regulates mtDNA copy number and mitocheckpoint pathway. Journal of carcinogenesis, 8, 8.

Lambeth, J.D. (2004) NOX enzymes and the biology of reactive oxygen. Nature reviews Immunology, 4, 181-9.

Lang, B.F., Gray, M.W. and Burger, G. (1999) Mitochondrial genome evolution and the origin of eukaryotes. Annu Rev Genet, 33, 351-97.

Lee, H.C.; Yin, P.H.; Yu, T.N.; Chang, Y.D.; Hsu, W.C.; Kao, S.Y.; Chi, C.W.; Liu, T.Y.; Wei, Y.H. (2001) Accumulation of mitochondrial DNA deletions in human oral tissues − effects of betel quid chewing and oral cancer. Mutat. Res., 493, 67-74.

Lee, H.-C. and Wei, Y.-H. (2005) Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. The international journal of biochemistry & cell biology, 37, 822-34.

Lee, H.-C., Yin, P.-H., Lin, J.-C., Wu, C.-C., Chen, C.-Y., Wu, C.-W., Chi, C.-W., Tam, T.-N. and Wei, Y.-H. (2005) Mitochondrial genome instability and mtDNA depletion in human cancers. Annals of the New York Academy of Sciences, 1042, 109-22.

Lee, H.-C., Yin, P.-H., Lin, J.-C., Wu, C.-C., Chen, C.-Y., Wu, C.-W., Chi, C.-W., Tam, T.-N. and Wei, Y.-H. (2005) Mitochondrial genome instability and mtDNA depletion in human cancers. Annals of the New York Academy of Sciences, 1042, 109-22.

Levkovitz, Y., Gil-Ad, I., Zeldich, E., Dayag, M. and Weizman, A. (2005) Differential induction of apoptosis by antidepressants in glioma and neuroblastoma cell lines: evidence for p-c-Jun, cytochrome c, and caspase-3 involvement. Journal of molecular neuroscience : MN, 27, 29-42.
Liang, B.C. (1996) Evidence for association of mitochondrial DNA sequence amplification and nuclear localization in human low-grade gliomas. Mutation research, 354, 27-33.

Liang, X., Le, W., Zhang, D. and Schulz, H. (2001) Impact of the intramitochondrial enzyme organization on fatty acid oxidation. Biochem Soc Trans, 29, 279-82.

Lievre, A., Blons, H., Houllier, A.M., Laccourreye, O., Brasnu, D., Beaune, P. and Laurent-Puig, P. (2006) Clinicopathological significance of mitochondrial D-Loop mutations in head and neck carcinoma. British journal of cancer, 94, 692-7.

Lievre, A., Chapusot, C., Bouvier, A.-M., Zinzindohoue, F., Piard, F., Roignot, P., Arnould, L., Beaune, P., Faivre, J. and Laurent-Puig, P. (2005) Clinical value of mitochondrial mutations in colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 23, 3517-25.

Liguori, L., Marques, B., Villegas-Mendez, A., Rothe, R. and Lenormand, J.-L. (2008) Liposomes-mediated delivery of pro-apoptotic therapeutic membrane proteins. Journal of controlled release : official journal of the Controlled Release Society, 126, 217-27.

Lin, P.-C., Lin, J.-K., Yang, S.-H., Wang, H.-S., Li, A.F.-Y. and Chang, S.-C. (2008) Expression of beta-F1-ATPase and mitochondrial transcription factor A and the change in mitochondrial DNA content in colorectal cancer: clinical data analysis and evidence from an in vitro study. International journal of colorectal disease, 23, 1223-32.

Liu, D., Mambo, E., Ladenson, P.W. and Xing, M. (2005) Letter re: uncommon mutation but common amplifications of the PIK3CA gene in thyroid tumors. The Journal of clinical endocrinology and metabolism, 90, 5509.

Liu, V.W., Shi, H.H., Cheung, A.N., Chiu, P.M., Leung, T.W., Nagley, P., Wong, L.C. and Ngan, H.Y. (2001) High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas. Cancer research, 61, 5998-6001.

Lo, S., Tolner, B., Taanman, J.-W., Cooper, J.M., Gu, M., Hartley, J.A., Schapira, A.H.V. and Hochhauser, D. (2005) Assessment of the significance of mitochondrial DNA damage by chemotherapeutic agents. International journal of oncology, 27, 337-44.

Losanoff, J.E., Zhu, W., Qin, W., Mannello, F. and Sauter, E.R. (2008) Can mitochondrial DNA mutations in circulating white blood cells and serum be used to detect breast cancer? Breast (Edinburgh, Scotland), 17, 540-2.

Lowry, O.H., Berger, S.J., Carter, J.G., Chi, M.M., Manchester, J.K., Knor, J. and Pusateri, M.E. (1983) Diversity of metabolic patterns in human brain tumors: enzymes of energy metabolism and related metabolites and cofactors. Journal of neurochemistry, 41, 994-1010.

Maitra, A., Cohen, Y., Gillespie, S.E.D., Mambo, E., Fukushima, N., Hoque, M.O., Shah, N., Goggins, M., Califano, J., Sidransky, D. and Chakravarti, A. (2004) The Human MitoChip: a high-throughput sequencing microarray for mitochondrial mutation detection. Genome research, 14, 812-9.

Mambo, E., Chatterjee, A., Xing, M., Tallini, G., Haugen, B.R., Yeung, S.-C.J., Sukumar, S. and Sidransky, D. (2005) Tumor-specific changes in mtDNA content in human cancer. International journal of cancer Journal international du cancer, 116, 920-4.

Matsuyama, W., Nakagawa, M., Wakimoto, J., Hirotsu, Y., Kawabata, M. and Osame, M. (2003) Mitochondrial DNA mutation correlates with stage progression and prognosis in non-small cell lung cancer. Human mutation, 21, 441-3.

Maximo, V., Soares, P., Lima, J., Cameselle-Teijeiro, J. and Sobrinho-Simoes, M. (2002) Mitochondrial DNA somatic mutations (point mutations and large deletions) and mitochondrial DNA variants in human thyroid pathology: a study with emphasis on Hurthle cell tumors. The American journal of pathology, 160, 1857-65.

Mayr, J.A., Meierhofer, D., Zimmermann, F., Feichtinger, R., Kogler, C., Ratschek, M., Schmeller, N., Sperl, W. and Kofler, B. (2008) Loss of complex I due to mitochondrial DNA mutations in renal oncocytoma. Clinical cancer research : an official journal of the American Association for Cancer Research, 14, 2270-5.

McBride, H.M., Neuspiel, M. and Wasiak, S. (2006) Mitochondria: more than just a powerhouse. Current biology : CB, 16, R551-60.

McFarland, R., Elson, J.L., Taylor, R.W., Howell, N. and Turnbull, D.M. (2004) Assigning pathogenicity to mitochondrial tRNA mutations: when "definitely maybe" is not good enough. Trends in genetics : TIG, 20, 591-6.

Melov, S., Coskun, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A.S., Zastawny, T.H., Dizdaroglu, M., Goodman, S.I., Huang, T.T., Miziorko, H., Epstein, C.J. and Wallace, D.C. (1999) Mitochondrial disease in superoxide dismutase 2 mutant mice. Proceedings of the National Academy of Sciences of the United States of America, 96, 846-51.

Memoli, B., Libetta, C., De Nicola, L., Rampino, T., Capuano, A., Guida, B. and Andreucci, V.E. (1996) Hemodialysis related interleukin-2 receptor release by peripheral blood mononuclear cells. ASAIO journal (American Society for Artificial Internal Organs : 1992), 42, 60-3.

Miranda, S., Foncea, R., Guerrero, J. and Leighton, F. (1999) Oxidative stress and upregulation of mitochondrial biogenesis genes in mitochondrial DNA-depleted HeLa cells. Biochemical and biophysical research communications, 258, 44-9.

Modica-Napolitano, J.S., Nalbandian, R., Kidd, M.E., Nalbandian, A. and Nguyen, C.C. (2003) The selective in vitro cytotoxicity of carcinoma cells by AZT is enhanced by concurrent treatment with delocalized lipophilic cations. Cancer letters, 198, 59-68.

Modica-Napolitano, J.S. and Singh, K.K. (2002) Mitochondria as targets for detection and treatment of cancer. Expert reviews in molecular medicine, 4, 1-19.

Montoya, J., Ojala, D. and Attardi, G. (1981) Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs. Nature, 290, 465-70.

Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A. and Saavedra, E. (2007) Energy metabolism in tumor cells. The FEBS journal, 274, 1393-418.

Naito, A., Carcel-Trullols, J., Xie, C.-h., Evans, T.T., Mizumachi, T. and Higuchi, M. (2008) Induction of acquired resistance to antiestrogen by reversible mitochondrial DNA depletion in breast cancer cell line. International journal of cancer Journal international du cancer, 122, 1506-11.

Nakagawa, T., Lomb, D.J., Haigis, M.C. and Guarente, L. (2009) SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell, 137, 560-70.

Nomoto, S., Yamashita, K., Koshikawa, K., Nakao, A. and Sidransky, D. (2002) Mitochondrial D-loop mutations as clonal markers in multicentric hepatocellular carcinoma and plasma. Clinical cancer research : an official journal of the American Association for Cancer Research, 8, 481-7.

Pang, C.Y.; Lee, H.C.; Yang, J.H.; Wei, Y.H. (1994) Human skin mitochondrial DNA deletions associated with light exposure. *Arch. Biochem. Biophys.* 312, 534-538.

Ojala, D., Montoya, J. and Attardi, G. (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature, 290, 470-4.

Okochi, O., Hibi, K., Uemura, T., Inoue, S., Takeda, S., Kaneko, T. and Nakao, A. (2002) Detection of mitochondrial DNA alterations in the serum of hepatocellular carcinoma patients. Clinical cancer research : an official journal of the American Association for Cancer Research, 8, 2875-8.

Papadopoulos, K. (2006) Targeting the Bcl-2 family in cancer therapy. Seminars in oncology, 33, 449-56.

Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapere, J.-J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.-R. and Gavish, M. (2006) Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends in pharmacological sciences, 27, 402-9.

Pappalardo, M., Rando, A., Cosenza, G., Capuano, M. and Ramunno, L. (1996) A BalI RFLP at the goat beta-casein gene. Animal genetics, 27, 123-4.

Parker, K.A. and Pilkington, G.J. (2006) Apoptosis of human malignant glioma-derived cell cultures treated with clomipramine hydrochloride, as detected by Annexin-V assay, Radiol Oncol 40**,** 87–93.

Park, S.Y., Chang, I., Kim, J.-Y., Kang, S.W., Park, S.-H., Singh, K. and Lee, M.-S. (2004) Resistance of mitochondrial DNA-depleted cells against cell death: role of mitochondrial superoxide dismutase. The Journal of biological chemistry, 279, 7512-20.

Parrella, P., Xiao, Y., Fliss, M., Sanchez-Cespedes, M., Mazzarelli, P., Rinaldi, M., Nicol, T., Gabrielson, E., Cuomo, C., Cohen, D., Pandit, S., Spencer, M., Rabitti, C., Fazio, V.M. and Sidransky, D. (2001) Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res, 61, 7623-6.

Pejovic, T., Ladner, D., Intengan, M., Zheng, K., Fairchild, T., Dillon, D., Easley, S., Marchetti, D., Schwartz, P., Lele, S., Costa, J. and Odunsi, K. (2004) Somatic D-loop mitochondrial DNA mutations are frequent in uterine serous carcinoma. Eur J Cancer, 40, 2519-24.

Penta, J.S., Johnson, F.M., Wachsman, J.T. and Copeland, W.C. (2001) Mitochondrial DNA in human malignancy. Mutat Res, 488, 119-33.

Petros, J.A., Baumann, A.K., Ruiz-Pesini, E., Amin, M.B., Sun, C.Q., Hall, J., Lim, S., Issa, M.M., Flanders, W.D., Hosseini, S.H., Marshall, F.F. and Wallace, D.C. (2005) mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci U S A, 102, 719-24.

Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J.K., Markowitz, S.D., Trush, M.A., Kinzler, K.W. and Vogelstein, B. (1998) Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet, 20, 291-3.

Racker, E. and Spector, M. (1981) Warburg effect revisited: merger of biochemistry and molecular biology. Science, 213, 303-7.

Reid, R.A. (1983) Can migratory mitochondrial DNA activate oncogenes? TIBS, 8,190–191.
Richard, S.M., Bailliet, G., Paez, G.L., Bianchi, M.S., Peltomaki, P. and Bianchi, N.O. (2000) Nuclear and mitochondrial genome instability in human breast cancer. Cancer research., 60, 4231-7.

Rogounovitch, T.I., Saenko, V.A., Shimizu-Yoshida, Y., Abrosimov, A.Y., Lushnikov, E.F., Roumiantsev, P.O., Ohtsuru, A., Namba, H., Tsyb, A.F., Yamashita, S. (2002) Large deletions in mitochondrial DNA in radiation-associated human thyroid tumors. Cancer Res., 62, 7031-7041.

Rube, D.A. and van der Bliek, A.M. (2004) Mitochondrial morphology is dynamic and varied. Mol Cell Biochem, 256-257, 331-9.

Santos, C., Martinez, M., Lima, M., Hao, Y.J., Simoes, N. and Montiel, R. (2008) Mitochondrial DNA mutations in cancer: a review. Curr Top Med Chem, 8, 1351-66.

Schwartz, M. and Vissing, J. (2002) Paternal inheritance of mitochondrial DNA. The New England journal of medicine, 347, 576-80.

Schwartz, M. and Vissing, J. (2004) No evidence for paternal inheritance of mtDNA in patients with sporadic mtDNA mutations. Journal of the neurological sciences, 218, 99-101.

Selvanayagam, P. and Rajaraman, S. (1996) Detection of mitochondrial genome depletion by a novel cDNA in renal cell carcinoma. Lab Invest, 74, 592-9.

Sharma, H., Singh, A., Sharma, C., Jain, S.K. and Singh, N. (2005) Mutations in the mitochondrial DNA D-loop region are frequent in cervical cancer. Cancer Cell Int, 5, 34.

Sharp, M.G., Adams, S.M., Walker, R.A., Brammar, W.J. and Varley, J.M. (1992) Differential expression of the mitochondrial gene cytochrome oxidase II in benign and malignant breast tissue. The Journal of pathology, 168, 163-8.

Shidara, Y., Yamagata, K., Kanamori, T., Nakano, K., Kwong, J.Q., Manfredi, G., Oda, H. and Ohta, S. (2005) Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. Cancer Res, 65, 1655-63.

Shigenaga, M.K., Hagen, T.M. and Ames, B.N. (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A, 91, 10771-8.

Sidransky, D. (2002) Emerging molecular markers of cancer. Nat Rev Cancer, 2, 210-9.
Simonnet, H., Alazard, N., Pfeiffer, K., Gallou, C., Beroud, C., Demont, J., Bouvier, R., Schagger, H. and Godinot, C. (2002) Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. Carcinogenesis, 23, 759-68.

Singh, K.K. (2004) Mitochondrial dysfunction is a common phenotype in aging and cancer. Ann N Y Acad Sci, 1019, 260-4.

Singh, K.K., Kulawiec, M., Still, I., Desouki, M.M., Geradts, J. and Matsui, S.-I. (2005) Inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene, 354, 140-6.

Singh, K.K., Kulawiec, M., Still, I., Desouki, M.M., Geradts, J. and Matsui, S.-I. (2005) Inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene, 354, 140-6.

Singh, K.K., Russell, J., Sigala, B., Zhang, Y., Williams, J. and Keshav, K.F. (1999) Mitochondrial DNA determines the cellular response to cancer therapeutic agents. Oncogene, 18, 6641-6.

Soboll, S. (1995) Regulation of energy metabolism in liver. Journal of bioenergetics and biomembranes, 27, 571-82.

Soboll, S. (1995) Regulation of energy metabolism in liver. Journal of bioenergetics and biomembranes, 27, 571-82.

Sorokin, A., Lapidus, A., Capuano, V., Galleron, N., Pujic, P. and Ehrlich, S.D. (1996) A new approach using multiplex long accurate PCR and yeast artificial chromosomes for bacterial chromosome mapping and sequencing. Genome research, 6, 448-53.

Strachan, T., and Read, A.P. (1999) Human Molecular Genetics. 2nd edn. John Wiley and Sons; New York.

Sui, G., Zhou, S., Wang, J., Canto, M., Lee, E.E., Eshleman, J.R., Montgomery, E.A., Sidransky, D., Califano, J.A. and Maitra, A. (2006) Mitochondrial DNA mutations in preneoplastic lesions of the gastrointestinal tract: a biomarker for the early detection of cancer. Molecular cancer, 5, 73.

Sun, X., Wong, J.R., Song, K., Hu, J., Garlid, K.D. and Chen, L.B. (1994) AA1, a newly synthesized monovalent lipophilic cation, expresses potent in vivo antitumor activity. Cancer research, 54, 1465-71.

Takeya, R. and Sumimoto, H. (2003) Molecular mechanism for activation of superoxide-producing NADPH oxidases. Molecules and cells, 16, 271-7.

Tamori, A., Nishiguchi, S., Nishikawa, M., Kubo, S., Koh, N., Hirohashi, K., Shiomi, S. and Inoue, M. (2004) Correlation between clinical characteristics and mitochondrial D-loop DNA mutations in hepatocellular carcinoma. Journal of gastroenterology, 39, 1063-8.

Tan, D.-J., Bai, R.-K. and Wong, L.-J.C. (2002) Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer. Cancer research, 62, 972-6.

Taylor, R.W., McDonnell, M.T., Blakely, E.L., Chinnery, P.F., Taylor, G.A., Howell, N., Zeviani, M., Briem, E., Carrara, F. and Turnbull, D.M. (2003) Genotypes from patients indicate no paternal mitochondrial DNA contribution. Annals of neurology, 54, 521-4.

Taylor, R.W. and Turnbull, D.M. (2005) Mitochondrial DNA mutations in human disease. Nature reviews Genetics, 6, 389-402.

Taylor, R.W. and Turnbull, D.M. (2005) Mitochondrial DNA mutations in human disease. Nat Rev Genet, 6, 389-402.
Torroni, A., Stepien, G., Hodge, J.A. and Wallace, D.C. (1990) Neoplastic transformation is associated with coordinate induction of nuclear and cytoplasmic oxidative phosphorylation genes. The Journal of biological chemistry, 265, 20589-93.

Tseng, L.-M., Yin, P.-H., Chi, C.-W., Hsu, C.-Y., Wu, C.-W., Lee, L.-M., Wei, Y.-H. and Lee, H.-C. (2006) Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. Genes, chromosomes & cancer, 45, 629-38.

Verma, M., Kagan, J., Sidransky, D. and Srivastava, S. (2003) Proteomic analysis of cancer-cell mitochondria. Nature reviews Cancer, 3, 789-95.

Verma, M. and Kumar, D. (2007) Application of mitochondrial genome information in cancer epidemiology. Clinica chimica acta; international journal of clinical chemistry, 383, 41-50.

Voet, D.J., Voet, J.G., Pratt, C.W. (2006). Fundamentals of Biochemistry: Life at the Molecular Level, 2nd Edition. John Wiley and Sons, Inc. pp. 547.

Wallace, D.C. (1982) Structure and evolution of organelle genomes. Microbiological reviews, 46, 208-40.

Wang, Y., Liu, V.W.S., Xue, W.-C., Tsang, P.C.K., Cheung, A.N.Y. and Ngan, H.Y.S. (2005) The increase of mitochondrial DNA content in endometrial adenocarcinoma cells: a quantitative study using laser-captured microdissected tissues. Gynecologic oncology, 98, 104-10.

Wang, Y.; Liu, V.W.; Xue, W.C.; Cheung, A.N.; Ngan, H.Y. (2006) Association of decreased mitochondrial DNA content with ovarian cancer progression. Br. J. Cancer, 95, 1087-1091

Warburg, O., Posener, K., Negelein, E. (1924) Ueber den Stoffwechsel der Tumoren; Biochemische Zeitschrift, 152: 319-344.

Warburg, O. (1956) On the origin of cancer cells. Science (New York, N Y), 123, 309-14.

Wardell, T.M., Ferguson, E., Chinnery, P.F., Borthwick, G.M., Taylor, R.W., Jackson, G., Craft, A., Lightowlers, R.N., Howell, N. and Turnbull, D.M. (2003) Changes in the human mitochondrial genome after treatment of malignant disease. Mutation research, 525, 19-27.

Weisberg, E.L., Koya, K., Modica-Napolitano, J., Li, Y. and Chen, L.B. (1996) In vivo administration of MKT-077 causes partial yet reversible impairment of mitochondrial function. Cancer research, 56, 551-5.

Weissig, V. and Torchilin, V.P. (2001) Drug and DNA delivery to mitochondria. Advanced drug delivery reviews, 49, 1-2.

Welter, C., Kovacs, G., Seitz, G. and Blin, N. (1989) Alteration of mitochondrial DNA in human oncocytomas. Genes, chromosomes & cancer, 1, 79-82.

Wilkie, D. (1979) Antimitochondrial drugs in cancer chemotherapy: preliminary communication. Journal of the Royal Society of Medicine, 72, 599-601.

Witte, J., Lehmann, S., Wulfert, M., Yang, Q. and Roher, H.D. (2007) Mitochondrial DNA mutations in differentiated thyroid cancer with respect to the age factor. World journal of surgery, 31, 51-9.

Wu, C.-W., Yin, P.-H., Hung, W.-Y., Li, A.F.-Y., Li, S.-H., Chi, C.-W., Wei, Y.-H. and Lee, H.-C. (2005) Mitochondrial DNA mutations and mitochondrial DNA depletion in gastric cancer. Genes, chromosomes & cancer, 44, 19-28.

Wu, C.W.; Yin, P.H.; Hung, W.Y.; Li, A.F.; Li, S.H.; Chi, C.W.; Wei, Y.H.; Lee, H.C. (2005) Mitochondrial DNA mutations and mitochondrial DNA depletion in gastric cancer. Gene. Chromosome. Cancer, 44, 19-28.

Yamamoto, A., Horai, S. and Yuasa, Y. (1989) Increased level of mitochondrial gene expression in polyps of familial polyposis coli patients. Biochemical and biophysical research communications, 159, 1100-6.

Yamada, S.; Nomoto, S.; Fujii, T.; Kaneko, T.; Takeda, S.; Inoue, S.; Kanazumi, N.; Nakao, A. (2006) Correlation between copy number of mitochondrial DNA and clinico-pathologic parameters of hepatocellular carcinoma. *Eur. J. Surg. Oncol.*, *32*, 303-307.

Yeh, J.J., Lunetta, K.L., van Orsouw, N.J., Moore, F.D., Mutter, G.L., Vijg, J., Dahia, P.L. and Eng, C. (2000) Somatic mitochondrial DNA (mtDNA) mutations in papillary thyroid carcinomas and differential mtDNA sequence variants in cases with thyroid tumours. Oncogene, 19, 2060-6.

Yin, P.H.; Lee, H.C.; Chau, G.Y.; Wu, Y.T.; Li, S.H.; Lui, W.Y.; Wei, Y.H.; Liu, T.Y.; Chi, C.W. (2004) Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. *Br. J. Cancer*, *90*, 2390-2396.

Yu, M., Zhou, Y., Shi, Y., Ning, L., Yang, Y., Wei, X., Zhang, N., Hao, X. and Niu, R. (2007) Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. IUBMB life, 59, 450-7.

Yu, M., Zhou, Y., Shi, Y., Ning, L., Yang, Y., Wei, X., Zhang, N., Hao, X. and Niu, R. (2007) Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. IUBMB life, 59, 450-7.

Zanssen, S. and Schon, E.A. (2005) Mitochondrial DNA mutations in cancer. PLoS medicine, 2, e401.

Zhang, Y., Marcillat, O., Giulivi, C., Ernster, L. and Davies, K.J. (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. The Journal of biological chemistry, 265, 16330-6.

Zhu, W., Qin, W., Bradley, P., Wessel, A., Puckett, C.L. and Sauter, E.R. (2005) Mitochondrial DNA mutations in breast cancer tissue and in matched nipple aspirate fluid. Carcinogenesis, 26, 145-52.

Part IV: Publications

Paper 1

Simultaneous quantitative assessment of circulating cell-free mitochondrial and nuclear DNA by multiplex real-time PCR

Peng Xia[1,2], Ramin Radpour[1], Rebecca Zachariah[1], Alex Xiu-Cheng Fan[1], Corina Kohler1, Sinuhe Hahn[1], Wolfgang Holzgreve[3] and Xiao Yan Zhong[1]

1Laboratory for Prenatal Medicine and Gynaecological Oncology, Women's Hospital, University of Basel, Switzerland.

2Department of Oncology Surgery, First Affiliated Hospital of the Medical College, Xi'an Jiaotong University, Xi'an, China.

3University Medical Center Freiburg, Germany.

Genetics and Molecular Biology, 32, 1, 20-24 (2009)

www.sbg.org.br

Research Article

Simultaneous quantitative assessment of circulating cell-free mitochondrial and nuclear DNA by multiplex real-time PCR

Peng Xia[1,2], Ramin Radpour[1], Rebecca Zachariah[1], Alex Xiu Cheng Fan[1], Corina Kohler[1], Sinuhe Hahn[1], Wolfgang Holzgreve[3] and Xiao Yan Zhong[1]

[1]*Laboratory for Prenatal Medicine and Gynaecological Oncology, Women's Hospital, University of Basel, Switzerland.*
[2]*Department of Oncology Surgery, First Affiliated Hospital of the Medical College, Xi'an Jiaotong University, Xi'an, China.*
[3]*University Medical Center Freiburg, Germany.*

Abstract

Quantification of circulating nucleic acids in plasma and serum could be used as a non-invasive diagnostic tool for monitoring a wide variety of diseases and conditions. We describe here a rapid, simple and accurate multiplex real-time PCR method for direct synchronized analysis of circulating cell-free (ccf) mitochondrial (mtDNA) and nuclear (nDNA) DNA in plasma and serum samples. The method is based on one-step multiplex real-time PCR using a FAM-labeled MGB probe and primers to amplify the mtDNA sequence of the ATP 8 gene, and a VIC-labeled MGB probe and primers to amplify the nDNA sequence of the glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) gene, in plasma and serum samples simultaneously. The efficiencies of the multiplex assays were measured in serial dilutions. Based on the simulation of the PCR reaction kinetics, the relative quantities of ccf mtDNA were calculated using a very simple equation. Using our optimised real-time PCR conditions, close to 100% efficiency was obtained from the two assays. The two assays performed in the dilution series showed very good and reproducible correlation to each other. This optimised multiplex real-time PCR protocol can be widely used for synchronized quantification of mtDNA and nDNA in different samples, with a very high rate of efficiency.

Key words: circulating cell-free DNA, mitochondrial DNA, nuclear DNA, real-time PCR, quantitative PCR.

Received: June 12, 2008; Accepted: July 24, 2008.

Introduction

Eukaryotic cells have nuclear DNA (nDNA) and additional cytoplasmic mitochondrial DNA (mtDNA). It has been demonstrated that cell-free nucleic acids, *i.e.*, cell-free (ccf) nuclear DNA (cf-nDNA) and ccf mtDNA exist in circulation (Sozzi *et al.*, 2003). Quantification of ccf nDNA and ccf mtDNA concentrations in plasma and serum has raised great interest as a tool for non-invasive diagnosis and monitoring of a wide variety of diseases and conditions, such as cancers (Zhong *et al.*, 2007c), pathological pregnancies (Zhong *et al.*, 2001), inflammatory disease (Zhong *et al.*, 2007d) and trauma (Lam *et al.*, 2003). It has been reported that both circulating plasma nDNA and mtDNA were increased after trauma (Lam *et al.*, 2003; Lam *et al.*, 2004). Many studies observed elevated levels of ccf nDNA in plasma or serum of various cancers (Allen *et al.*, 2004; Gormally *et al.*, 2004; Sozzi *et al.*, 2003; Taback *et al.*, 2004). Elevated levels of mtDNA were detected in plasma of prostate cancer patients using quantitative real-time PCR amplification (Mehra *et al.*, 2007). Recently, Ellinger *et al.* (2008) observed that mtDNA in serum of patients with prostate cancer has a predictive value of biochemical recurrence after prostatectomy. The observations suggested that ccf DNA might be a potentially valuable prognostic marker for these patients. The similar increase of ccf mtDNA and ccf nDNA in cancers, and in patients after trauma, implies that both the nDNA and mtDNA might be released from the same tissues of origin and by similar mechanisms. Since the total amount of mtDNA per cell is unknown, determining both species in a single reaction would be the most effective and accurate method to compare relative mtDNA quantities with nDNA genome equivalents. Furthermore, the multiplexed assay for simultaneous testing of two parameters can reduce the time consumed by the diagnostic procedures.

In our study, we developed a rapid, simple and accurate multiplex real-time PCR method for direct synchronized analysis of mtDNA and nDNA in paired plasma and serum samples. This method is based on a single-step real-time PCR, using a FAM- and a VIC-labelled probe for

Send correspondence to Xiao Yan Zhong. Laboratory for Prenatal Medicine and Gynaecological Oncology, Women's Hospital, Department of Biomedicine, University of Basel, Hebelstrasse 20, CH 4031 Basel, Switzerland. E-mail: xzhong@uhbs.ch.

determining selected mtDNA and nDNA regions of interest. We optimised the multiplex assays for amplifying nDNA and mtDNA simultaneously and efficiently. Since most methods for DNA extraction are established for extracting and purifying nDNA, in our study we also compared the mtDNA and nDNA quantities by using three commercial kits for ccf DNA extraction.

Materials and Methods

Sample collection

Paired plasma and serum samples were obtained from 25 healthy blood donors, with informed consent. The study was approved by the local institutional review board.

Processing of blood samples

The 10 mL of peripheral blood samples for coagulant serum and 10 mL of peripheral blood samples for EDTA plasma were taken from blood donors. The blood samples were processed immediately by centrifugation at 1600 g for 10 min. The plasma and serum layers were transferred to new Eppendorf tubes and centrifuged again at maximum speed (16000 g) for 10 min. Plasma and serum samples were divided into aliquots of 400 µL each and stored at -80 °C.

DNA extraction

Since there are no commercial kits for ccf mtDNA extraction from serum and plasma, we firstly compared three different DNA extraction methods for co-extracting nDNA and mtDNA using plasma and serum samples from five individuals. DNA extraction from the five paired serum and plasma samples was performed using the QIAamp DNA mini kit (QIAGEN) for a first aliquot. For a second aliquot, we used the High pure PCR template preparation kit (Roche Applied Science), and for a third aliquot the automated method with the MagNA Pure LC DNA Isolation Kit – large Volume (Roche Applied Science) and the MagNA Pure LC Instrument. Visually, the automated method with MagNA Pure LC DNA Isolation Kit seemed to yield larger amounts of mtDNA and nDNA, however no significant differences were observed in the quantities using the different commercial kits (Kruskal-Wallis-Test: $p = 0.32$ for nDNA; and $p = 0.194$ for mtDNA, respectively). Using the automated method, ccf DNA was extracted from each 400 µL plasma and serum sample, and the DNA preparations were eluted in 100 µL elution buffer according to the MagNA Pure LC software.

Quantitative analysis of ccf DNA in plasma and serum samples

Five µL of DNA elution were used as template for the real-time PCR analysis. For testing nDNA, the GAPDH housekeeping gene was used with forward 5'-CCCCAC ACACATGCACTTACC-3' and reverse 5'-CCTAGTCCC AGGGCTTTGATT-3' primers and 5'-MGB-TAGGAAG GACAGGCAAC – VIC-3' as the probe. For determining mtDNA, a sequence of the MTATP 8 gene starting at locus 8446 was amplified, with forward primer 5'-AATATTAA ACACAAACTACCACCTACC-3', reverse primer 5'-TGGTTCTCAGGGTTTGTTATAA-3' and a 5'-6-FAM-CCTCACCAAAGCCCATA-MGB-3' probe (Walker *et al.*, 2005). PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI) in a total reaction volume of 25 µL, containing 5 µL of DNA, 12.5 µL of TaqMan® Universal PCR Master Mix, 4 primers and 2 probes, using a 2 min incubation at 50 °C, followed by an initial denaturation step at 95 °C for 10 min and 40 cycles of 1 min at 60 °C and 15 s at 95 °C. For the simultaneous multiplex TaqMan amplification of the two species, we optimised the concentration of primers and probes, which were: 0.6 µM for each primer and 0.4 µM for each probe.

Efficiency Measurements of the multiplex assays

The efficiency of the multiplex assay for amplifying both nDNA and mtDNA was measured with standard curves generated by dilution series. Two kinds of dilution series were used for the measurements: 1) HPLC-purified single-stranded synthetic DNA oligonucleotides (Microsynth) specifying a 79-bp mtDNA amplicon and a 97 GAPDH amplicon with 6 concentration points ranging from 5×10^7 copies to 5×10^2 copies; 2) a known concentration of human genomic DNA with six points ranging from 3.125×10^4 to 10 pg/µL (including 31250, 6250, 1250, 250, 50 and 10 pg/µL). The latter dilution series showed higher reproducible standard dilution curves than the former, and was therefore used for the further experiments.

Quantitative assessment of ccf mtDNA and nDNA

The concentrations of ccf nDNA were estimated according to the standard curves, using the known concentration of human genomic DNA, and were expressed as genome-equivalents (GE) per mL of plasma or serum. A conversion factor of 6.6 pg of DNA per cell was used to calculate the GE (Garcia Moreira *et al.*, 2006), as shown in our previous studies on ccf nDNA (Zhong *et al.*, 2007a; Zhong *et al.*, 2007b; Zhong *et al.*, 2007c). Fold change of ccf mtDNA could be calculated using two methods (Liu & Saint, 2002):

$$1)\ 2^{\Delta}CT = 2^{C_{tnDNA} - C_{tmtDNA}}$$

$$2)\ \frac{R_{0mtDNA}}{R_{0nDNA}} = \frac{(1+E_{nDNA})^{C_{tnDNA}}}{(1+E_{mtDNA})^{C_{tmtDNA}}}$$

Relative quantities of ccf mtDNA could be estimated using an equation of GE_{nDNA} x $\text{fold-change}_{mtDNA}$ and expressed also as GE per mL of plasma or serum.

Statistical analysis

Data were analysed using the SPSS software (Statistical Software Package for Windows v. 15.0). Quantities of ccf mtDNA and ccf nDNA are expressed as median, range and fold change. The Spearman Rank Test was applied to analyse the relationship between mtDNA and nDNA amplifications. Mann-Whitney and Krurkal-Wallis tests were used to determine the statistical significance of the differences between the measured concentrations of nDNA and mtDNA.

Results

Optimised experimental design and conditions for the multiplex assays

Ccf DNA extracted by two different manual methods and one automated method was amplified and compared for differences in the quantification of nDNA and mtDNA. Using the three different kits, it was possible to co-extract nDNA and mtDNA from plasma and serum samples. The automated method showed greater advantages, as it proved less time- and labour-consuming, and minimized the risk of contamination, and was therefore used for further experiments.

The primers and probes for amplifying GAPDH have been successfully used in our many previous studies, and the specificity of the assay has been confirmed (Lapaire *et al.*, 2007; Zanetti-Dallenbach *et al.*, 2007). To assess the specificity of the assay for amplifying mtDNA, the ρ0 cell line without mtDNA was tested. There was no false-positive amplification for mtDNA in the ρ0 cells observed (Xiu-Cheng Fan *et al.*, 2008).

Amplification efficiencies of the multiplex assays

We analysed 10 standard curves, using a known concentration of human genomic DNA containing six concentration points for both mtDNA and nDNA. The standard curves with average slopes at approximately -3.3 (~100% efficiency) were obtained using our optimised TaqMan PCR conditions. The two assays on the dilution series were very similar and showed very good correlation to each other and reproducibility. The average correlation coefficient of the 10 standard curves using the Spearman Rank Test was 0.99 (range: 0.989-0.999, $p < 0.001$) Figure 1 shows two examples of the standard curves with a correlation coefficient of 0.994 and 0.997, respectively.

Quantitative assessment of ccf nDNA and ccf mtDNA in serum and plasma

The ccf nDNA equivalents were calculated according to the standardised method, using very reproducible standard dilution curves, which have been described in our previous studies (Lapaire *et al.*, 2007; Zanetti-Dallenbach *et al.*, 2007; Zhong *et al.*, 2007a). Based on the comparative amplifications of nDNA and mtDNA with an efficiency close to 100%, the fold changes of ccf mtDNA were calculated using the equation of $2^{CtnDNA-CtmtDNA}$ ($R_{0mtDNA} / R_{0nDNA} = (1 + E_{nDNA})^{CtnDNA} / (1 + E_{mtDNA})^{CtmtDNA} = (1 + E_{nDNA})^{CtnDNA- CtmtDNA}$, if $E_{nDNA} = E_{mtDNA}$; $(1 + E_{nDNA})^{CtnDNA-CtmtDNA}$ $2^{CtnDNA-CtmtDNA}$, if E close to 100%).

The relative equivalents of ccf mtDNA were estimated, and both the quantities of ccf nDNA and ccf mtDNA in the 20 paired plasma and serum samples are shown in Table 1.

Ccf mtDNA and nDNA were determined by multiplex real-time PCR, with a mean concentration of 208,184 GE/mL and 6,106 GE/mL in the plasma samples, respectively, and of 2,491,364 GE/mL and 273,337 GE/mL in the serum samples, respectively. The ccf nDNA and ccf mtDNA levels in the serum samples were significantly higher than those of the plasma samples (12-45 fold). This may be a result of the release of cellular DNA artefacts during blood clotting procedures. The ccf mtDNA levels in the

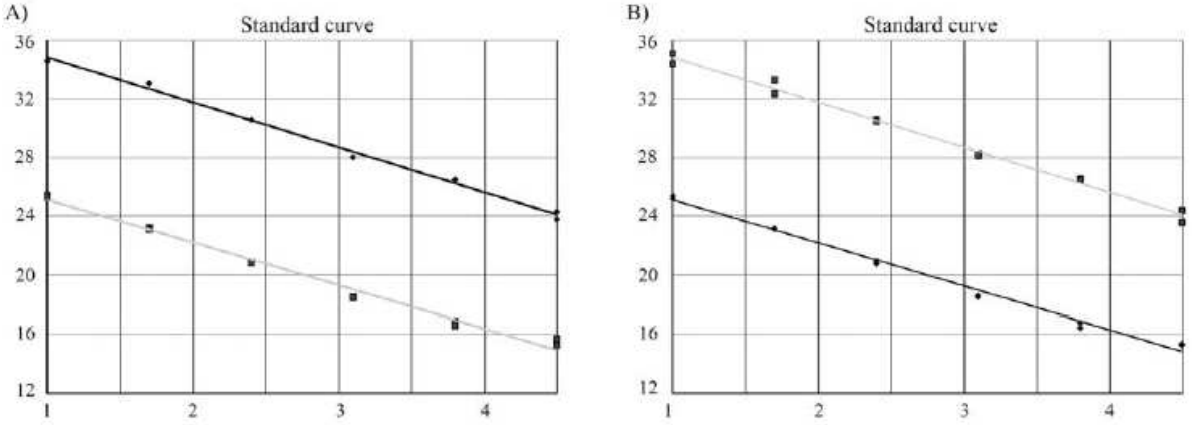


Figure 1 - Simulation of real-time PCR kinetics for amplifying mtDNA and nDNA on serial dilutions. The figure shows reproducible standard dilution curves for identification of the mtDNA and nDNA. The upper lines are nDNA standard dilution curves and the lower lines are mtDNA standard dilution curves. The numbers on the y axis represent the values of cycle threshold (Ct) and numbers on the x axis represent the dilution points.

Table 1 - Ccf mtDNA and nDNA represented in genome equivalent (GE)/mL in serum and plasma

	mtDNA	nDNA	mtDNA/nDNA	p- value
Serum	2491364 (33787-11720914)	273337 (14845-1426767)	9 fold	< 0.001
Plasma	208184 (8027-2618508)	6106 (465-61722)	34 fold	< 0.001
Serum/plasma	12 fold	45 fold		
p-value	< 0.001	< 0.001		

plasma and serum samples were significantly higher than those of ccf nDNA (9-34 fold).

Discussion

In this study, we described the multiplex assays used to analyse ccf nDNA and ccf mtDNA simultaneously. Using the optimised PCR protocol, the amplification of nDNA and mtDNA in a single reaction tube was very similar and showed a simulation of real-time PCR kinetics. With the comparative efficiencies of two assays in a single tube, we could use the nDNA level as a reference to assess the relative quantities of mtDNA. The method is simple and does not require generating standard curves, which often result in errors due to dilution inaccuracy. Using the multiplex assays, we could rapidly and accurately determine the levels of ccf nDNA and ccf mtDNA in serum and plasma samples. The levels of ccf mtDNA in circulation are significantly higher than those of ccf nDNA, due to the fact that the number of mitochondrial genomes in a cell ranges from several hundreds to more than 10,000 copies, and each mitochondrion contains between two and 10 mtDNA molecules (Higuchi, 2007). The serum samples showed a significantly higher concentration of ccf DNA because of cellular DNA release during the blood clotting procedures (Zanetti-Dallenbach, 2008; Zhong *et al.*, 2007a).

Quantitative alterations of ccf nDNA and mtDNA have been observed in many conditions, especially in aging, apoptosis and carcinogenesis (Goebel *et al.*, 2005; Liu *et al.*, 2003; Mehra *et al.*, 2007; Takeuchi *et al.*, 2004). So far, the exact content of human mtDNA in different cells and tissues remains unclear. Two studies developed multiplex assays to analyse nDNA and mtDNA simultaneously for forensic medicine (Alonso *et al.*, 2004; Walker *et al.*, 2005). In our study, we were able to use the GAPDH gene as a housekeeping gene to analyse the quantities of mtDNA. The aim of this study was to develop a rapid, simple and accurate multiplex real-time PCR for the direct synchronized analysis of ccf mtDNA and ccf nDNA, which may provide a platform for further investigations leading to a better understanding of the biology of ccf mtDNA and ccf nDNA on large-scale sample sizes.

It is known that ancient mtDNA sequences, also termed as nuclear pseudogenes, are present in the human nuclear genome as multiple copies. Woischnik and Moraes (2002) found up to 612 nuclear integrations. Their homology with the current mtDNA was up to 99%. An accidental co-amplification of these nuclear copies of mitochondrial genes might bias the results (Wallace *et al.*, 1997). We tested the specificity by using the mitochondria-negative cell line ρ0, and no mtDNA signals were detected in this cell line by real-time multiplex PCR.

Based on the importance of quantification of both mtDNA and nDNA in life science, we developed a rapid, accurate, simple and low-cost approach that enables the simultaneous identification of physiological and pathogenic mtDNA and nDNA variants. Since there are no commercial kits for ccf mtDNA extraction from serum and plasma samples, we, for the first time, compared three different DNA extraction methods for co-extracting nDNA and mtDNA from plasma and serum. We were also the first ones to examine the efficiencies of the two assays in a single tube. After calculating the comparative efficiencies we could use the nDNA level as a reference to assess the relative quantities of mtDNA, which can simplify the calculation of mtDNA content. The method is simple and does not require generating standard curves, which often result in errors due to dilution inaccuracy. This method can be considered a standard approach for widely quantifying both mtDNA and nDNA in different kinds of samples.

Acknowledgments

This work was supported in part by the Swiss National Science Foundation (320000-119722/1), Swiss Cancer League, Krebsliga Beider Basel and the Dr Hans Altschueler Stiftung. We thank Mrs Vivian Kiefer and Mrs Nicole Chiodetti for their help. We are grateful to Mrs Regan Geissmann for proofreading the text.

References

Allen D, Butt A, Cahill D, Wheeler M, Popert R and Swaminathan R (2004) Role of cell-free plasma DNA as a diagnostic marker for prostate cancer. Ann NY Acad Sci 1022:76-80.

Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, Garcia-Hirschfeld J, Sancho M, de La Rua C and Fernandez-Piqueras J (2004) Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Sci Int 139:141-149.

Garcia Moreira V, de la Cera Martinez T, Gago Gonzalez E, Prieto Garcia B and Alvarez Menendez FV (2006) Increase in and clearance of cell-free plasma DNA in hemodialysis quantified by real-time PCR. Clin Chem Lab Med 44:1410-1415.

Goebel G, Zitt M, Zitt M and Muller HM (2005) Circulating nucleic acids in plasma or serum (CNAPS) as prognostic and predictive markers in patients with solid neoplasias. Dis Markers 21:105-120.

Gormally E, Hainaut P, Caboux E, Airoldi L, Autrup H, Malaveille C, Dunning A, Garte S, Matullo G, Overvad K, *et al.* (2004) Amount of DNA in plasma and cancer risk: A prospective study. Int J Cancer 111:746-749.

Higuchi M (2007) Regulation of mitochondrial DNA content and cancer. Mitochondrion 7:53-57.

Lam NY, Rainer TH, Chan LY, Joynt GM and Lo YM (2003) Time course of early and late changes in plasma DNA in trauma patients. Clin Chem 49:1286-1291.

Lam NY, Rainer TH, Chiu RW, Joynt GM and Lo YM (2004) Plasma mitochondrial DNA concentrations after trauma. Clin Chem 50:213-216.

Lapaire O, Volgmann T, Huang D, Hahn S, Holzgreve W and Zhong XY (2007) Maternal smoking: Effect on circulating cell-free fetal and total DNA levels in maternal plasma from the second trimester. Obstet Gynecol 110:1358-1363.

Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS and Wei YH (2003) Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. Free Radic Res 37:1307-1317.

Liu W and Saint DA (2002) A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem 302:52-59.

Mehra N, Penning M, Maas J, van Daal N, Giles RH and Voest EE (2007) Circulating mitochondrial nucleic acids have prognostic value for survival in patients with advanced prostate cancer. Clin Cancer Res 13:421-426.

Sozzi G, Conte D, Leon M, Ciricione R, Roz L, Ratcliffe C, Roz E, Cirenei N, Bellomi M, Pelosi G, *et al.* (2003) Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol 21:3902-3908.

Taback B, O'Day SJ and Hoon DS (2004) Quantification of circulating DNA in the plasma and serum of cancer patients. Ann NY Acad Sci 1022:17-24.

Takeuchi H, Fujimoto A and Hoon DS (2004) Detection of mitochondrial DNA alterations in plasma of malignant melanoma patients. Ann NY Acad Sci 1022:50-54.

Walker JA, Hedges DJ, Perodeau BP, Landry KE, Stoilova N, Laborde ME, Shewale J, Sinha SK and Batzer MA (2005) Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: Application in human identification. Anal Biochem 337:89-97.

Wallace DC, Stugard C, Murdock D, Schurr T and Brown MD (1997) Ancient mtDNA sequences in the human nuclear genome: A potential source of errors in identifying pathogenic mutations. Proc Natl Acad Sci USA 94:14900-14905.

Xiu-Cheng Fan A, Garritsen HS, Tarhouny SE, Morris M, Hahn S, Holzgreve W and Zhong XY (2008) A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med 46:299-305.

Zanetti-Dallenbach RA, Schmid S, Wight E, Holzgreve W, Ladewing A, Hahn S and Zhong XY (2007) Levels of circulating cell-free serum DNA in benign and malignant breast lesions. Int J Biol Markers 22:95-99.

Zanetti-Dallenbach RA, Wight E, Fan AXC, Lapaire O, Hahn S, Holzgreve W and Zhong XY (2008) Positive correlation of cell-free dna in plasma/serum in patients with malignant and benign breast. Disease Anticancer Research 28:921-926.

Zhong XY, Hahn S, Kiefer V and Holzgreve W (2007a) Is the quantity of circulatory cell-free DNA in human plasma and serum samples associated with gender, age and frequency of blood donations? Ann Hematol 86:139-143.

Zhong XY, Hahn S, Steinborn A and Holzgreve W (2007b) Quantitative analysis of intact fetal cells in maternal plasma by real-time PCR. Eur J Obstet Gynecol Reprod Biol 133:20-24.

Zhong XY, Ladewig A, Schmid S, Wight E, Hahn S and Holzgreve W (2007c) Elevated level of cell-free plasma DNA is associated with breast cancer. Arch Gynecol Obstet 276:327-331.

Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W and Hahn S (2001) Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 184:414-419.

Zhong XY, von Muhlenen I, Li Y, Kang A, Gupta AK, Tyndall A, Holzgreve W, Hahn S and Hasler P (2007d) Increased concentrations of antibody-bound circulatory cell-free DNA in rheumatoid arthritis. Clin Chem 53:1609-1614.

Associate Editor: Carlos F.M. Menck

Paper 2.

A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry

Alex Xiu-Cheng Fan[1,a], Henk S.P. Garritsen[2,a], Shereen E.L. Tarhouny[1,3], Michael Morris[4], Sinuhe Hahn[1], Wolfgang Holzgreve[1] and Xiaoan Zhong[1],*

1 Laboratory for Prenatal Medicine and GynecologicOncology, Women's Hospital/Department of Research, University of Basel, Basel, Switzerland

2 Institute for Clinical Transfusion Medicine, Staedtisches Klinikum Braunschweig GmbH, Braunschweig, Germany

3 Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Clin Chem Lab Med 2008;46(3):299–305 © 2008 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2008.071

A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry

Alex Xiu-Cheng Fan[1,a], Henk S.P. Garritsen[2,a], Shereen E.L. Tarhouny[1,3], Michael Morris[4], Sinuhe Hahn[1], Wolfgang Holzgreve[1] and Xiao Yan Zhong[1,*]

[1] Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department of Research, University of Basel, Basel, Switzerland
[2] Institute for Clinical Transfusion Medicine, Staedtisches Klinikum Braunschweig GmbH, Braunschweig, Germany
[3] Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt
[4] Laboratoire de Diagnostic Moleculaire, Service de Medicine Genetique, Geneva, Switzerland

Abstract

Background: Single nucleotide polymorphisms (SNPs) of mitochondrial DNA (mtDNA) are involved in physiological and pathological conditions. We developed a rapid, accurate, highly sensitive and high-throughput approach with low cost to identify mtDNA SNPs.

Methods: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to detect 18 SNPs of mtDNA by uniplex and multiplex assays. The sensitivity and specificity of the MALDI-TOF MS were evaluated. The accuracy of the approach was validated by the comparison of using the robust sequencing analysis.

Results: The detection limit achieved with the assays corresponded to the identification of five-genome equivalence of mtDNA per reaction after first round PCR amplification. The testing system enabled the discrimination of as little as 5% of mtDNA polymorphism in the predominating background of mtDNA not containing the SNP. No false positive and false negative results were obtained using the uniplex and multiplex MALDI-TOF MS assays for the analysis of the 18 SNPs compared with those obtained by sequencing analysis.

Conclusions: Possible fields which could benefit from this powerful and sensitive tool include forensic medicine, tracing of matrilineage, transplantation immunology, transfusion medicine, the diagnosis of mtDNA mutation related disorders, and the research regarding aging, apoptosis and carcinogenesis based on physiologic and pathogenic alterations of mtDNA for the analysis of large-scale samples, multiple SNPs or rare mtDNA.
Clin Chem Lab Med 2008;46:299–305.

Keywords: high-throughput analysis; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); mitochondrial DNA (mtDNA); multiplex assay; single nucleotide polymorphisms (SNPs); uniplex assay.

Introduction

Mitochondrial DNA (mtDNA) is a circular cytoplasmic double stranded DNA of 16.6 kb, including the coding regions and the hypervariable non-coding D-loop region (1, 2). The features of mtDNA, such as matrilineal inheritance, high copy numbers per cell and lack of recombination make it a powerful tool in life science.

Physiologically, mtDNA typing has been applied routinely in forensic medicine (3). Maternal inheritance of mtDNA enables the tracing of matrilineage far back in time (4). Single nucleotide polymorphisms (SNPs) can also be used as markers in transfusion and transplantation medicine (5).

Pathologically, the properties of mtDNA, such as reactive oxygen species enhanced aggression, high sensitivity to damage, deficient repair of damage and high rate of mutations suggest their potential importance in mtDNA related disorders, aging, apoptosis and carcinogenesis (6–8). Somatic mutations in mtDNA have been identified in various human cancers and in a large spectrum of clinically important disorders (9, 10).

Based on the importance of mtDNA SNPs in life science, a rapid, accurate, highly sensitive and high-throughput approach with low cost to identify physiologic and pathogenic mtDNA variants is needed for the analysis of large-scale samples, multiple SNPs or rare mtDNA.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) proved to be a superior, high-throughput technology for the robust detection of single nucleotide variations with low cost on nuclear DNA (nDNA). It could permit the reliable detection of SNPs (11) from a very rare target, including fetal point mutations from cell-free fetal

[a] The first two authors contributed equally to this work.
*Corresponding author: Dr. Xiao Yan Zhong, Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department of Research, University of Basel, Hebelstraße 20, Room Nr. 416, 4031 Basel, Switzerland
Phone: +41-61-2659224/9595, Fax: +41-61-2659399,
E-mail: xzhong@uhbs.ch
Received October 2, 2007; accepted November 8, 2007; previously published online February 6, 2008

DNA in maternal plasma and donor derived cell-free DNA in organ transplant recipients urine, which cannot be reliably detected by more conventional PCR-based approaches (12, 13).

In this study, we developed a MALDI-TOF MS-based method to detect 18 SNPs in mtDNA on mtDNA hypervariable regions in platelet-apheresis products by uniplex and multiplex assays. This novel application enables the rapid, sensitive and accurate identification of single or multiple mtDNA SNP variants in a single reaction on mtDNA hypervariable regions.

Materials and methods

DNA extraction

Platelet-apheresis products containing mtDNA from three blood donors with informative mtDNA SNPs were provided by the Institute for Clinical Transfusion Medicine, Staedtisches Klinikum Braunschweig gGmbH, Germany. This study was approved by the Local Ethics Committee "Ethikkommission beider Basel". Written informed consent was obtained from all three individuals. A total of 18 mtDNA SNPs on three hypervariable regions (HVR1, HVR2 and HVR3) within the displacement loop (D-loop) region of the mtDNA were identified using a traditional sequencing approach by the Institute (5).

For our study, mtDNA was isolated from 800 μL of plateletapheresis products using the MagNA LC Isolation Kit-Large Volume protocol with the MagNA Pure LC Instrument (Roche Applied Science, Basel, Switzerland) according to the manufacturer's introduction. The DNA preparations were eluted in 200 μL elution buffer according to this protocol.

Quantification of mtDNA and contamination of nDNA in the samples

The amounts of nDNA and mtDNA were quantified by multiplex TaqMan real-time PCR for both glyceraldehype-3-phosphodehydrogenase (GAPDH) gene and mtDNA sequence.

The GAPDH and mtDNA primer and probe sequences are shown as follows: GAPDH (14): forward, 5′-CCCCACACACATGCACTTACG-3′, reverse, 5′-CCTAGTCCCAGGGCTTTGATT-3′, probe, 5′-(MGB) GTGAACGTGGATGAAGTTGG (VIC)-3′; mtDNA (15): forward, 5′-AATATTAAACACAAACTACCACCTACC-3′, reverse, 5′-TGGTTCTCAGGGTTTGTTATA-3′, probe, 5′-(MGB) CCTCACCAAAGCCCATA (FAM)-3′.

The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI, Rotkreuz, Switzerland). DNA (5 μL) was used as template for the PCR analysis. The TaqMan assays were carried out in 25 μL of total reaction volume using 2 min incubation at 50°C, followed by an initial denaturation step at 95°C for 10 min, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

To determine the quantification of mtDNA and nDNA present in platelet-apheresis product samples, standard dilution curves using HPLC-purified single-stranded synthetic DNA oligonucleotides (Microsynth, Balgach, Switzerland) specifying a 79-bp mtDNA amplicon and a 97-GAPDH amplicon with concentration ranging from 5×10^7 copies to 5×10^2 copies were used. Absolute concentrations of mtDNA and GAPDH were expressed as genome equivalence (GE/mL) of platelet-apheresis products.

Detection of mtDNA polymorphisms on hypervariable regions using MALDI-TOF MS

The PCR and extension primers used to analyze the mtDNA SNPs were designed using MassArray Assay Design v.3.1 (Sequenom, San Diego, CA, USA) and are listed in Table 1.

The uniplex and multiplex PCR reactions were carried out in 50 μL PCR cocktail mixes containing 5 μL DNA, 1.626 mM $MgCl_2$, 500 μM dNTP mix, 0.5 U Hot-star Taq Gold polymerase and primer pairs. The amplification was performed under the following conditions: incubation at 94°C for 15 min, followed by 50 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min.

To remove the non-incorporated dNTPs, a shrimp alkaline phosphatase treatment was performed after the PCR reaction under the following conditions: 37°C for 20 min, 85°C for 5 min and cooling to 4°C.

The iPLEX reaction was performed using the iPLEX cocktail mix (Sequenom, San Diego, CA, USA), which contains buffer, termination mix, enzyme and extension primers, under the following conditions: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 52°C for 5 s, 80°C for 5 s, final extension at 72°C for 5 min, then cooling to 4°C.

The iPLEX reaction products were desalted using clean resin to optimize mass spectrometric analysis and dispensed onto a 384-format SpectroCHIP bioarray using a nanodispenser for MALDI-TOF MS analysis. MassARRAY Workstation version 3.4 software (Sequenom) was used to process and analyze iPLEX SpectroCHIP bioarrays. The call rate, extension rate and peak area for all allele-specific analyses in any given assay were calculated using the software provided by the manufacturer. Positive and negative control samples were run at each step and on each chip.

The specificity of the assay for mtDNA was assessed through the use of a mitochondria negative cell line, which was obtained by subculturing that cell line in the presence of low concentration of ethidium bromide until the cells were devoid of any mitochondria. The sensitivity of the assay for detecting mtDNA polymorphisms was assessed through the five-fold dilution of mtDNA samples containing informative polymorphisms with concentrations as low as 1 GE/μL, as well as through mixing known mtDNA SNPs into a predominant background of mtDNA molecules not containing those polymorphisms.

Results

Co-extraction of mtDNA and nDNA from platelet-apheresis

mtDNA and nDNA were co-extracted from the platelet-apheresis products (Table 2). The GAPDH level, representing cell-free total nDNA, ranged from 1073 to 4039 GE/mL of platelet-apheresis concentrate in the platelet-apheresis samples, and the mtDNA level ranged from 869,667 to 3,492,084 GE/mL of platelet-apheresis products. The levels of mtDNA were found to be at least 774- up to 2713-fold higher than nDNA levels.

Specificity of MALDI-TOF MS for detecting mtDNA polymorphisms

The specificity of the MALDI-TOF MS for detecting the 18 mtDNA polymorphisms was assessed through the use of a mitochondria negative cell line (143b rho0).

Table 1 SNPs in the study cases and sequences of primers.

	Sequencing	PCR primers
1	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
	309 C-CC	Reverse primer: ACGTTGGATGCCACTTTCCACACAGACATC Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Extension primer: TTTCCACCAAACCCCCCC
	315 C-CC	Forward primer: ACGTTGGATGGCTGGTGTTAGGGTTCTTTG Reverse primer: ACGTTGGATGAAATTTCCACCAAACCCCCC Extension primer: CAAACCCCCCCTCCCCC
2	16189 T-C	Forward primer: ACGTTGGATGGGGTTGATTGCTGTACTTGC Reverse primer: ACGTTGGATGCTTGACCACCTGTAGTACAT Extension primer: CAATCCACATCAAAACCCCC
	152 T-C	Forward primer: ACGTTGGATGGTTCGCCTGTAATATTGAACG Reverse primer: ACGTTGGATGCCTATGTCGCAGTATCTGTC Extension primer: TGATTCCTGCCTCATCC
	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
3	16069 C-T	Forward primer: ACGTTGGATGGTGGCTGGCAGTAATGTACG Reverse primer: ACGTTGGATGGAAGCAGATTTGGGTACCAC Extension primer: CCACCCAAGTATTGACT
	16324 T-C	Forward primer: ACGTTGGATGTCATCCATGGGGACGAGAAG Reverse primer: ACGTTGGATGAACCTACCCACCCTTAACAG Extension primer: ATTTACCGTACATAGCACAT
	16366 C-T	Forward primer: ACGTTGGATGGCGGGATATTGATTTCACGG Reverse primer: ACGTTGGATGGCACATTACAGTCAAATCCC Extension primer: AAATCCCTTCTCGTCCC
	16390 G-A	Forward primer: ACGTTGGATGAGTCAAATCCCTTCTCGTCC Reverse primer: ACGTTGGATGGCGGGATATTGATTTCACGG Extension primer: GATGGTGGTCAAGGGAC
	73 A-G	Forward primer: ACGTTGGATGTATTAACCACTCACGGGAGC Reverse primer: ACGTTGGATGAGCGTCTCGCAATGCTATCG Extension primer: TCGCAATGCTATCGCGTGCA
	185 G-A	Forward primer: ACGTTGGATGGTGCAGACATTCAATTGTTA Reverse primer: ACGTTGGATGTATCGCACCTACGTTCAATA Extension primer: CACCTACGTTCAATATTACAG
	188 A-G	Forward primer: ACGTTGGATGCGCACCTACGTTCAATATTA Reverse primer: ACGTTGGATGGCTGTGCAGACATTCAATTGT Extension primer: TTAACACACTTTAGTAAGTATGT
	228 G-A	Forward primer: ACGTTGGATGAAGTGGCTGTGCAGACATTC Reverse primer: ACGTTGGATGGGCGAACATACTTACTAAAG Extension primer: TGTGTTAATTAATTAATGCTTGTAG
	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
	295 C-T	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGCCACTTTCCACACAGACATC Extension primer: ACAGACATCATAACAAAAAATTT
	315 C-CC	Forward primer: ACGTTGGATGGCTGGTGTTAGGGTTCTTTG Reverse primer: ACGTTGGATGAAATTTCCACCAAACCCCCC Extension primer: CAAACCCCCCCTCCCCC
	462 C-T	Forward primer: ACGTTGGATGTGTGTGTGCTGGGTAGGATG Reverse primer: ACGTTGGATGTTTAACAGTCACCCCCCAAC Extension primer: AACACATTATTTTCCCCTC
	489 T-C	Forward primer: ACGTTGGATGTTAGCAGCGGTGTGTGTGTG Reverse primer: ACGTTGGATGTTATTTTCCCCTCCCACTCC Extension primer: CCCATACTACTAATCTCATCAA
	522 C-del	Forward primer: ACGTTGGATGTCTCATCAATACAACCCCCG Reverse primer: ACGTTGGATGTTTGGTTGGTTCGGGGTATG Extension primer: TATGGGGTTAGCAGCGGT
	523 A-del	Forward primer: ACGTTGGATGTTTGGTTGGTTCGGGGTATG Reverse primer: ACGTTGGATGTCTCATCAATACAACCCCCG Extension primer: CTACCCAGCACACACAC

Table 2 Quantities of mtDNA and nDNA in the samples of platelet-apheresis products.

Case	mtDNA quantities, GE/mL	GAPDH, GE/mL	Ratio mtDNA/nDNA
1	2,911,332	1073	2713
2	869,667	1123	774
3	3,492,084	4039	865

No positive amplifications were obtained from the cell line using the 18 primer pairs listed in Table 1.

Sensitivity of MALDI-TOF MS for detecting mtDNA polymorphisms

To determine the sensitivity of MALDI-TOF MS for analyzing mtDNA SNPs, we designed experiments by diluting known amounts of mtDNA five-fold with informative polymorphisms in a series from 5×10^4 GE to 5 GE (1 GE/μL) per PCR reaction. Analysis for informative polymorphisms was performed using MALDI-TOF MS. The assay enabled the reliable identification of the polymorphisms using as little as 5 GE per PCR reactions. In our other study, where we used MALDI-TOF MS to identify point mutations on single cell level, close to 70% of the successful analysis was achieved (data not shown).

A dilution series (2.5×10^4, 5×10^4, 10×10^4, 25×10^4, 50×10^4 GE) of mtDNA with an informative polymorphism was mixed with mtDNA molecules (1×10^6 GE) not carrying the same polymorphism, to give a ratio ranging from 2.5%, 5%, 10%, 25% and to 50% of mtDNA molecules with the polymorphism to those without the polymorphism. MALDI-TOF MS could discriminate down to the level of 5% of polymorphism-containing mtDNA in a 95% background of mtDNA lacking the polymorphism (Figure 1). We compared the sensitivity using MALDI-TOF with those using sequencing analysis (Figure 2).

Detection of mtDNA polymorphisms in three individuals using uniplex and multiplex assays

From three individuals, 18 mtDNA polymorphisms were identified by sequencing analysis. The positions and nature of the polymorphisms in the three individuals are listed in Table 1.

A total of 18 uniplex assays for each polymorphism and 3–12 multiplexed assays using MALDI-TOF MS were developed. The results were compared with those obtained by sequencing analysis. No false positive and false negative results were obtained using the MALDI-TOF MS assays. The uniplex assays and the multiplex assays showed good concordance regarding the determination of the polymorphisms.

Discussion

Rapid and accurate identification of physiologic and pathogenic mtDNA variants is an important issue in life science. In this study, we evaluated the sensitivity and specificity of MALDI-TOF MS for the detection of SNPs mtDNA on hypervariable regions.

To evaluate the specificity of MALDI-TOF MS for detection of mtDNA SNPs, the assays for 18 mtDNA SNPs in this study were tested by using a mitochondria negative cell line. No mtDNA was detectable from the cell line by both MALDI-TOF MS and sequencing. In our study, we co-extracted mtDNA with nDNA using commercial kits. The contaminated co-extraction of nDNA did not alter our results regarding the detection of mtDNA SNPs.

The detection limit achieved with the assay corresponded to the identification of 5 GE of mtDNA per reaction after first round PCR amplification. As a human eukaryotic cell contains hundreds or thousands of mtDNA (16), the sensitive MALDI-TOF MS established in our study could enable the analysis of mtDNA SNPs from less than one cell.

In our study, MALDI-TOF MS enabled the discrimination of as little as 5% of the mtDNA polymorphism in the predominating background of mtDNA not containing the SNP. Several studies using MALDI-TOF MS for genetic analysis support our results regarding the sensitivity of this assay for mtDNA detection. In our group, Li et al. could detect very rare fetal genetic materials in maternal blood (12). The concentration of fetal DNA in maternal circulation is considerably low, comprising 3.7% of total DNA as quantified by real-time quantitative analysis (17). A previous study from our laboratory also showed that donor-derived genetic materials could be sensitively detected in the urine of kidney transplant recipients using MALDI-TOF MS (13).

In our group, Li et al. compared the MALDI-TOF MS assay with a well-established TaqMan real-time PCR assay for the detection of rare fetal genetic material in the maternal circulation. The MALDI-TOF MS assay and the TaqMan real-time PCR assay had similar detection rates in terms of non-invasive prenatal identification of fetal gender (18). However, identification of multiple SNPs by individual allele-specific TaqMan real-time PCR is a time-consuming and laborious process. Furthermore, SNPs, which only differ minimally from the wild-type DNA of interest, make the design of allele-specific PCR especially challenging (19).

The iPLEX Assay developed by Sequenom, Inc. enables up to 36–40 multiplex PCR and primer extension reactions per assay, so that 36–40 mutations can be detected simultaneously in a single reaction. The multiplexed genotyping assays rely on the natural molecular weight differences of DNA bases. In our study, we analyzed the 18 SNPs using uniplex and multiplex assays in parallel. The precision and accuracy of the mass determination by multiplex iPLEX assay at 3–12 levels were comparable with those by uniplex assay. There were no false positive or false negative signals from the MALDI-TOF MS method, as compared to the results obtained by sequencing. The method demonstrated a high concordance of results and 100% accuracy when compared with capillary sequencing, suggesting that

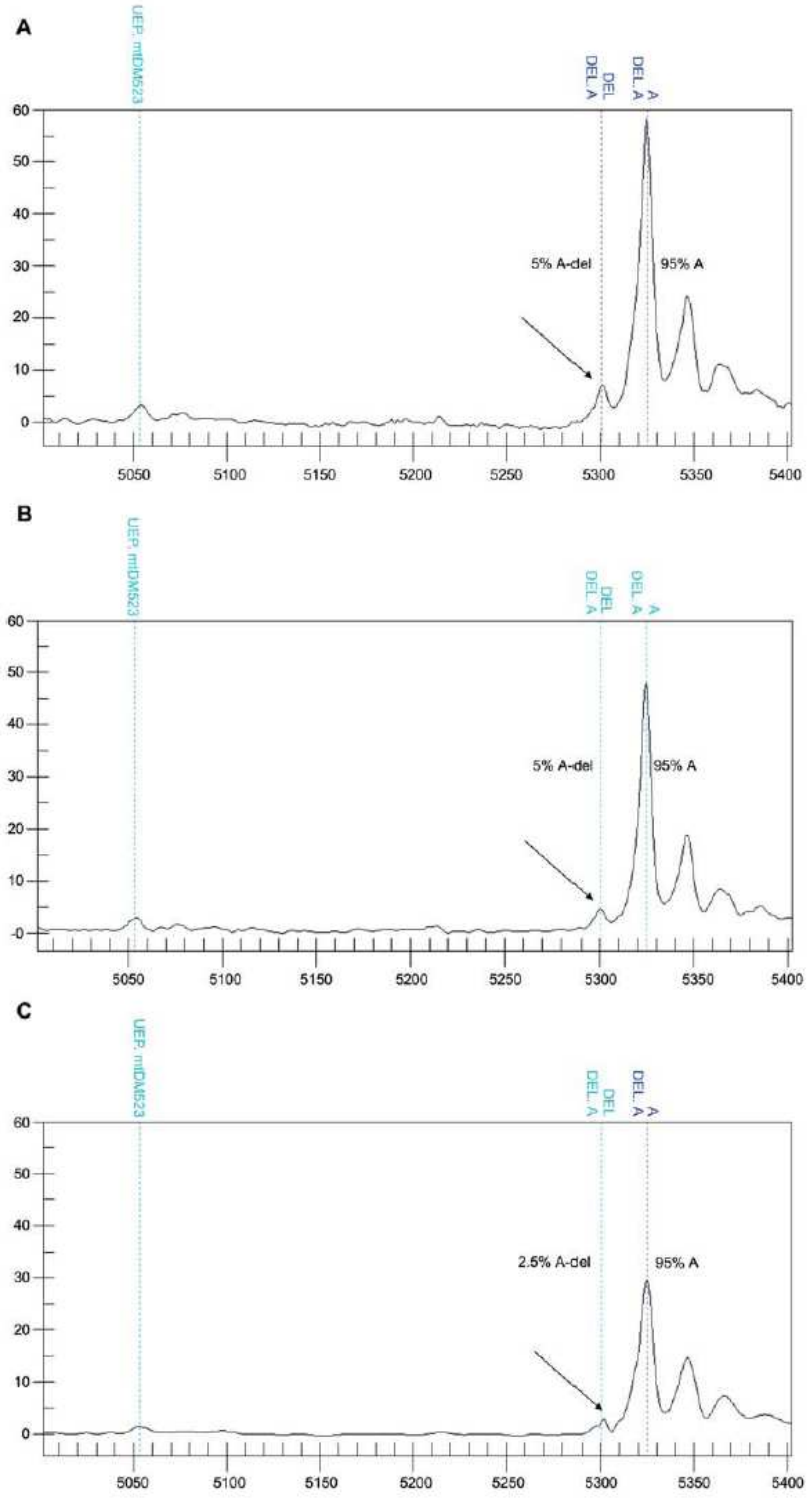


Figure 1 MALDI-TOF MS discriminates rare SNP-containing mtDNA in the background of mtDNA lacking the polymorphism. mtDNA sample containing the SNP of an "A-deletion" at the position of 523 was mixed into the mtDNA sample without an "A-deletion" at the same position. Panels (A) and (B) show the reproducible detections of as little as 5% of the "A-deletion"-containing mtDNA in the 95% background molecules by MALDI-TOF MS. Panel (C) shows a mini-peak on MALDI-TOF MS for the detection of as little as 2.5% of the "A-deletion"-containing mtDNA in the 97.5% background molecules.

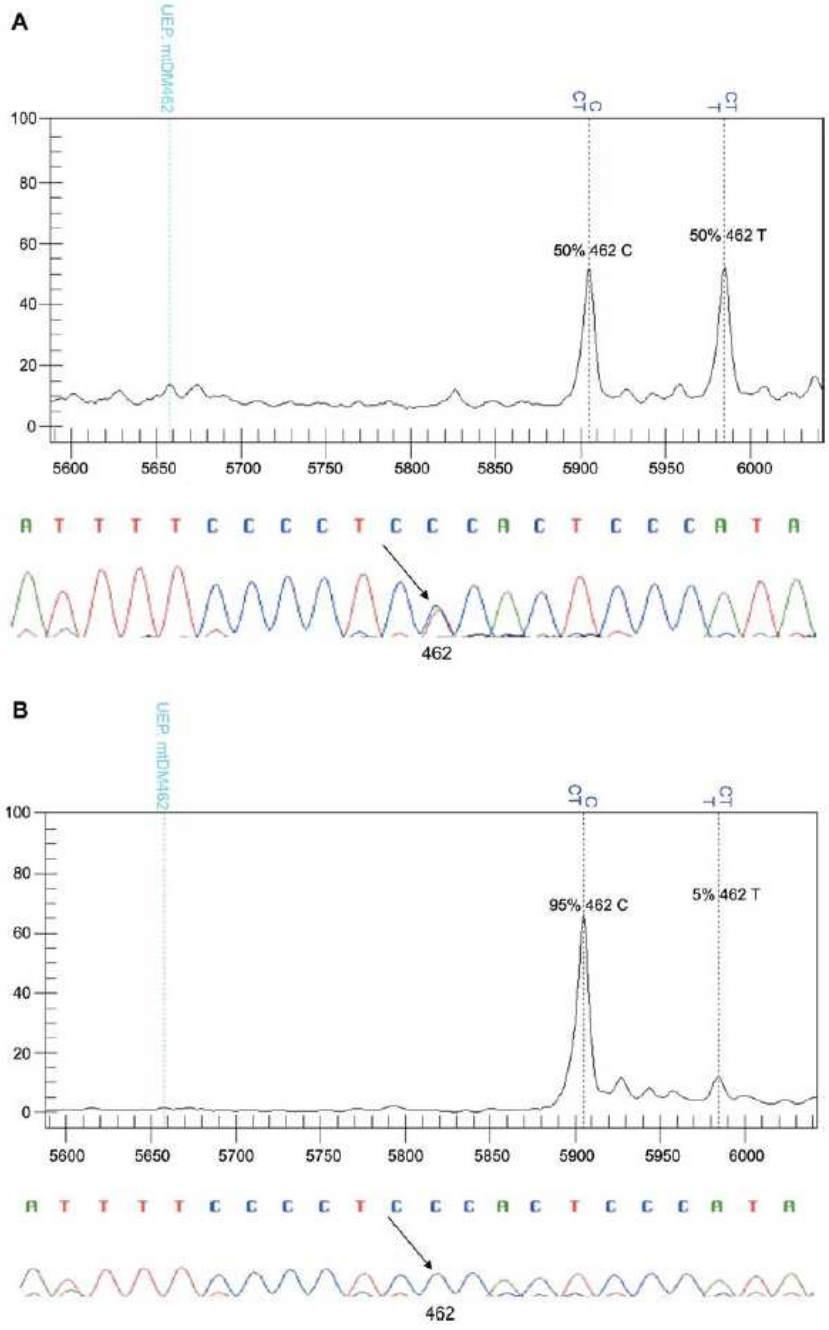


Figure 2 Comparison of MALDI-TOF and sequencing analysis for the sensitivity test.
mtDNA sample containing the SNP of "T" at the position of 462 was mixed into the mtDNA sample containing a "C" at the same position. Panel (A) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 50%–50%. Panel (B) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 5%–95%.

mass spectrometric analysis of SNPs is a robust and reproducible technique for the detection of mtDNA mutations. Multiplex assays enable the rapid detection of polymorphisms and reduce the amount of DNA input required and the costs for multiple genotyping. The possibility of analyzing up to 384 samples on a single chip enables high-throughput detections in large-scale studies.

Possible fields which could benefit from this powerful and sensitive tool include forensic medicine and transplantation immunology, particularly when the target mtDNA input is limited (20). This approach can

also be used for the tracing of matrilineage with large-scale multiple genotyping. Further potential applications may be possible in the field of transfusion medicine, in the diagnosis of mtDNA mutation related disorders, and in research regarding aging, apoptosis and carcinogenesis based on physiologic and pathogenic alterations of mtDNA.

Acknowledgements

This work was supported in part by the Swiss Cancer League and Krebsliga Beider Basel. We thank Prof. Dr. Rudolf J. Wiesner for kindly providing us with 143B rho 0 cell line and Ms. Sabrina Eckertz for managing this post for us. We thank Dr. Ying Li for technical support. We thank Mrs. Vivian Kiefer and Ms. Nicole Chiodetti for their excellent assistance. We thank Dr. Dorothy Huang for her helpful comments and reviewing the English.

References

1. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457–65.
2. Fernandez-Silva P, Enriquez JA, Montoya J. Replication and transcription of mammalian mitochondrial DNA. Exp Physiol 2003;88:41–56.
3. Andreasson H, Nilsson M, Budowle B, Lundberg H, Allen M. Nuclear and mitochondrial DNA quantification of various forensic materials. Forensic Sci Int 2006;164:56–64.
4. Alonso A, Albarran C, Martin P, Garcia P, Capilla J, Garcia O, et al. Usefulness of microchip electrophoresis for the analysis of mitochondrial DNA in forensic and ancient DNA studies. Electrophoresis 2006;27:5101–9.
5. Garritsen HS, Hellenkamp F, Hoerning A, Mittmann K, Sibrowski W. Identifying allogeneic platelets by resolution of point mutations in mitochondrial DNA using single-stranded conformational polymorphism PCR. Transfusion 2001;41:1531–8.
6. Augenlicht LH, Heerdt BG. Mitochondria: integrators in tumorigenesis? Nat Genet 2001;28:104–5.
7. Bartnik E, Lorenc A, Mroczek K. Human mitochondria in health, disease, ageing and cancer. J Appl Genet 2001; 42:65–71.
8. Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. Mutat Res 2001; 488:9–23.
9. Zhu W, Qin W, Bradley P, Wessel A, Puckett CL, Sauter ER. Mitochondrial DNA mutations in breast cancer tissue and in matched nipple aspirate fluid. Carcinogenesis 2005;26:145–52.
10. Filosto M, Tomelleri G, Tonin P, Scarpelli M, Vattemi G, Rizzuto N, et al. Neuropathology of mitochondrial diseases. Biosci Rep 2007;27:23–30.
11. Huang DJ, Nelson MR, Zimmermann B, Dudarewicz L, Wenzel F, Spiegel R, et al. Reliable detection of trisomy 21 using MALDI-TOF mass spectrometry. Genet Med 2006;8:728–34.
12. Li Y, Wenzel F, Holzgreve W, Hahn S. Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation. Electrophoresis 2006;27:3889–96.
13. Li Y, Hahn D, Wenzel F, Holzgreve W, Hahn S. Detection of SNPs in the plasma of pregnant women and in the urine of kidney transplant recipients by mass spectrometry. Ann NY Acad Sci 2006;1075:144–7.
14. Zhong XY, Ladewig A, Schmid S, Wight E, Hahn S, Holzgreve W. Elevated level of cell-free plasma DNA is associated with breast cancer. Arch Gynecol Obstet 2007; 276:327–31.
15. Walker JA, Hedges DJ, Perodeau BP, Landry KE, Stoilova N, Laborde ME, et al. Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: application in human identification. Anal Biochem 2005;337: 89–97.
16. Robin ED, Wong R, Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J Cell Physiol 1988;136:507–13.
17. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
18. Li Y, Holzgreve W, Kiefer V, Hahn S. MALDI-TOF mass spectrometry compared with real-time PCR for detection of fetal cell-free DNA in maternal plasma. Clin Chem 2006;52:2311–2.
19. Ding C, Lo YM. MALDI-TOF mass spectrometry for quantitative, specific, and sensitive analysis of DNA and RNA. Ann NY Acad Sci 2006;1075:282–7.
20. Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, et al. Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Sci Int 2004;28:139: 141–9.

Paper 3.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for genotyping of human platelet-specific antigens

Henk S.P. Garritsen, Alex Xiu-Cheng Fan, Nicole Bosse, Horst Hannig, Reinhard Kelsch, Hartmut Kroll, Wolfgang Holzgreve, and Xiao Yan Zhong

From

The Institute for Clinical Transfusion Medicine, Städtisches Klinikum Braunschweig gGmbH, Braunschweig, Germany;

The Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department Research, University of Basel, Basel, Switzerland;

The Institute of Transfusion Medicine and Transplantation Immunology, Westfaelische Wilhelms-University Münster, Germany; and the Institute for Transfusion Medicine Dessau, Red Cross Blood Transfusion Service NSTOB, Dessau, Germany.

Correspondence to

Prof. Dr Xiao Yan Zhong,

Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department Research, University of Basel, Hebelstrasse 20, Room Nr. 416, CH 4031 Basel, Switzerland; e-mail: xzhong@uhbs.ch.

*** The first two authors contributed equally to this work.**

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for genotyping of human platelet-specific antigens

Henk S.P. Garritsen, Alex Xiu-Cheng Fan, Nicole Bosse, Horst Hannig, Reinhard Kelsch, Hartmut Kroll, Wolfgang Holzgreve, and Xiao Yan Zhong

BACKGROUND: Genotyping of single-nucleotide polymorphisms (SNPs) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging technique, where finally tools for end users have become available to design primers and analyze SNPs of their own interest. This study investigated the potential of this technique in platelet (PLT) genotyping and developed a validated method for genotyping of clinical relevant human PLT antigens (HPAs).

STUDY DESIGN AND METHODS: A multiplex assay using MALDI-TOF MS to analyze six HPA loci (HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15) simultaneously in a single reaction was applied for the genotyping of 100 DNA samples from a cohort of plateletpheresis donors and a patient population (n = 20) enriched for rare alleles. The genotyping results using MALDI-TOF MS were validated by the comparison with the results from typing by polymerase chain reaction with sequence-specific primers and conventional DNA sequencing.

RESULTS: Both homozygous and heterozygous genotypes of HPA-1 to -5 and -15 of the 120 individuals were easily identified by a six-plexed assay on MALDI-TOF MS. The three approaches achieved a 100 percent concordance for the genotyping results of the six HPA loci.

CONCLUSION: Compared to conventional methods, the MALDI-TOF MS showed several advantages, such as a high velocity, the ability to perform multiplexed assays in a single reaction, and automated high-throughput analysis of samples. This enables cost-efficient large-scale PLT genotyping for clinical applications.

Allelic variants of immunogenic glycoproteins on human platelets (PLTs) have been defined by serology. To facilitate the research on these serologically defined alloantigens on PLTs, a consensus nomenclature was introduced: human PLT antigens (HPAs). HPAs are a group of biallelic PLT specific antigens. To date 24 HPAs have been defined by serology, of which 12 are grouped in six biallelic systems (HPA-1, -2, -3, -4, -5, and -15) followed by either an "a" for the major allele or a "b" for the minor allele.[1,2] The molecular basis of 22 of the 24 serologically defined antigens has been resolved.[1] In most cases, the difference of HPA alleles in a certain locus is defined by a single-amino-acid substitution owing to a single-nucleotide polymorphism

ABBREVIATIONS: HPA(s) = human platelet antigen(s); MALDI-TOF MS = matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NAITP = neonatal alloimmune thrombocytopenia; PCR-SSP = polymerase chain reaction with sequence-specific primers; SNP(s) = single-nucleotide polymorphism(s).

From the Institute for Clinical Transfusion Medicine, Städtisches Klinikum Braunschweig gGmbH, Braunschweig, Germany; the Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department Research, University of Basel, Basel, Switzerland; the Institute of Transfusion Medicine and Transplantation Immunology, Westfaelische Wilhelms-University Münster, Germany; and the Institute for Transfusion Medicine Dessau, Red Cross Blood Transfusion Service NSTOB, Dessau, Germany.

Address reprint requests to: Dr Xiao Yan Zhong, Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital/Department Research, University of Basel, Hebelstrasse 20, Room Nr. 416, CH 4031 Basel, Switzerland; e-mail: xzhong@uhbs.ch.

The first two authors contributed equally to this work.

Received for publication May 23, 2008; revision received August 18, 2008; and accepted August 18, 2008.

doi: 10.1111/j.1537-2995.2008.01953.x

TRANSFUSION 2009;49:252-258.

(SNP) in the gene encoding the relevant membrane glycoprotein.[3]

The clinical relevance of these polymorphisms is demonstrated by several immune-mediated PLT disorders.[4,5] In prenatal medicine, the most well-known alloimmune thrombocytopenic syndrome is the neonatal alloimmune thrombocytopenia (NAITP), which is caused by passively transmitted maternal antibodies specific against paternal inherited fetal HPAs.[6] In transfusion medicine, mismatches between donor and recipient for HPAs can cause refractoriness to PLT transfusions and posttransfusion purpura.[7] Matching of HPAs can improve the success of PLT transfusion and stem cell transplantation.[8-10]

Several technical approaches have been postulated and published for typing the relevant PLT antigens either by serology, for example, the monoclonal antibody–specific immobilization of PLT antigens, or by genotyping the relevant SNPs from the HPA system.[11-15] Several molecular methods, such as high-resolution amplicon melting, closed-tube fluorescent assay, or polymerase chain reaction with sequence-specific primers (PCR-SSP), are available for HPA genotyping.[12-15] However, those methods are time- and cost-consuming. Therefore, it is necessary to develop robust, cost-effective diagnostic techniques for HPA typing.

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers a solution for high-throughput qualitative and quantitative analysis of up to 36 to 40 multiple SNPs in a single reaction. MALDI-TOF MS is a relatively new technique. It has been adapted for creating ionized gas-phase DNA molecules, which are accelerated in an electric field, followed by a flight through a vacuum chamber to a detector. This detection method is based on the time taken by each particle to fly to a detector ("time of flight"). The time of flight is proportional to the mass to charge ratio (m/z) of a particle and inversely related to its velocity. The time of flight is a specific characteristic of each DNA fragment and depends mainly on its base composition. This situation allows us to discriminate DNA fragments that differ in only one base (SNPs) and by specific design of the size of the DNA fragments to test multiple SNPs in one reaction. In our group, MALDI-TOF MS has been used successfully for genotyping fetal SNPs in maternal blood for noninvasive prenatal diagnosis of genetic diseases.[16] MALDI-TOF MS can differentiate between two alleles differing by as little as one base with high automation.

In this study, we developed a multiplex assay by using MALDI-TOF MS for typing the six HPAs (HPA-1 to -5 and -15) simultaneously in a single reaction. We validated the genotyping results using MALDI-TOF MS by comparison with the results from genotyping by PCR-SSP and conventional DNA sequencing.

MATERIALS AND METHODS

Donors

Five-milliliter ethylenediaminetetraacetate-blood samples were obtained from 100 unrelated plateletpheresis donors from the Institute for Clinical Transfusion Medicine at the Staedtisches Klinikum Braunschweig in Braunschweig, Germany. To overcome the relative low frequency of rare HPA alleles in our samples, we added 20 DNA samples from patients.

DNA extraction

DNA was extracted from 150 µL of peripheral blood by using a magnetic bead-based system (Invitek, Berlin, Germany) according to the instructions supplied by the manufacturer. The concentration of DNA in each sample was measured using a spectrophotometer (NanoDrop-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE).

HPA genotyping using PCR-SSP

PCR-SSP was performed using a commercially available PCR-SSP kit (HPA-Type, BAG Systems, Lich, Germany). PCR preparation was performed according to the recommendations of the manufacturer. Briefly a master mix was prepared consisting of 10× PCR buffer, DNA solution, *Taq* polymerase (Q-biogene, MP-Biomedicals, Heidelberg, Germany) and aqua dest.

The isolated DNA was of excellent purity, as judged by the A_{260}/A_{280} value of approximately 1.8. We therefore reduced the recommended amount of DNA (50-100 ng per reaction) to 10 ng per reaction. The PCR conditions were as follows: 5 minutes at 96°C followed by 5 cycles of 10 seconds at 96°C (denaturation), 60 seconds at 70°C (annealing + extension); 10 cycles of 10 seconds at 96°C (denaturation), 50 seconds at 65°C (annealing), and 45 seconds and 72°C (extension); 15 cycles of 10 seconds at 96°C (denaturation), 50 seconds at 61°C (annealing), and 45 seconds at 72°C (extension); and a final extension step for 5 minutes at 72°C.

PCR amplification products were visualized by ultraviolet illumination in 2 percent agarose gel stained with ethidium bromide. The gels were then analyzed by comparing the bands of control samples, donor samples, and size markers.

DNA sequencing

In a small number of cases,[5] we checked the results of PCR-SSP and MALDI-TOF MS by DNA sequencing. For sample preparation, a PCR purification kit (MinElute, Qiagen, Hilden, Germany) was used. Purified PCR

(SNP) in the gene encoding the relevant membrane glycoprotein.[3]

The clinical relevance of these polymorphisms is demonstrated by several immune-mediated PLT disorders.[4,5] In prenatal medicine, the most well-known alloimmune thrombocytopenic syndrome is the neonatal alloimmune thrombocytopenia (NAITP), which is caused by passively transmitted maternal antibodies specific against paternal inherited fetal HPAs.[6] In transfusion medicine, mismatches between donor and recipient for HPAs can cause refractoriness to PLT transfusions and posttransfusion purpura.[7] Matching of HPAs can improve the success of PLT transfusion and stem cell transplantation.[8-10]

Several technical approaches have been postulated and published for typing the relevant PLT antigens either by serology, for example, the monoclonal antibody–specific immobilization of PLT antigens, or by genotyping the relevant SNPs from the HPA system.[11-15] Several molecular methods, such as high-resolution amplicon melting, closed-tube fluorescent assay, or polymerase chain reaction with sequence-specific primers (PCR-SSP), are available for HPA genotyping.[12-15] However, those methods are time- and cost-consuming. Therefore, it is necessary to develop robust, cost-effective diagnostic techniques for HPA typing.

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers a solution for high-throughput qualitative and quantitative analysis of up to 36 to 40 multiple SNPs in a single reaction. MALDI-TOF MS is a relatively new technique. It has been adapted for creating ionized gas-phase DNA molecules, which are accelerated in an electric field, followed by a flight through a vacuum chamber to a detector. This detection method is based on the time taken by each particle to fly to a detector ("time of flight"). The time of flight is proportional to the mass to charge ratio (m/z) of a particle and inversely related to its velocity. The time of flight is a specific characteristic of each DNA fragment and depends mainly on its base composition. This situation allows us to discriminate DNA fragments that differ in only one base (SNPs) and by specific design of the size of the DNA fragments to test multiple SNPs in one reaction. In our group, MALDI-TOF MS has been used successfully for genotyping fetal SNPs in maternal blood for noninvasive prenatal diagnosis of genetic diseases.[16] MALDI-TOF MS can differentiate between two alleles differing by as little as one base with high automation.

In this study, we developed a multiplex assay by using MALDI-TOF MS for typing the six HPAs (HPA-1 to -5 and -15) simultaneously in a single reaction. We validated the genotyping results using MALDI-TOF MS by comparison with the results from genotyping by PCR-SSP and conventional DNA sequencing.

MATERIALS AND METHODS

Donors

Five-milliliter ethylenediaminetetraacetate-blood samples were obtained from 100 unrelated plateletpheresis donors from the Institute for Clinical Transfusion Medicine at the Staedtisches Klinikum Braunschweig in Braunschweig, Germany. To overcome the relative low frequency of rare HPA alleles in our samples, we added 20 DNA samples from patients.

DNA extraction

DNA was extracted from 150 µL of peripheral blood by using a magnetic bead-based system (Invitek, Berlin, Germany) according to the instructions supplied by the manufacturer. The concentration of DNA in each sample was measured using a spectrophotometer (NanoDrop-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE).

HPA genotyping using PCR-SSP

PCR-SSP was performed using a commercially available PCR-SSP kit (HPA-Type, BAG Systems, Lich, Germany). PCR preparation was performed according to the recommendations of the manufacturer. Briefly a master mix was prepared consisting of 10× PCR buffer, DNA solution, *Taq* polymerase (Q-biogene, MP-Biomedicals, Heidelberg, Germany) and aqua dest.

The isolated DNA was of excellent purity, as judged by the A_{260}/A_{280} value of approximately 1.8. We therefore reduced the recommended amount of DNA (50-100 ng per reaction) to 10 ng per reaction. The PCR conditions were as follows: 5 minutes at 96°C followed by 5 cycles of 10 seconds at 96°C (denaturation), 60 seconds at 70°C (annealing + extension); 10 cycles of 10 seconds at 96°C (denaturation), 50 seconds at 65°C (annealing), and 45 seconds and 72°C (extension); 15 cycles of 10 seconds at 96°C (denaturation), 50 seconds at 61°C (annealing), and 45 seconds at 72°C (extension); and a final extension step for 5 minutes at 72°C.

PCR amplification products were visualized by ultraviolet illumination in 2 percent agarose gel stained with ethidium bromide. The gels were then analyzed by comparing the bands of control samples, donor samples, and size markers.

DNA sequencing

In a small number of cases,[5] we checked the results of PCR-SSP and MALDI-TOF MS by DNA sequencing. For sample preparation, a PCR purification kit (MinElute, Qiagen, Hilden, Germany) was used. Purified PCR

products were applied to the fluorescent dye terminator cycle sequencing reaction with a cycle sequencing kit (Big Dye Terminator, Applied Biosystems, Darmstadt, Germany). Thereafter, the sequencing products were purified with a spin kit (DyeEx, Qiagen) and analyzed on a sequencer (ABI 3130, Applied Biosystems). Nucleotide sequence alignment was performed with computer software (DNA Star, DNASTAR, Inc., Madison, WI). All sequencing reactions were performed in both directions and tested for concordance.

HPA genotyping using MALDI-TOF MS

Primer design

The MALDI-TOF MS assay is based on an allele-specific primer extension reaction, which enables the differentiation of homozygous and heterozygous samples. The positions and natures of the HPA polymorphisms are shown in Table 1. According to the polymorphisms, the PCR primers and extension primers for use in determining HPA-1a, HPA-1b, HPA-2a, HPA-2b, HPA-3a, HPA-3b, HPA-4a, HPA-4b, HPA-5a, HPA-5b, HPA-15a, and HPA-15b in a single multiplex reaction were designed using computer software (MassArray Assay Design v.3.4, Sequenom, Inc., San Diego, CA). The primer sequences are listed in Table 1 and were synthesized and purified by high-performance liquid chromatography by Microsynth (Balgach, Switzerland).

PCR amplification

The multiplex PCR reactions for amplifying the six HPAs in a single tube were carried out in 10-µL PCR cocktail mixes containing 5 to 10 ng of DNA, 1.626 mmol per L $MgCl_2$, 500 µmol per L dNTP mix, 0.5 U of Hot-star *Taq* Gold polymerase, and six primer pairs. The amplification was incubated at 94°C for 15 minutes, followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute. A final extension at 72°C for 5 minutes was performed.

TABLE 1. Sequences of PCR primers and extension primers for the MALDI-TOF MS assay

Position of HPA polymorphisms	Primer sequences
HPA-1	Forward: ACGTTGGATGTTGCTGGACTTCTCTTTGGG
176 T (a)-C (b)	Reverse: ACGTTGGATGCAGATTCTCCTTCAGGTCAC
	Extension primer: CTTACAGGCCCTGCCTC
HPA-2	Forward: ACGTTGGATGACCTGAAAGGCAATGAGCTG
482 C (a)-T (b)	Reverse: ACGTTGGATGTTAGCCAGACTGAGCTTCTC
	Extension primer: CTGCCCCCAGGGCTCCTGA
HPA-3	Forward: ACGTTGGATGTGGGCCTGACCACTCCTTTG
2621 T (a)-G (b)	Reverse: ACGTTGGATGTGCGATCCCGCTTGTGATG
	Extension primer: GTGGACTGGGGGCTGCCCA
HPA-4	Forward: ACGTTGGATGATCTGTGGAGCATCCAGAAC
506 G (a)-A (b)	Reverse: ACGTTGGATGGAAGCCAATCCGCAGGTTAC
	Extension primer: GGTTACTGGTGAGCTTT
HPA-5	Forward: ACGTTGGATGAGGAAGGAAGAGTCTACCTG
1600 G (b)-A (a)	Reverse: ACGTTGGATGGCAAGTTAAATTACCAGTAC
	Extension primer: AGTCTACCTGTTTACTATCAAA
HPA-15	Forward: ACGTTGGATGCAAAATGTATCAGTTCTTGG
2108 C (a)-A (b)	Reverse: ACGTTGGATGGAATCAGGTACAGTTACTTC
	Extension primer: TTCAAATTCTTGGTAAATCCTG

TABLE 2. Genotype distribution in 100 German plateletpheresis donors as determined by PCR-SSP/sequencing and MALDI-TOF MS

	a/a		a/b		b/b	
HPA	SSP/sequencing	MALDI-TOF	SSP/sequencing	MALDI-TOF	SSP/sequencing	MALDI-TOF
1	69	69	30	30	1	1
2	85	85	14	14	1	1
3	36	36	45	45	19	19
4	100	100	0	0	0	0
5	82	82	18	18	0	0
15	28	28	51	51	21	21

TABLE 3. Allele frequencies (%) in 100 German plateletpheresis donors as determined by SSP-PCR/sequencing and MALDI-TOF MS

HPA	a	b
1	77	23
2	87	13
3	56	44
4	100	0
5	85	15
15	52	48

TABLE 4. Genotype distribution in 20 patients enriched for rare HPA alleles as determined by SSP-PCR/sequencing and MALDI-TOF MS

	b/b	
HPA	SSP/sequencing	MALDI-TOF
1	16	16
2	1	1
3	4	4
4	2	2
5	1	1
15	9	9

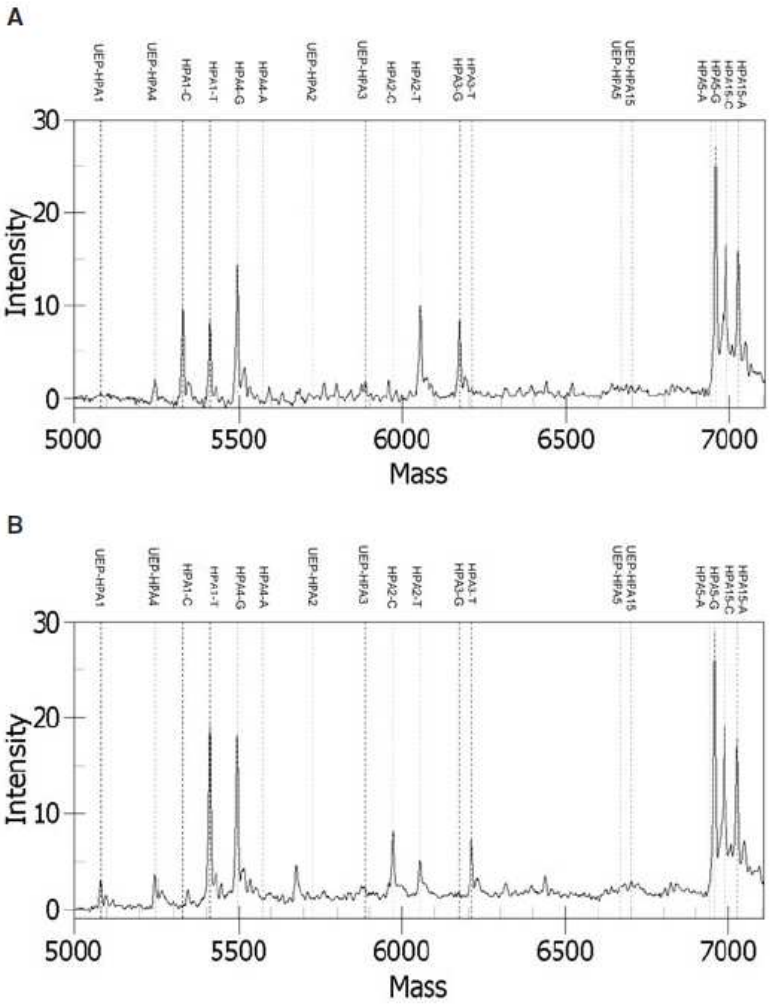


Fig. 1. (A-D) Four example data of six-plexed MALDI-TOF MS analysis of HPA-1 to -5 and -15. The genotypes of HPA-1 to -5 and -15 from the example cases are displayed separately on the graphs A to D. The peaks corresponding to the HPA alleles are indicated with different letters (HPA-1 C/T, HPA-2 C/T, HPA-3 G/T, HPA-4 G/A, HPA-5 A/G and HPA-15 A/C). The A, T, G, or C represents the allele, where the polymorphic site has an A, T, G, or C residue, respectively. The HPA genotypes of Case A are HPA-1ab, -2bb, -3bb, -4aa, -5aa, and -15ab; the HPA genotypes of Case B are HPA-1aa, HPA-2ab, HPA-3aa, HPA-4aa, HPA-5aa, and HPA-15ab; the HPA genotypes of Case C are HPA-1aa, HPA-2aa, HPA-3bb, HPA-4aa, HPA-5aa, and HPA-15ab; and the HPA genotypes of Case D are HPA-1ab, HPA-2aa, HPA-3ab, HPA-4aa, HPA-5aa, and HPA-15ab.

Shrimp alkaline phosphatase treatment

To remove the non-incorporated dNTPs, shrimp alkaline phosphatase treatment was performed after the PCR procedure. Four microliters of shrimp alkaline phosphatase solution (Sequenom, Inc.) was added into the 10 µL of PCR product. The mixture was incubated under the following conditions: 37°C for 20 minutes, 85°C for 5 minutes, and cooling to 4°C.

Primer extension reaction

The iPLEX Gold reactions were performed using the iPLEX gold cocktail mix (Sequenom, Inc.), which contains buffer, termination mix, enzyme, and extension primers. The following conditions were used: 94°C for 30 seconds, followed by 45 cycles of 94°C for 5 seconds, 52°C for 5 seconds, 80°C for 5 seconds, final extension at 72°C for 5 minutes, and then cooling to 4°C.

The iPLEX Gold reaction products were desalted by adding additional 32 µL of distilled H_2O and 6 mg of resin (SpectroCLEAN, Sequenom, Inc.) to optimize mass-spectrometric analysis according to the protocol developed by Sequenom.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed using a mass array compact system (Bruker Daltonics, Inc., Billerica, MA).

Fifteen to twenty-five nanoliters of the desalted iPLEX Gold reaction products were dispensed onto a 384-format bioarray (SpectroCHIP, Sequenom, Inc.) using a nanodispenser (MassARRAY, Samsung, Daegu, S. Korea) for MALDI-TOF MS analysis. Software (MassARRAY Workstation, Version 3.4, Sequenom, Inc.) was used to process and analyze the iPLEX SpectroCHIP bioarrays.

To perform MALDI-TOF MS analysis, the system is equipped with an N_2 laser with 337-nm wavelength (pulse max energy of 100 µJ and 0.5-ns pulse width) for use with matrix components absorbing light of this wavelength. For our analysis the laser power was turned to 41 percent.

Internal and external calibration was performed using a 3-oligo calibrant (Sequenom, Inc.) and H_2O. The mass accuracy is higher than 95 percent. Peaks were considered significant if they were 50 percent higher than surrounding salt peaks.

The call rate, extension rate, and peak area for all allele-specific analytes in any given assay were calculated using the software provided by the manufacturer. The data were recorded and interpreted by software (MassARRAY TYPER, Sequenom, Inc.).

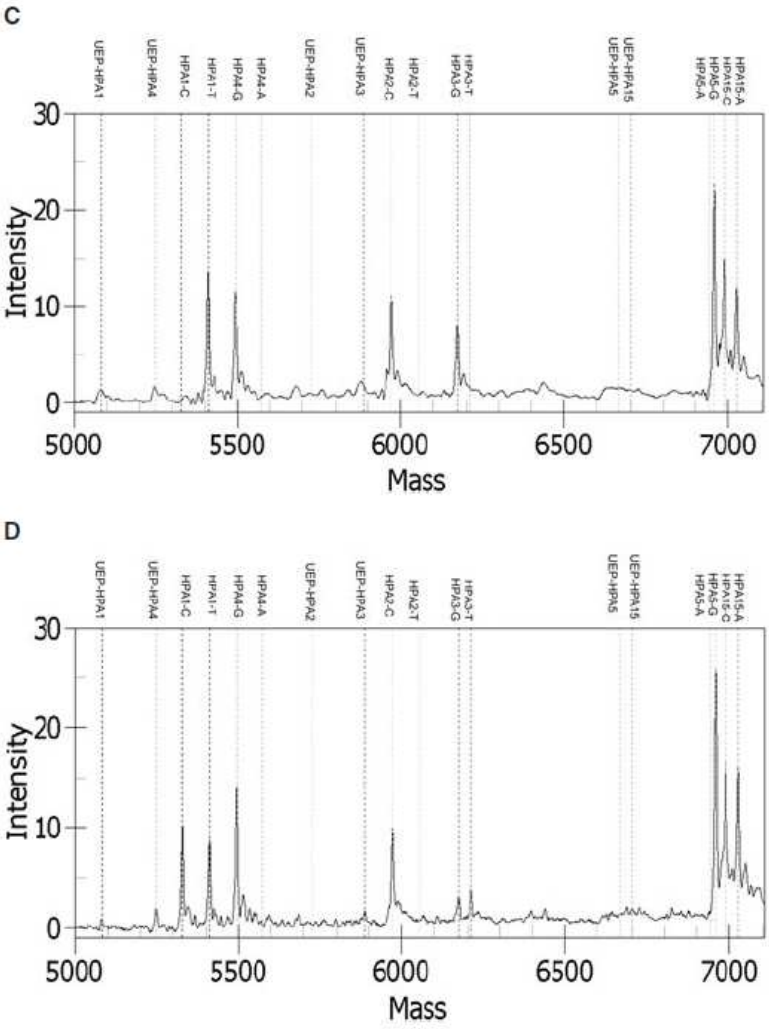


Fig. 1. Continued.

RESULTS

HPA genotyping using PCR-SSP and DNA sequencing

The results of the HPA genotyping are presented in Tables 2 through 4. In total we analyzed 720 SNPs. In three cases we performed DNA sequencing to check the PCR-SSP results. Tables 2 and 3 show the HPA genotype distribution and the HPA allele frequencies that are expected in this donor population. In Table 4 we present the results of the patient population (n = 20) enriched for rare alleles.

HPA genotyping using MALDI-TOF MS

After designing and testing the primer sets for specific HPA SNPs presented in Table 1, we combined those six primer sets into a single reaction using a multiplex assay. We could detect alleles of the six HPAs in all samples of the 100 donors. The individuals have at least 6 alleles and a maximum of 10 alleles. Figure 1 shows example records of HPA genotypes from four donors, which are interpreted by the MassARRAY TYPER software. At 176, 482, 2621, 506, 1600, and 2108, the possible nucleotides are listed in Table 1. Each peak displayed on the graphs represents an HPA allele (HPA-1 to -5 and -15, a and/or b alleles). Homozygous samples (a or b) show single peaks whereas heterozygous samples (a and b) contain both peaks. The "a" allele was identified in 79 to 100 percent of the donors, representing the major allele, and the "b" allele was identified in 0-75 percent of the cases, almost representing the minor allele. Zero to fifty-two percent of the individuals are heterozygous for HPA-1 to -5 and -15 and 48 to 100 percent of the donors are homozygous for those HPAs (Tables 2 and 3).

Validation studies

We validated our genotyping results by comparison with the results from PCR-SSP and DNA sequencing analysis. No discrepant results were observed for the HPA -1, -2, -3, -4, -5, and -15, showing a 100 percent concordance between the novel MALDI-TOF MS and the conventional methods. In three cases (1× HPA-3, 1× HPA-5, 1× HPA-15) we repeated the MALDI-TOF MS analysis because of poor quality of MS data. After repeating the three cases, a 100 percent concordance (720/720 SNPs) for the genotyping of HPA-1 to -5 and -15 between the MALDI-TOF MS assay and sequencing analysis was achieved. In the case of HPA-15, where a homozygous type was found by conventional methods (HPA-15aa), the MALDI-TOF MS analysis initially showed a potential heterozygous pattern. However the second peak was small and was shown not to be in the exact position for the second SNP (HPA-15b). The height of the peak did not fulfill our criteria for a positive peak. We therefore concluded that the second peak did not derive from a HPA-15b allele (Fig. 2). The 100 percent concordancy of MALDI-TOF MS results and conventional methods was also shown for the 20 samples enriched for rare alleles (Table 3).

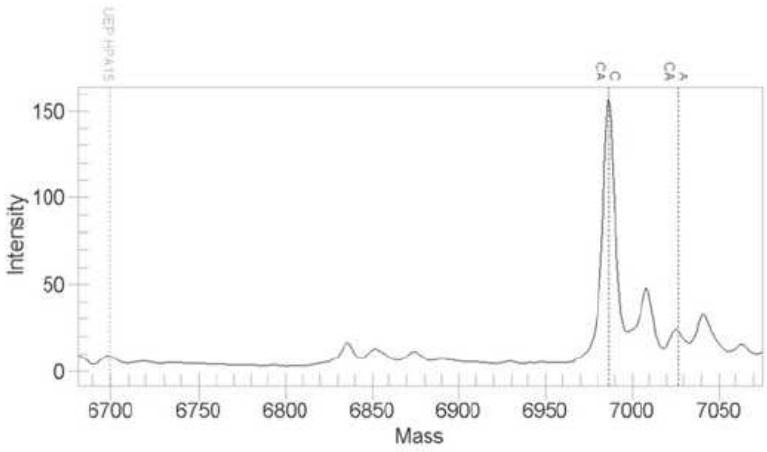


Fig. 2. HPA-15 retesting: the figure shows a very small peak around allele b position but it is not the exact position and it is even smaller than the other two salt peaks around it. We therefore reported the sample as HPA-15a homozygous.

DISCUSSION

We developed a multiplexed assay for genotyping of HPA-1 to -5 and -15 using high-throughput MALDI-TOF MS. Blood samples from 100 donors and 20 patients were analyzed by the assay.

Our data show that most donors are positive for the presence of HPA-1a (99%), HPA-2a (99%), HPA-4a (100%), and HPA-5a (100%). Most individuals are homozygous for these alleles and, as expected, the "b" alleles show a much lower frequency in the donor population. Each donor carries at least 6 alleles and a maximum 10 alleles. In transfusion medicine, PLT genotyping has evolved to an important diagnostic tool to aid in diagnosis and therapy of relevant PLT disorders. Transfusions with HPA-1a–negative PLTs are important therapeutic options.[17] In prenatal medicine, it was reported that the NAITP is caused in the majority of cases by fetomaternal mismatches of HPA-1 or HPA-5 in Caucasians and HPA-3 or HPA-4 mismatches are frequently in Asians.[5,18-20] Therefore, HPA genotyping for HPA-1 incompatibility alone is not sufficient to fully evaluate NAITP cases. In our study, the multiplexed assay using MALDI-TOF MS enabled simultaneous genotyping of six HPAs and 12 alleles in a single reaction. Comparison of the genotypes of the 100 donors and 20 patients by the multiplexed assay using MALDI-TOF MS with those derived from single assays using PCR-SSP and the conventional sequencing method showed a 100 percent concordance of results.

The MALDI-TOF MS assay is sensitive in the discrimination of homozygous and heterozygous bases in SNP positions. In our group, using the approach, Li and colleagues[16] could successfully determinate rare fetal-derived and paternal inherited mutations in the maternal circulation, suggesting that this method can be applied in the risk-free noninvasive prenatal diagnosis of NAITP before delivery based on cell-free fetal DNA. In prenatal medicine, the amount of material for analysis is mostly a limiting factor. The technique proposed here uses nanoliter amounts for analysis. In fact, one of the rare samples (HPA-4bb) was available only in 1-µL volume. We did not have any problem performing a complete six-antigen analysis with this limited amount of sample.

The MS approach is completely automated and suitable for high-throughput analysis of as much as 384 samples running on one chip. The stability of this automated method makes it suitable as a routine testing tool for PLT genotyping. Detection of multiple HPAs in a single reaction enhances velocity and reduction of reaction volume will lead to a cost reduction in clinical laboratories. Although the initial purchase of mass-spectrometric equipment is at this moment rather costly, the running costs for assays like the one we present here can be very low (as low as 3.5 cents per genotype). Our data demonstrated that it is possible to perform genotyping of six different HPA loci (12 alleles) in a single tube, eliminating the need of the time- and cost-consuming gel electrophoresis or fluorescent labeling. The data suggest that this technique could be a serious competitor in the market for SNP analysis in transfusion medicine.

ACKNOWLEDGMENT

The authors thank Dr Sentot Santoso, Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany, for providing us with an HPA-4b/b patient sample.

REFERENCES

1. Metcalfe P, Watkins NA, Ouwehand WH, Kaplan C, Newman P, Kekomaki R, De Haas M, Aster R, Shibata Y, Smith J, Kiefel V, Santoso S. Nomenclature of human platelet antigens. Vox Sang 2003;85:240-5.
2. Santoso S. Human platelet alloantigens. Transfus Apher Sci 2003;28:227-36.
3. Kroll H, Carl B, Santoso S, Bux J, Bein G. Workshop report on the genotyping of blood cell alloantigens. Transfus Med 2001;11:211-9.
4. Carl B, Kroll H, Bux J, Bein G, Santoso S. B-lymphoblastoid cell lines as a source of reference DNA for human platelets and neutrophil antigen genotyping. Transfusion 2000;40:62-8.

5. Kroll H, Yates J, Santoso S. Immunization against a low-frequency human platelet alloantigen in fetal alloimmune thrombocytopenia is not a single event: characterization by the combined use of reference DNA and novel allele-specific cell lines expressing recombinant antigens. Transfusion 2005;45:353-8.
6. Kaplan C. Neonatal alloimmune thrombocytopenia: a 50-year story. Immunohematology 2007;23:9-13.
7. Rosenberg N, Dardik R. Post-transfusion purpura—when and why? Isr Med Assoc J 2006;8:709-10.
8. Cooling L. ABO and platelet transfusion therapy. Immunohematology 2007;23:20-33.
9. Golovkina LL, Kutyina RM, Savchenko VG. Genetic differences by platelet-specific antigens used for monitoring allomyelotransplant engraftment. Bull Exp Biol Med 2006; 141:507-12.
10. Leitner GC, Stiegler G, Kalhs P, Greinix HT, Rabitsch W, Sillaber C, Hoecker P, Panzer S. The influence of human platelet antigen match on the success of allogeneic peripheral blood progenitor cell transplantation following a reduced-intensity conditioning regimen. Transfusion 2005; 45:195-201.
11. Hurd CM, Cavanagh G, Schuh A, Ouwehand WH, Metcalfe P. Genotyping for platelet-specific antigens: techniques for the detection of single nucleotide polymorphisms. Vox Sang 2002;83:1-12.
12. Meyer O, Agaylan A, Bombard S, Kiesewetter H, Salama A. A novel antigen-specific capture assay for the detection of platelet antibodies and HPA-1a phenotyping. Vox Sang 2006;91:324-30.
13. Liew M, Nelson L, Margraf R, Mitchell S, Erali M, Mao R, Lyon E, Wittwer C. Genotyping of human platelet antigens 1 to 6 and 15 by high-resolution amplicon melting and conventional hybridization probes. J Mol Diagn 2006;8:97-104.
14. Meyer O, Abou-Chaker K, Heymann G, Bombard S, Kiesewetter H, Salama A. Human platelet antigen genotyping by using sequence-specific primers and the particle gel agglutination assay. Vox Sang 2005;88:271-4.
15. Prager M. Molecular genetic blood group typing by the use of PCR-SSP technique. Transfusion 2007;47 1 Suppl:54S-9S.
16. Li Y, Wenzel F, Holzgreve W, Hahn S. Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation. Electrophoresis 2006;27:3889-96.
17. Kroll H, Kiefel V, Mueller-Eckhardt C. [Clinical and serologic studies in 34 patients with post-transfusion purpura]. Beitr Infusionsther 1992;30:403-7.
18. Blanchette VS, Johnson J, Rand M. The management of alloimmune neonatal thrombocytopenia. Baillieres Best Pract Res Clin Haematol 2000;13:365-90.
19. Ohto H. [Neonatal alloimmune thrombocytopenia]. Nippon Rinsho 1997;55:2310-4.
20. Mueller-Eckhardt C, Kiefel V, Grubert A, Kroll H, Weisheit M, Schmidt S, Mueller-Eckhardt G, Santoso S. 348 cases of suspected neonatal alloimmune thrombocytopenia. Lancet 1989;1:363-6.

Paper 4.

Levels of Plasma Circulating Cell Free Nuclear and Mitochondrial DNA as Potential Biomarkers for Breast Tumours

Corina Kohler[1], Ramin Radpour[1], **Alex Xiu-Cheng Fan[1]**, Zeinab Barekati[1],Reza Asadollahi[1], Johannes Bitzer[1], Wolfgang Holzgreve[2], Xiao Yan Zhong[1]

1. **Laboratory for Prenatal Medicine and Gynaecologic Oncology, Women's Hospital / Department Biomedicine, University of Basel, Switzerland.**
2. **University Medical Centre of Freiburg, Germany.**

Correspondence to:

Prof. Dr. Xiao Yan Zhong

Laboratory for Prenatal Medicine and Gynaecologic Oncology

Women's Hospital / Department Biomedicine

University of Basel

Hebelstrasse 20, Room Nr. 416

CH 4031 Basel

Switzerland

Tel: ++41 61 265 9224 / 9595

Fax: ++41 61 265 9399

e-mail:zhongx@uhbs.ch

Abstract

With the aim to simplify cancer management, cancer research lately dedicated itself more and more to discover and develop non-invasive biomarkers. In this connection, circulating cell-free (ccf) DNA seems to be a promising candidate. Altered levels of ccf nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have been found in several cancer types, suggesting that these two species might have a diagnostic value.

Using multiplex real-time PCR we investigated the levels of ccf nDNA and mtDNA in plasma samples from patients with malignant and benign breast tumors, and from healthy controls. To evaluate the applicability of plasma ccf nDNA and mtDNA as a biomarker for distinguishing between the three study-groups we performed ROC (Receiver Operating Characteristic) curve analysis. We also compared the levels of both species in the cancer group with clinicopathological parameters.

While the level of ccf nDNA in the cancer group were significantly higher in comparison with the benign tumour group and the healthy control group, the level of ccf mtDNA was found to be significantly lower in the two tumour-groups. The level of ccf nDNA was also associated with tumor-size. Using ROC curve analysis, we were able to distinguish between the breast cancer cases and the healthy controls using ccf nDNA as marker and between the tumour group and the healthy controls using ccf mtDNA as marker.

Our data suggest that both species might have a potential as biomarkers in breast tumour management. However, ccf nDNA seems to be the stronger biomarker regarding sensitivity and specificity.

Key words: Circulating cell-free DNA, mitochondrial DNA, breast cancer, real-time PCR

Introduction

In several branches of biomedical research the quest for new disease-related biomarkers has become one of the main objectives [1-3]. When it comes to discover and develop new biomarkers, oncology seems to be the most ambitious field. During the last few years a lot of research has been done finding new cancer biomarkers with the aim to identify high risk individuals, detect cancer at an early stage, predict outcome, monitor and screen for disease recurrence [4]. In this respect the focus is now mainly directed towards the identification of non-invasive cancer biomarkers [5, 6].

In the case of breast cancer, there are a few non-invasive biomarkers for screening, predicting prognosis and monitoring that have come to routine clinical application [7]. Current established methods for routine breast cancer screening encompass in the first place non-invasive methods including clinical breast examination and imaging techniques like, mammography and ultrasonography [8]. However, in case of suspicion of breast cancer all these techniques have to be followed by histopathological analysis for which invasive procedures, such as biopsies, are needed.

Lately, the discovery of circulating cell-free DNA (ccf-DNA) has sparked the interest of cancer scientists as it opens up a new possibility for non-invasive analysis of tumor derived genetic materials. Both, ccf nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have become a matter of research and qualitative as well as quantitative alterations in these species have been implicated with cancer [9]. Changes in the level of ccf nDNA and mtDNA have been found in plasma and serum of patients with various cancer types [10, 11]. In breast cancer patients it has been shown that the ccf nDNA level is elevated in plasma as well as in serum when compared to healthy controls [12, 13]. On the other hand the mtDNA level was mostly found to be decreased in breast cancer patients in comparison with healthy controls [14, 15].

To investigate the potential of ccf nuclear and mitochondrial DNA as a marker for clinical application we examined the level of both species in the malignant and benign tumour groups and healthy controls.

Material and Methods

The study was performed at the Laboratory for Prenatal Medicine and Gynaecological Oncology/Department of Biomedicine, Women's Hospital Basel and approved by the local institutional review board (Ethic commission beider Basel). Written consent forms were collected from all patients who were involved in this study.

Study cohort and sampling procedure

The blood samples used in this study were collected in a time period from 2005 to 2007 in either the Women's Hospital of the University of Basel or the Women's Hospital of Liestal. In total 148 women were included in the study. Most of the women were European Caucasians. All blood samples were taken before any surgical interventions or therapeutic treatments. Patients' data (age, tumour size, lympmph node involvement, extend of metastasis, estrogen receptor, progesterone receptor and Her2neu - status) were obtained from the pathological reports. The blood samples were processed according to a standardized protocol which was described previously elsewhere [16].

The study cohort (n=148) was divided into 3 groups: 1) malignant disease group (n=52); 2) benign disease group (n=26) and 3) healthy control group (n=70). For groups 1 and 2 the diagnoses were all biopsy-confirmed. The healthy control group used in the study did neither have a history of cancer nor suffered from any other severe disease.

Real-time PCR

For the simultaneous quantification of ccf nDNA and mtDNA from plasma a multiplex real-time PCR was performed using the Glyceraldehyd-3-phosphat-dehydrogenase (*GAPDH*) gene and the mtDNA encoded ATPase 8 (*MTATP 8*) gene. The sequences of primers and probes for the *GAPDH* and the *MTATP 8* gene are shown as follows: *GAPDH* (forward): 5' CCC CAC ACA CAT GCA CTT ACC3'; (reverse): 5'CCT AGT CCC AGG GCT TTG ATT 3'; probe5' (MGB) TAG GAA GGA CAG GCA AC (VIC) 3'. *MTATP 8* (forward): 5' AAT ATT AAA CAC AAA CTA CCA CCT ACC 3'; (reverse): 5' TGG TTC TCA GGG TTT GTT ATA 3'; probe: 5' (MGB) CCT CAC CAA AGC CCA TA (FAM) 3'.

The real-time RT-PCR was carried out in 25 µl of total reaction volume containing 7 µl H_2O, 12.5 µl TaqMan® Universal PCR Master Mix, 0.75 µl of each of the above mentioned primers, 1 µl of the FAM-labeled *MTATP 8*-probe and 0.5 µl of the VIC-labeled *GAPDH*-probe. For each reaction 1 µl of DNA was added resulting in a total reaction volume of 25 µl. The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) under the following conditions: an initiation step for 2 minutes at 50°C is followed by a first denaturation for 10 minutes at 95°C and a further step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

For calibration a standard calibrator curve with known genomic DNA concentrations ranging from 3.125×10^4 to 10 pg/µL with a dilution factor of 5 (including 31250, 6250, 1250, 250, 50 and 10 pg/µL) was used.

Statistical Analysis

All statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, USA). The normality distribution of the data was determined using the Shapiro-Wilk-Test. The data were not

normally distributed. For the comparison of cff nDNA and mtDNA levels between the three groups (malignant disease group, benign disease group and healthy control group) the Mann-Whitney-U-Test was performed. For the comparison of the ccf nDNA and mtDNA levels with other established prognostic factors the Mann-Whitney-U-Test and the Kruskal-Wallis-Test were used. *P*-values ≤ 0, 05 were considered statistically significant.

Results

Comparison of plasma ccf nDNA and mtDNA levels between the three study-groups

We compared the levels of plasma ccf nDNA and mtDNA, analyzed by multiplex real-time PCR, between the malignant disease group, the benign disease group and the healthy control group. The level of ccf nDNA in the malignant disease group was significantly higher in comparison with the benign disease group (4678 vs. 1359, Mann-Whitney: $P<0.001$) and the healthy control group (4678 vs.1298, Mann-Whitney: $P<0.001$). No significant difference could be found in the level of nDNA between the benign disease group and the healthy controls (1359 vs. 1298 Mann-Whitney: $P=0.830$).

In contrast to the ccf nDNA determination, a decreased level of ccf mtDNA was found in the malignant disease group when compared with the healthy control group (205013 vs. 522115, Mann-Whitney; $P=0.022$) and the benign disease group (205013 vs. 73977; Mann-Whitney: $P<0.001$). However, the level of ccf mtDNA in the benign disease group is even significantly lower than that in the malignant disease group (73977 vs. 205013; Mann-Whitney: $P=0.001$). Median and range of plasma ccf nDNA and mtDNA are shown in Table 1. The comparison of the ccf nDNA and mtDNA levels between the study groups is depicted in Fig.1.

Correlation between the level of plasma ccf nDNA and mtDNA with clinicopathological parameters

For the malignant disease group, the association between the level of ccf DNA and other established clinical parameters, including tumor size, lymph node involvement, extend of metastasis and the receptor status of estrogen receptor (ER), progesterone receptor (PR) and Her2/neu were analyzed.

Association between plasma ccf DNA level and tumor size in the malignant disease group

The level of ccf nDNA was significantly lower in the patients with breast cancer with a tumour size of less than two centimetres (<2 cm; n=21) than in those with a tumour size between 2 and 5 centimetres (>2cm<5cm; n=25) (2250 vs. 6658; Mann-Whitney-U-Test: $P = 0.034$). Only four patients with a tumour size of more than five centimetres (>5 cm) were recruited. There was no significant difference in the level of ccf nDNA between patients with a tumour size of more than five centimetres (>5 cm) and a tumour size from 2 to 5 centimetres (2 cm-5 cm). No association between the levels of ccf mtDNA and the tumor size could be found. The association between the ccf nDNA level and the tumor size is depicted in Fig.2.

Association between plasma ccf DNA level and lymph node involvement, extend of metastasis, receptor status of ER, PR and Her2/neu amplification in the malignant disease group

In the malignant disease group no statistical significance in the level of neither ccf nDNA nor mtDNA between node negative and node positive patients, extend of metastasis, receptor status of ER, PR and Her2/neu amplification could be found.

The applicability of plasma ccf nDNA and mtDNA as marker for the discrimination between the three study groups

To evaluate the applicability of ccf plasma nDNA and mtDNA as a marker for distinguishing between the malignant, the benign disease groups and the healthy control group, we performed ROC (Receiver Operating Characteristic) curve analysis. For the identification of the optimal cut-off point we used the Youden index (J). J is the maximum vertical distance between the ROC-curve and the diagonal reference line and is defined as J = maximum (sensitivity) + (specificity) – 1. The Youden index allows the selection of an optimal cut-off point under the assumption that sensitivity and specificity are equally weighted [17].

ROC curve analysis using ccf nDNA for the discrimination between the malignant disease group and the control group

Level of ccf nDNA in the malignant disease group was significantly higher in comparison with the the healthy control group, but no significant difference was found in the level of ccf nDNA between the benign disease group and the healthy controls. For discriminating between the malignant disease group and the healthy control group, an optimal cut-off point was indicated at 1866 GE/ml for plasma ccf nDNA with a sensitivity of 81 % and a specificity of 69 % (AUC=0.80, $P<0.001$, 95% confidence interval=0.732-0.885). The ROC-curve for discrimination between the malignant disease group and the healthy control group using ccf nDNA is shown in Fig.3.

ROC curve analysis using ccf mtDNA for the discrimination between the breast tumour group and the healthy control group

Decreased levels of ccf mtDNA was found in both, the benign disease group and the malignant disease group, when compared to the healthy control group. For discriminating between the breast tumour group (malignant and benign) and the healthy control group an optimal cut-off point was indicated at 463282 GE/ml for ccf nDNA with a sensitivity 53 % and a specificity of 87 % (AUC=0.68, P<0.001, 95% confidence interval=0.589-0.768). The ROC-curve for discrimination between the breast tumour group and the healthy control group using ccf mtDNA is shown in Fig.3.

Discussion

In our study, according to our knowledge, for the first time we found increased levels of ccf nDNA and simultaneously decreased levels of ccf mtDNA in plasma samples from patients with breast tumour compared to normal controls. The former shows a probable diagnostic value in discriminating between breast cancer and normal controls with a sensitivity of 81 % and specificity of 69 % , the latter reveals possible relevance in distinguishing between breast tumours (malignant and benign) and normal controls with a sensitivity of 53 % and specificity of 87 %.

For ccf nDNA, in our previous work, we observed that in comparison with other potential circulating biomarkers involved in malignancy, such as nucleosomes, vascular endothelial growth factor (VEGF) and its soluble receptor (sVEGFR1), the ccf DNA showed more sensitivity and specificity in discriminating between breast cancer and normal controls [18 , 19]. Recently, Diehl et al, explored the possibility of using ccf tumour derived DNA for the management of colorectal cancer [20]. Patients with detectable ccf tumour DNA suffered from a relapse, whereas subjects without circulating tumour DNA did not experience tumour recurrence. The ccf tumour DNA

detection seems to be more reliable for predicting a relapse than the standard biomarker, carcinoembryonic antigen (CEA), used for the management of colorectal cancer [21]. It was also reported that the levels of ccf DNA could be changed after therapy in breast cancer [22, 23]. The observations suggest that determination of ccf DNA in cancer may be an useful tool in the management of the condition.

In this study, we found high levels of ccf plasma DNA related to tumour size. The finding can be supported by the investigations in the field of prenatal medicine. Placenta has been regarded as a "pseudomalignant". Placental derived ccf fetal DNA in maternal circulation can be used for risk-free prenatal diagnosis [24-27]. The concentration of placental derived ccf fetal DNA in maternal blood increases with the progress in gestational weeks regarding placental size [28]. Using fetal specific DNA sequences, ccf fetal DNA could be detected from the 5th gestational week, and the results were reliable by the 8th gestational week with an accuracy of 100% in fetal DNA determination [29, 30]. The results imply that using tumour specific genetic alteration as marker, tumour derived ccf DNA may be detectable in early stage with small tumour size for early diagnosis.

For mtDNA, either down-or up-regulation in cancer patients has been shown before, and many attempts to explain both events have been made. While up-regulation of mtDNA in cancer patients was only shown in a few cases [31], many studies including this one found decreased mtDNA levels in cancer patients [32-34]. One explanation for lower mtDNA copy numbers in cancer patients might be ascribed to mutations or deletions occurring as a consequence of exposure of mtDNA to reactive oxygen species (ROS) which are a by-product of respiration and oxidative phosphorylation. Especially in the D-Loop region which controls replication and transcription of mtDNA, such mutations and deletions may lead to changes in transcription and replication rate and finally result in a decrease of mtDNA levels in cancer patients [35]. In this

study we found lower levels of mtDNA in the benign group when compared with the cancer group. In benign tumors depletion of mtDNA could be a mechanism of tumour cells to escape apoptosis and to finally promote cancer progression [36]. On the other hand the relative increase of mtDNA levels in the cancer group compared to the benign disease group might be a compensatory mechanism of the cells, to respond to the decline in respiratory function [37].

In this study we showed that higher levels of ccf nDNA where significantly elevated in breast cancer patients in comparison with a benign disease group and a healthy control group while lower levels of ccf mtDNA were significantly elevated in the breast tumour group (malignant and benign) when compared to the healthy control group. Regarding ccf nDNA levels, our results confirmed the findings of other studies which also found altered levels of ccf nDNA in cancer patients. For ccf mtDNA the fact that down- as well as up- regulation of ccf mtDNA levels in cancer patients has been found in other studies makes it necessary to further investigate mtDNA content in different cancer and tumor types, to see if mtDNA content might be cancer type or tumor specific. However, both ccf nDNA and mtDNA could be used to discriminate between the different study groups. While ccf nDNA could be used for discriminating between patients with breast cancer and healthy controls, ccf mtDNA could be used for distinguishing between patients with breast tumors (malignant and benign) and healthy controls. Altogether this suggests that ccf nDNA might be used as a cancer specific biomarker, whereas ccf mtDNA might be rather used as a tumour biomarker.

Acknowledgements

This work was supported in part by Swiss National Science Foundation (320000-119722/1) and Swiss Cancer League, Krebsliga Beider Basel and Dr Hans Altschueler Stiftung. We thank Vivian Kiefer and Nicole Chiodetti for their help.

References

1. Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, Jacques PF, Rifai N, Selhub J, Robins SJ, Benjamin EJ, D'Agostino RB, Vasan RS (2006). Multiple biomarkers for the prediction of first major cardiovascular events and death. N Engl J Med 355: 2631-2639.

2. Antoniades CA, Barker RA (2008). The search for biomarkers in Parkinson's disease: a critical review. Expert Rev Neurother 8: 1841-1852. doi:10.1586/14737175.8.12.1841

3. Chou YY, Leporé N, Avedissian C, Madsen SK, Parikshak N, Hua X, Shaw LM, Trojanowski JQ, Weiner MW, Toga AW, Thompson PM; the Alzheimer's Disease Neuroimaging Initiative (2009). Mapping correlations between ventricular expansion and CSF amyloid and tau biomarkers in 240 subjects with Alzheimer's disease, mild cognitive impairment and elderly controls. Neuroimage [Epub ahead of print]. doi:10.1016/j.neuroimage.2009.02.015

4. Hartwell L, Mankoff D, Paulovich A, Ramsey S, Swisher E (2006). Cancer biomarkers: a systems approach. Nat Biotechnol 24: 905-908. doi:10.1038/nbt0806-905

5. Nicolini A, Tartarelli G, Carpi A, Metelli MR, Ferrari P, Anselmi L, Conte M, Berti P, Miccoli P (). Intensive post-operative follow-up of breast cancer patients with tumour markers: CEA, TPA or CA15.3 vs MCA and MCA-CA15.3 vs CEA-TPA-CA15.3 panel in the early detection of distant metastases. BMC Cancer 6:269. doi:10.1186/1471-2407-6-269.

6. Martínez L, Castilla JA, Blanco N, Perán F, Herruzo A (1995). CA 125, CA 15.3, CA 27.29, CEA, β-hCG and α-fetoprotein levels in cyst fluid of breast macrocysts. Int J Gynaecol Obstet 48: 187-192. doi:10.1016/0020-7292(94)02279-8.

7. Molina R, Barak V, van Dalen A, Duffy MJ, Einarsson R, Gion M, Goike H, Lamerz R, Nap M, Sölétormos G, Stieber P (2005). Tumor Markers in Breast Cancer – European Group on Tumor Markers Recommendations. Tumour Biol 26: 281-293. doi: 10.1159/000089260

8. Berg WA, Blume JD, Cormack JB, Mendelson EB, Lehrer D, Böhm-Vélez M, Pisano ED, Jong RA, Evans WP, Morton MJ, Mahoney MC, Larsen LH, Barr RG, Farria DM, Marques HS, Boparai K; ACRIN 6666 Investigators (2008). Combined screening with ultrasound and mammography vs mammography alone in women at elevated risk of breast cancer. JAMA 299: 2151-2163. doi:10.1001/jama.299.18.2151

9. Tseng LM, Yin PH, Chi CW, Hsu CY, Wu CW, Lee LM, Wei YH, Lee HC (2006). Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. Genes Chromosomes Cancer 45:629-638. doi:10.1002/gcc.20326

10. Wu TL, Zhang D, Chia JH, Tsao KH, Sun CF, Wu JT (2002). Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. Clin Chim Acta 321:77-87. doi:10.1016/S0009-8981(02)00091-8.

11. Zachariah RR, Schmid S, Buerki N, Radpour R, Holzgreve W, Zhong X (2008). Levels of circulating cell-free nuclear and mitochondrial DNA in benign and malignant ovarian tumors. Obstet Gynecol 112:843-50. doi: 10.1097/AOG.0b013e3181867bc0.

12. Zanetti-Dällenbach R, Wight E, Fan AX, Lapaire O, Hahn S, Holzgreve W, Zhong XY (2008). Positive correlation of cell-free DNA in plasma/serum in patients with malignant and benign breast disease. Anticancer Res 28:921-925.

13. Zanetti-Dällenbach RA, Schmid S, Wight E, Holzgreve W, Ladewing A, Hahn S, Zhong XY (2007). Levels of circulating cell-free serum DNA in benign and malignant breast lesions. Int J Biol Markers 22: 95-99.

14. Wang Y, Liu VW, Xue WC, Cheung AN, Ngan HY (2006). Association of decreased mitochondrial DNA content with ovarian cancer progression. Br J Cancer 95: 1087-1091. doi:10.1038/sj.bjc.6603377.

15. Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung SC, Sukumar S, Sidransky D (2005). Tumor-specific changes in mtDNA content in human cancer. Int J Cancer 116:920-924. doi: 10.1002/ijc.21110.

16. Zhong XY, Ladewig A, Schmid S, Wight E, Hahn S, Holzgreve W (2007). Elevated level of cell-free plasma DNA is associated with breast cancer. Arch Gynecol Obstet 276: 327-331. doi:10.1007/s00404-007-0345-1

17. Akobeng AK (2006). Understanding diagnostic tests 3: receiver operating characteristic curves. Acta Paediatr 96: 644-647 doi:10.1111/j.1651-2227.2006.00178.

18. Seefeld M, El Tarhouny S, Fan AX, Hahn S, Holzgreve W, Zhong XY (2008). Parallel assessment of circulatory cell-free DNA by PCR and nucleosomes by ELISA in breast tumors. Int J Biol Markers 23: 69-73.

19. El Tarhouny S, Seefeld M, Fan AX, Hahn S, Holzgreve W, Zhong XY (2008). Comparison of serum VEGF and its soluble receptor sVEGFR1 with serum cell-free DNA in patients with breast tumor. Cytokine 44: 65-9. doi:10.1016/j.cyto.2008.06.008.

20. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA, Kinzler KW, Vogelstein B, Diaz LA, Jr. (2008) Circulating mutant DNA to assess tumor dynamics. Nat Med 14: 985-90. doi:10.1038/nm.1789

21. Catarino R, Ferreira MM, Rodrigues H, Coelho A, Nogal A, Sousa A, Medeiros R (2008). Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer. DNA Cell Biol 27: 415-21. doi:10.1089/dna.2008.0744.

22. Deligezer U, Eralp Y, Akisik EZ, Akisik EE, Saip P, Topuz E, Dalay N (2008). Effect of adjuvant chemotherapy on integrity of free serum DNA in patients with breast cancer. Ann N Y Acad Sci 1137: 175-9. doi:10.1196/annals.1448.010.

23. Deligezer U, Eralp Y, Akisik EE, Akisik EZ, Saip P, Topuz E, Dalay N (2008). Size distribution of circulating cell-free DNA in sera of breast cancer patients in the course of adjuvant chemotherapy. Clin Chem Lab Med 46:311-7. doi:10.1016/S0140-6736(05)71754-2.

24. Zhong XY, Hahn S, Holzgreve W. Prenatal identification of fetal genetic traits (2001). Lancet 357: 310-1.

25. Zhong XY, Holzgreve W, Hahn S. Risk free simultaneous prenatal identification of fetal Rhesus D status and sex by multiplex real-time PCR using cell free fetal DNA in maternal plasma (2001). Swiss Med Wkly 131:70-4.

26. Zhong XY, Holzgreve W, Hahn S (2001). Circulatory fetal and maternal DNA in pregnancies at risk and those affected by preeclampsia. Ann N Y Acad Sci 945:138-40 doi:10.1111/j.1749-6632.2001.tb03874.

27. Zhong XY, Volgmann T, Hahn S, Holzgreve W (2007). Large scale analysis of circulatory fetal DNA concentrations in pregnancies which subsequently develop preeclampsia using two Y chromosome specific real-time PCR assays. JTGGA 8:135-139.

28. Lo YM (2000). Fetal DNA in maternal plasma: biology and diagnostic applications. Clin Chem 46:1903-6.

29. Deng Z, Wu G, Li Q, Zhang X, Liang Y, Li D, Gao S, Lan Y (2006). Noninvasive genotyping of 9 Y-chromosome specific STR loci using circulatory fetal DNA in maternal plasma by multiplex PCR. Prenat Diagn 26: 362-8. doi:10.1002/pd.1422.

30. Deng ZH, Li Q, Wu S, Li DC, Yang BC (2008). [Application of 17 Y-chromosome specific STR loci in paternity testing]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 16: 699-703.

31. Mizumachi T, Muskhelishvili L, Naito A, Furusawa J, Fan CY, Siegel ER, Kadlubar FF, Kumar U, Higuchi M (2008). Increased distributional variance of mitochondrial DNA

content associated with prostate cancer cells as compared with normal prostate cells. Prostate 68:408-417. doi:10.1002/pros.20697.

32. Selvanayagam P, Rajaraman S (1996). Detection of mitochondrial genome depletion by a novel cDNA in renal cell carcinoma. Lab Invest 74:592-599.

33. Jiang WW, Rosenbaum E, Mambo E, Zahurak M, Masayesva B, Carvalho AL, Zhou S, Westra WH, Alberg AJ, Sidransky D, Koch W, Califano JA (2006). Decreased mitochondrial DNA content in posttreatment salivary rinses from head and neck cancer patients. Clin Cancer Res 2:1564-1569. doi:10.1158/1078-0432.CCR-05-1471.

34. Wang Y, Liu VW, Xue WC, Cheung AN, Ngan HY (2006). Association of decreased mitochondrial DNA content with ovarian cancer progression. Br J Cancer 95:1087-1091. doi:10.1038/sj.bjc.6603377.

35. Lee HC, Hsu LS, Yin PH, Lee LM, Chi CW (2007). Heteroplasmic mutation of mitochondrial DNA D-loop and 4977-bp deletion in human cancer cells during mitochondrial DNA depletion. 7: 157-163. doi:10.1016/j.mito.2006.11.016.

36. Higuchi M (2007). Regulation of mitochondrial DNA content and cancer. Mitochondrion 7:53-57. doi:10.1016/j.mito.2006.12.001.

37. Barrientos A, Casademont J, Cardellach F, Estivill X, Urbano-Marquez A, Nunes V (1997). Reduced steady-state levels of mitochondrial RNA and increased mitochondrial DNA amount in human brain with aging. *Brain Res Mol Brain Res* 52:284-289. doi:10.1016/S0169-328X(97)00278-7

Group		Ccf nDNA (GE/mL)	Ccf mtDNA (GE/mL)
Malignant disease group	Median	4678	205013
	Range	758- 23263	6508 - 6603330
Benign disease group	Median	1359	73977
	Range	88 - 483209	7887 - 541739
Control group	Median	1298	522115
	Range	95 - 30437	3427 - 28327810

Table 1: Concentrations (GE/mL) of plasma ccf nDNA and ccf mtDNA in the 3 study-groups; expressed as median and range.

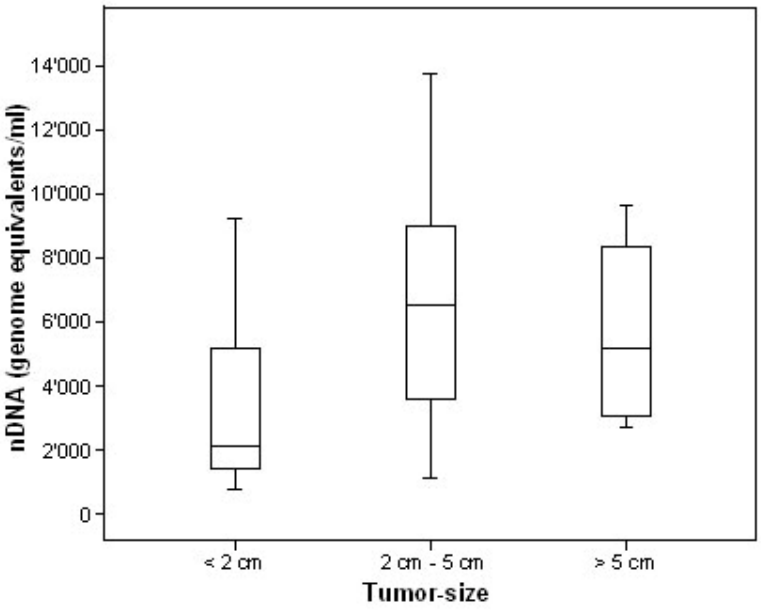


Fig.2 Boxplot for correlating levels of ccf nDNA between breast cancer patients with a tumour size of more than five centimetres (> 5 cm; 4 cases), two to five centimetres (2 cm–5 cm; 25 cases) and less than 2 centimetres (< 2 cm; 21 cases). Significant difference in the levels of ccf nDNA could be found between tumours with a tumour size of two to five centimetres and tumours with a tumour size of less than two centimetres. For the group of the tumour size bigger than 5 cm, only 4 cases were recruited.

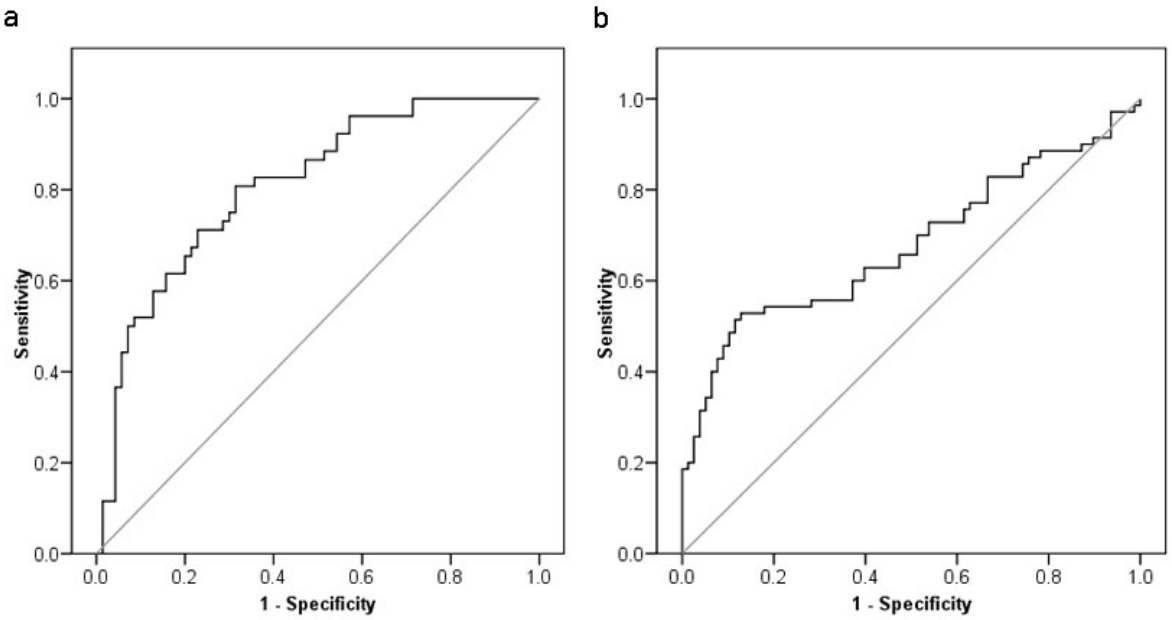


Fig.3 ROC curves using ccf nDNA and mtDNA for discrimination between the study-groups. A) ROC curve of ccf nDNA for discrimination between the cancer group and the healthy control group (sensitivity = 81 %; specificity = 69 %). B) ROC curve of ccf mtDNA for discrimination between the tumour group and the healthy control group (sensitivity = 53 %; specificity = 87 %).

Paper 5.

Mitochondrial DNA content in paired normal and cancerous breast tissue samples from patients with breast cancer

Alex Xiu-Cheng Fan,
Ramin Radpour,Mahdi Montazer Haghighi, Corina Kohler, Peng Xia,
Sinuhe Hahn, Wolfgang Holzgreve, Xiao Yan Zhong

1 Laboratory for Prenatal Medicine and Gynecologic Oncology,
Department of Biomedicine, Women's Hospital,
University of Basel, Hebelstrasse 20,
4031 Basel, Switzerland

2 Azad University, East of Tehran Branch, Tehran, Iran

3 University Medical Center, University of Freiburg,
Hugstetter Str. 49, 79106 Freiburg, Germany

Correspondent to:

Prof. Dr. X.Y. Zhong

Laboratory for Prenatal Medicine and Gynecologic Oncology,
Department of Biomedicine, Women's Hospital,
University of Basel, Hebelstrasse 20,
Room Nr. 416, 4031 Basel, Switzerland
e-mail: xzhong@uhbs.ch

J Cancer Res Clin Oncol
DOI 10.1007/s00432-008-0533-9

ORIGINAL PAPER

Mitochondrial DNA content in paired normal and cancerous breast tissue samples from patients with breast cancer

Alex Xiu-Cheng Fan · Ramin Radpour · Mahdi Montazer Haghighi · Corina Kohler · Peng Xia · Sinuhe Hahn · Wolfgang Holzgreve · Xiao Yan Zhong

Received: 19 May 2008 / Accepted: 8 December 2008

Abstract
Introduction We develop a multiplex quantitative real-time PCR for synchronized analysis of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) to investigate relative mtDNA abundance in paired normal and cancerous breast tissues.
Materials and methods The amounts of nDNA and mtDNA in 102 tissue samples were quantified for both glyceraldehype-3-phosphodehydrogenase (GAPDH) gene and mtDNA encoded ATPase (MTATP) 8 gene. The average threshold cycle (Ct) number values of the nDNA and mtDNA were used to calculate relative mtDNA content in breast tissues.
Results The median delta Ct (ΔCt) and the median mtDNA content for normal and cancerous breast tissues were 6.73 and 2.54, as well as 106.50 and 5.80 ($P = 0.000$, respectively). The mtDNA content was decreased in 82% of cancerous breast tissues compared with the normal ones. The changes were associated with hormone receptor status.
Conclusion Our finding suggests that decreased mtDNA content in breast cancer may have diagnostic and prognostic value for the disease.

A. X.-C. Fan · R. Radpour · C. Kohler · P. Xia · S. Hahn · W. Holzgreve · X. Y. Zhong (✉)
Laboratory for Prenatal Medicine and Gynecologic Oncology, Department of Biomedicine, Women's Hospital, University of Basel, Hebelstrasse 20, Room Nr. 416, 4031 Basel, Switzerland
e-mail: xzhong@uhbs.ch

M. M. Haghighi
Azad University, East of Tehran Branch, Tehran, Iran

W. Holzgreve
University Medical Center, University of Freiburg, Hugstetter Str. 49, 79106 Freiburg, Germany

Published online: 06 January 2009

Keywords Quantitative alteration · Mitochondrial DNA · Breast cancer · Breast tissue

Introduction

Human cells have a nuclear genome and additional cytoplasmic genomes that are compartmentalised in the mitochondria. In comparison to nuclear genomic DNA, mitochondrial DNA (mtDNA) reveals high mutation rates caused by constant exposure to mutagenic oxygen radicals and lacks the protective mechanisms of DNA repair. These properties of mtDNA suggest their potential importance in ageing, apoptosis and especially carcinogenesis (Zhang and Qi 2008; Zhang et al. 2008).

Qualitative aberrations of mtDNA, such as mutations, have been found in solid tumours, such as colon, stomach, liver, kidney, bladder, prostate, skin and lung cancer (Copeland et al. 2002; Penta et al. 2001), and in haematological malignancies, such as leukaemia and lymphoma (Fontenay et al. 2006). Quantitative aberrations of mtDNA have been observed in various sample types from patients with cancers (Mambo et al. 2005). While increased mtDNA content has been found in prostate (Mizumachi et al. 2008a), head and neck (Kim et al. 2004), endometrial adenocarcinoma (Wang et al. 2005), etc., reduced mtDNA content in renal (Meierhofer et al. 2004) and liver cancers (Yin et al. 2004) has been reported.

Because of these mutations and the quantitative aberrations involved in the development of human cancers, mtDNA may have promising clinical applications for cancers (Jiang et al. 2005; Jain 2007). Decreased mitochondrial DNA copy number is correlated with tumour progression and prognosis in Chinese breast cancer patients (Yu et al. 2007). Increased mtDNA content in saliva is associated

with head and neck cancer (Mizumachi et al. 2008b). High levels of cell free circulating mtDNA in prostate cancer patients with poor prognosis could be a valuable predictor of prognosis (Ellinger et al. 2008; Mehra et al. 2007). Furthermore, mtDNA aberrations play important roles in response to cancer therapy, for example, causing resistance to therapeutic agents (Mizumachi et al. 2008b; Chen et al. 2007).

In the present study, we developed a simple and accurate multiplex real-time PCR method for synchronised quantification of nuclear DNA (nDNA) and mtDNA in paired adjacent normal and cancerous breast tissue samples from 51 patients with breast cancer. The content of MTATP8 gene on mtDNA in the cancerous tissues was compared with that in normal tissues from the same patients. We analysed the correlation between the content of mtDNA in tissues and the traditional pathological parameters and clinical predictive markers.

Materials and methods

Samples

The study was approved by the local institutional review board. Paraffin-embedded sections from adjacent normal and cancerous breast tissue samples were examined by two experienced pathologists. Breast cancer characteristics, the histological grading; hormone receptor status and biomarkers from the breast cancer patients are listed in Table 1. DNA was extracted from three to five sections of each 10 µm thick paraffin-embedded sample (around 0.01–0.02 g of tissue) using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) and eluted into 150 µl of elution buffer. The eluted DNA was stored at −20°C until further use.

Real-time PCR

The amounts of nDNA and mtDNA were quantified by multiplex TaqMan real-time PCR for both GAPDH gene of nDNA and MTATP 8 gene of mtDNA starting at locus 8446. The GAPDH and MTATP 8 primer and probe sequences are shown as follows: GAPDH (forward):5′ CCC CAC ACA CAT GCA CTT ACC3′; (reverse): 5′CCT AGT CCC AGG GCT TTG ATT 3′; probe 5′ (MGB) TAG GAA GGA CAG GCA AC (VIC) 3′. MTATP 8 (forward): 5′ AAT ATT AAA CAC AAA CTA CCA CCT ACC 3′; (reverse): 5′ TGG TTC TCA GGG TTT GTT ATA 3′; probe: 5′ (MGB) CCT CAC CAA AGC CCA TA (FAM) 3′.

The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI). A total of 2.5 µl of DNA were used as template for the PCR analysis. The real-time PCR were carried out in 25 µl of total reaction volume using a 2 min incubation at 50°C, followed by an initial denaturation step at 95°C for 10 min and 40 cycles of 1 min at 60°C and 15 s at 95°C.

To determine the quantities of mtDNA and nDNA present in tissue samples, standard dilution curves using HPLC-purified single-stranded synthetic DNA oligonucleotides (Microsynth) specifying a 79-bp MTATP 8 gene amplicon and a 97-bp GAPDH amplicon with concentration ranging from 5×10^7 copies to 5×10^2 copies, were used. We examined the amplification efficiency for both GAPDH and MTATP 8 on experimental serial dilutions. The amplifications of mtDNA and nDNA on serial dilutions showed a good correlation with comparable efficiencies. A threshold cycle (Ct) reflects the cycle number at which a fluorescence signal within a reaction crosses a threshold. In our study, the average threshold cycle number (Ct) values of the nDNA and mtDNA were obtained from each case. The content of mtDNA was calculated using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt = CtnDNA − CtmtDNA) in the same well as an exponent of 2 ($2^{\Delta Ct}$).

Statistical analysis

The data were analysed using SPSS software (Statistical Software Package for Windows v. 15.0). Content of mtDNA is given as the median, the range and the fold difference. Wilcoxon signed ranks test was used to compare the differences between ranks of each paired samples. The Mann–Whitney *U* test and Kruskal–Wallis test was used to compare the content of mtDNA in normal adjacent normal tissues and cancerous tissues. The Spearman rank test was applied to analyse the relationship of mtDNA content between normal tissues and cancerous tissues.

Results

Co-extraction of mtDNA and nDNA from the paired breast tissue samples

Nuclear DNA and mtDNA were co-extracted from the paraffin-embedded sections. The average Ct values for GAPDH sequence, representing total nDNA, ranged from 24.42 to 37.08 in cancerous tissues and from 25.19 to 38.38 in normal tissues, respectively. The average Ct values for MTATP 8 gene sequence, representing total mtDNA, ranged from 18.48 to 32.54 in cancerous tissues and from 19.28 to 33.42 in normal tissues, respectively. The average Ct values of mtDNA were less than those of nDNA in all cases, represented higher amounts of mtDNA than those of nDNA in the breast tissues. While the average Ct values of GAPDH amplification in the normal tissues are correlated with those of MTATP8 gene amplification (Spearman rank

Table 1 Patients data as well as relationships between decreased content of mtDNA in cancerous tissues and clinical factors

Variables	Group (cases)	mtDNA content	*P* value
Age	<50 (32)	195.36 (6.70–2,460.95)	0.596*
	≥50 (19)	23.48 (6.43–284.05)	
Histological type	Ductal (41)	53.82 (1.35–9,877.98)	0.337*
	Lobular (10)	201.39 (7.31–823.14)	
Primary tumour	T1 (23)	149.09 (6.43–749.61)	0.824**
	T2 (15)	23.48 (6.70–861.08)	
	T3 (9)	2382.54 (2,304.12–2,460.95)	
Lymph node involvement	Positive (41)	149.09 (6.43–2,460.95)	0.201*
	Negative (9)	57.82 (9.48–106.15)	
Distant metastasis	M0 (40)	137.44 (6.70–2,460.95)	0.585*
	M1 (10)	53.82 (6.43–584.07)	
Stage	I (5)	57.82(9.48–106.15)	0.140**
	II (27)	137.44 (6.70–861.08)	
	III (6)	2382.54 (2,304.12–2,460.95)	
	IV (9)	53.82 (6.43–584.07)	
Histological grading	G1 (8)	397.97 (9.48–2,460.95)	0.696**
	G2 (19)	195.36 (6.43–861.08)	
	G3 (15)	34.22 (7.31–225.97)	
Nuclear grading	1 (3)	57.82 (9.48–106.15)	0.418**
	2 (14)	195.36 (9.99–2,304.12)	
	3 (16)	137.44 (6.43–2,460.95)	
ER	Positive (14)	12.46 (1.18–69,272.73)	0.613*
	Negative (18)	6.57 (1.18–8,451.55)	
PR	Positive (18)	20.83 (1.18–69,272.73)	0.037*
	Negative (14)	3.77 (1.18–394.81)	
Her2	Positive (15)	53.82 (6.70–2,460.95)	
	Negative (17)	210.67 (6.43–2,304.12)	
P53	Positive (3)	1205.14 (106.15–2,304.12)	0.153*
	Negative (29)	125.80 (6.43–2,460.95)	
PS2	Positive (11)	106.15 (6.43–2,304.12)	0.699*
	Negative (20)	137.44 (6.70–2,460.95)	
Ki62	Positive (0)		
	Negative (31)	125.80 (6.43–2,460.95)	

ER Oestrogen receptor, *PR* progesterone receptor
* Mann–Whitney *U* test,
** Kruskal–Wallis test

test $P < 0.001$, $r = 0.63$) (Fig. 1a), no similar phenomenon was observed in cancerous tissues ($P > 0.05$, $r = 0.261$) (Fig. 1b), suggesting an alteration of the relationship between nDNA and mtDNA in cancerous tissues.

Content of mtDNA in paired normal and cancerous breast tissues

The ΔCt values between GAPDH amplification and MTATP 8 gene amplification show a 6.73 cycle difference in normal tissues and 2.54 cycle difference in cancerous tissues. The ΔCt values between normal and cancerous tissues shows a 4.2 cycle difference (ΔΔCt) (Mann–Whitney *U* test $P < 0.001$) (Table 2). We calculated the content of mtDNA in the tissues using a formula of $2^{\Delta Ct}$. The content of mtDNA in cancerous tissues is significantly lower than that in normal tissues (Mann–Whitney *U* test $P < 0.001$) (Fig. 2). Out of 51 paired samples, 42 pairs show mtDNA in normal tissues > mtDNA in cancerous tissues (Wilcoxon signed ranks test $P < 0.001$).

Relationship between decreased content of mtDNA and other established prognostic factors

In this study, associations between the content of mtDNA in breast tissues and traditional clinical parameters, such as age, tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, receptor status and pathological biomarkers (HER-2/neu and PS2), were analysed (Table 1).

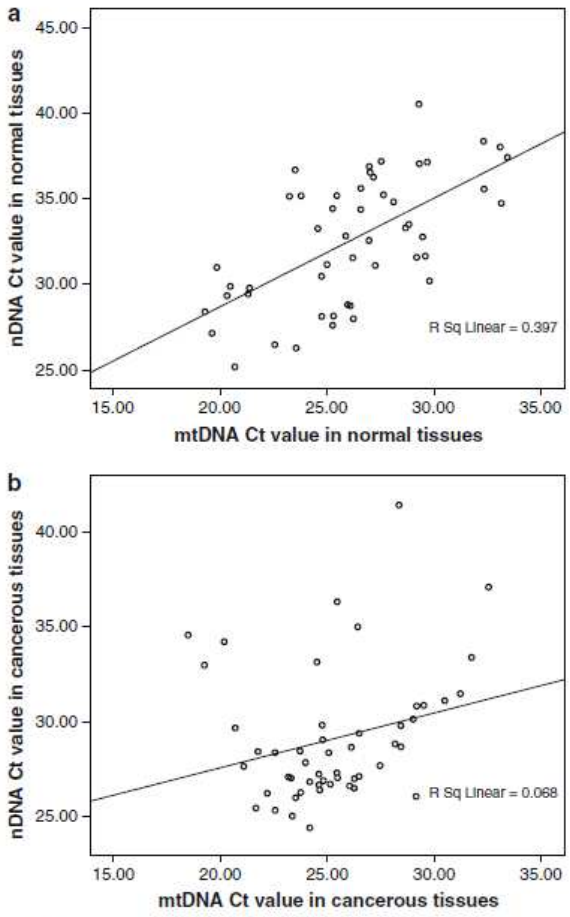


Fig. 1 a mtDNA amplifications are correlated with nDNA amplifications in normal tissues ($P = 0.000$, $r = 0.63$); b mtDNA amplifications are not correlated with nDNA amplifications in cancerous tissues ($P = 0.065$, $r = 0.261$)

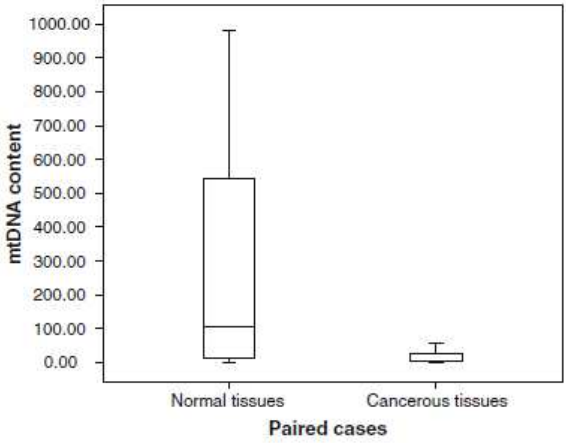


Fig. 2 mtDNA content in paired adjacent normal and cancerous breast tissues

Decreased content of mtDNA in breast cancer was not associated with age (≥50 vs. <50), tumour type (ductal vs. lobular), tumour size (T1, T2, T3), lymph node involvement (N0 and N1), extent of metastasis (M0 vs. M1), stage, histological grading (I, II, III), HER-2/neu amplifications and PS2 detections (Table 1). However, while we found significantly decreased mtDNA content in normal tissues of an oestrogen receptor (ER) negative detection compared with that of an ER positive detection (mtDNA in normal tissues 89.81 vs. 756.46, $P = 0.041$; mtDNA in cancerous tissues 6.57 vs. 12.46, $P = 0.613$) (Fig. 3a), a significantly decreased content of mtDNA in both normal and cancerous tissues of a progesterone receptor (PR) negative detection compared with that of PR positive tissues was observed (mtDNA in normal tissues 37.67 vs. 434.06, $P = 0.016$; mtDNA in cancerous tissues 3.77 vs. 20.83, $P = 0.037$) (Fig. 3b).

Discussion

In this study, we found that mtDNA content of MTATP 8 gene for encoding ATP synthase was decreased in 42 of 51 (82%) cancerous breast tissues compared to their normal breast tissues collected from same individuals (Table 2).

Table 2 Comparison of mtDNA content in normal and cancerous breast tissues

	Normal (N) 51	Cancerous (C) 51	Fold	Significances/ correlation (P value)
$\Delta CT = Ct_{nDNA} - CtmtDNA$	6.73	2.54	$\Delta\Delta CT$:	0.000*
	0.43–13.27	0.23–16.08	6.73–2.54 = 4.2	
Content = $2^{\Delta CT}$	106.20	5.80	Fold	0.000*
	1.30–9877.90	1.20–69272.70	106.20/5.80 = 18.3	0.242** (C:N)
N > C or C > N	42	9		0.000***
Correlation (Ct_{nDNA}:Ct_{mtDNA})	**$P = 0.000$	**$P = 0.065$		
	$r = 0.63$	$r = 0.26$		

* Mann–Whitney U test; ** Spearman Rank test; *** Wilcoxon Signed Ranks test

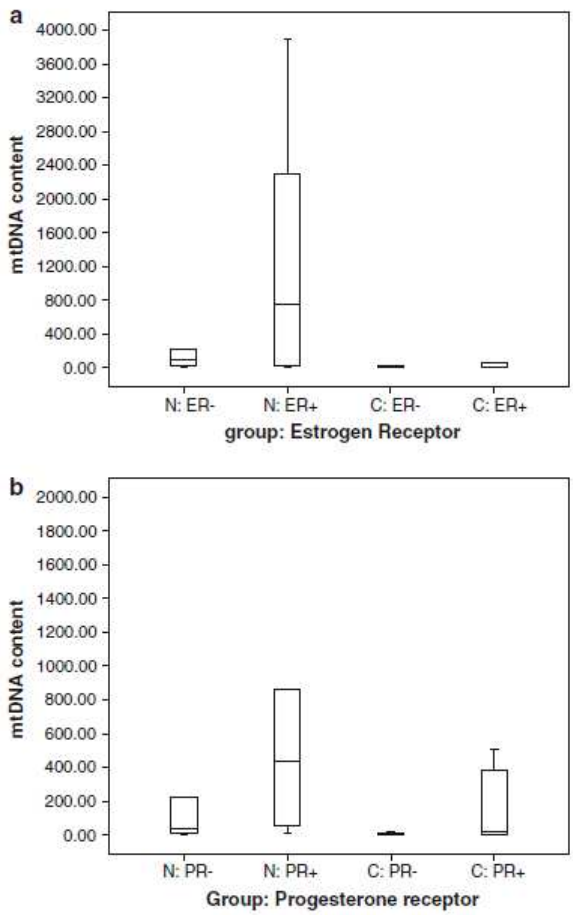


Fig. 3 a mtDNA content in ER positive and ER negative normal and cancerous tissues; b mtDNA content in PR positive and PR negative normal and cancerous tissues. *ER+* estrogen receptor positive, *ER−* estrogen receptor negative, *N* normal tissues, *C*cancerous tissues, *PR+* progesterone receptor positive, *PR−* progesterone receptor negative

Using different primers and methods to amplify different regions on mtDNA, Yu et al. (2007) (on D-loop), Mambo et al. (2005) (on Co I region) and Tseng et al. (2006) (on ND1 gene) found the similar phenomenon. This suggests that, as it has been reported for other cancer types, quantitative alterations of mtDNA may be a potential biomarker for breast cancer. Both decrease and increase of mtDNA content in cancers have been shown in many studies, varying with tissue type or origin of tumours, because of different patterns of mitochondrial transcripts coding for proteins involved in oxidative phosphorylation (Lebrecht et al. 2005; Masuyama et al. 2005).

Decrease of mtDNA has been observed in renal cancer (Meierhofer et al. 2004) and hepatocellular carcinoma (Yin et al. 2004). However, the pathogenesis of mtDNA decrease in cancers remains unclear. It was hypothesised that mitochondrial respiratory dysfunction and damage of mtDNA, such as mutation, deletion or depletion, in carcinogenesis may course the quantitative alterations (Ye et al. 2008). Those alterations of mtDNA could correlate with increased risk (Mosquera-Miguel et al. 2008; Bai et al. 2007) and increased invasiveness of cancer (Simonnet et al. 2002). Decreased mtDNA content can cause decreased oxidative phosphorylation capacity that could increase cancer cell growth under hypoxic conditions during cancer development and cancer progression (Rossignol et al. 2004). Depletion of mtDNA can also lead to cells resistant to a certain apoptosis pathway during cancer development (Higuchi 2007).

In our study, mtDNA content was significantly decreased in cancerous breast tissues. However, high copy numbers of mtDNA per cell compared to those of nDNA (from $2^{2.535}$ to $2^{6.73}$ is from 5.8- to 106.2-fold higher in our study) may still increase the sensitivity of testing mtDNA alterations, thus having more promising clinical applications in cancers.

Using QuantiTest SYBR Green PCR, Yu et al. (2007) found that, on a 59 case study, mtDNA copy number alteration is correlated with tumour progression and prognosis in Chinese breast cancer patients. They showed that the reduced copy number in mtDNA was associated with an older onset age ($\geq$50 years old) and a higher histological grade. We compared the decrease in mtDNA content with other traditional clinical parameters, such as age, tumour size, lymph node involvement, extent of metastasis, and predictive markers, such as histological grades, HER-2/*neu* amplification and PS2 detection. No relationship was found between the decrease and those parameters. It was likewise shown by Mambo et al. (2005) that changes in mtDNA content in breast cancer did not correlate with tumour grade and metastasis, suggesting that these alterations may occur in the early stages of tumorigenesis.

However, in our study, we did find associations between mtDNA content and hormone receptor status. MtDNA content is significantly higher in ER positive normal breast tissues than that in ER negatives, suggesting that ER status in normal breast tissue may play a part in mtDNA function. Chen et al. (2004) observed that ER is present in mitochondria of human MCF7 cells, implying that oestrogens can have an effect on mitochondrial via ER in regulation of mitochondrial respiratory chain structure and function (Chen et al. 2005; Yager and Chen 2007). A similar phenomenon was not shown in the cancerous tissues. We presume that mtDNA dependent ER action may be altered during cancer development in vivo.

In our study, we also observed that changes in mtDNA content are associated with PR in both normal and cancerous tissues (mtDNA in PR positive > that in PR negative). We assume that the changes may be tissue specific rather than tumour specific. Several studies showed that mitochondria respiration varies during the menstrual cycle and

is associated with pregnancy (Buffenstein et al. 1995; Webb 1986). Mitochondria respiration increases during the progesterone-dominant lucteal phase.

In conclusion, our data suggest that using a robust Taqman real-time PCR, the content of mtDNA in breast tissues can be easily quantified. MtDNA content in breast cancer tissues decreased dramatically, and the changes are not associated with most traditional prognostic parameters. Many studies in vitro, using animal models and cell culture models, showed that reduced mtDNA content could lead to cells resistant to apoptosis, favour cancer cell growth, increase invasiveness and contribute to progression and metastasis of cancer cells. Therefore, decrease of mtDNA content in breast cancer may have diagnostic and prognostic value. In this study, we also found hormone receptor related changes in mtDNA content, which may assist us in understanding the biology of mtDNA in gender and age manner.

Acknowledgments This work was supported in part by Swiss National Science Foundation (320000-119722/1), Swiss Cancer League, Krebsliga Beider Basel and Dr. Hans Altschueler Stiftung. We thank Mrs Vivian Kiefer and Mrs Nicole Chiodetti for their help. We are grateful to Mrs Regan Geissmann for proofreading the text.

References

Bai RK, Leal SM, Covarrubias D, Liu A, Wong LJ (2007) Mitochondrial genetic background modifies breast cancer risk. Cancer Res 67:4687–4694. doi:10.1158/0008-5472.CAN-06-3554

Buffenstein R, Poppitt SD, McDevitt RM, Prentice AM (1995) Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. Physiol Behav 58:1067–1077. doi:10.1016/0031-9384(95)02003-9

Chen JQ, Delannoy M, Cooke C, Yager JD (2004) Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. Am J Physiol Endocrinol Metab 286:E1011–E1022. doi:10.1152/ajpendo.00508.2003

Chen JQ, Yager JD, Russo J (2005) Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim Biophys Acta 1746:1–17. doi:10.1016/j.bbamcr.2005.08.001

Chen Z, Lu W, Garcia-Prieto C, Huang P (2007) The Warburg effect and its cancer therapeutic implications. J Bioenerg Biomembr 39:267–274. doi:10.1007/s10863-007-9086-x

Copeland WC, Wachsman JT, Johnson FM, Penta JS (2002) Mitochondrial DNA alterations in cancer. Cancer Invest 20:557–569. doi:10.1081/CNV-120002155

Ellinger J, Muller SC, Wernert N, von Ruecker A, Bastian PJ (2008) Mitochondrial DNA in serum of patients with prostate cancer: a predictor of biochemical recurrence after prostatectomy. BJU Int 102(5):628–632

Fontenay M, Cathelin S, Amiot M, Gyan E, Solary E (2006) Mitochondria in hematopoiesis and hematological diseases. Oncogene 25:4757–4767. doi:10.1038/sj.onc.1209606

Higuchi M (2007) Regulation of mitochondrial DNA content and cancer. Mitochondrion 7:53–57. doi:10.1016/j.mito.2006.12.001

Jain KK (2007) Cancer biomarkers: current issues and future directions. Curr Opin Mol Ther 9:563–571

Kim MM, Clinger JD, Masayesva BG, Ha PK, Zahurak ML, Westra WH et al (2004) Mitochondrial DNA quantity increases with histopathologic grade in premalignant and malignant head and neck lesions. Clin Cancer Res 10:8512–8515. doi:10.1158/1078-0432.CCR-04-0734

Lebrecht D, Kokkori A, Ketelsen UP, Setzer B, Walker UA (2005) Tissue-specific mtDNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. J Pathol 207:436–444. doi:10.1002/path.1863

Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung SC et al (2005) Tumor-specific changes in mtDNA content in human cancer. Int J Cancer 116:920–924. doi:10.1002/ijc.21110

Masuyama M, Iida R, Takatsuka H, Yasuda T, Matsuki T (2005) Quantitative change in mitochondrial DNA content in various mouse tissues during aging. Biochim Biophys Acta 1723:302–308

Mehra N, Penning M, Maas J, van Daal N, Giles RH, Voest EE (2007) Circulating mitochondrial nucleic acids have prognostic value for survival in patients with advanced prostate cancer. Clin Cancer Res 13:421–426. doi:10.1158/1078-0432.CCR-06-1087

Meierhofer D, Mayr JA, Foetschl U, Berger A, Fink K, Schmeller N et al (2004) Decrease of mitochondrial DNA content and energy metabolism in renal cell carcinoma. Carcinogenesis 25:1005–1010. doi:10.1093/carcin/bgh104

Mizumachi T, Suzuki S, Naito A, Carcel-Trullols J, Evans TT, Spring PM et al (2008a) Increased mitochondrial DNA induces acquired docetaxel resistance in head and neck cancer cells. Oncogene 27:831–838. doi:10.1038/sj.onc.1210681

Mizumachi T, Naito MLA, Furusawa J, Fan CY, Siegel ER et al (2008b) Increased distributional variance of mitochondrial DNA content associated with prostate cancer cells as compared with normal prostate cells. Prostate 68:408–417. doi:10.1002/pros.20697

Mosquera-Miguel A, Alvarez-Iglesias V, Carracedo A, Salas A, Vega A, Carracedo A et al (2008) Is mitochondrial DNA variation associated with sporadic breast cancer risk? Cancer Res 68:623–625. doi:10.1158/0008-5472.CAN-07-2385 author reply 624

Penta JS, Johnson FM, Wachsman JT, Copeland WC (2001) Mitochondrial DNA in human malignancy. Mutat Res 488:119–133. doi:10.1016/S1383-5742(01)00053-9

Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 64:985–993. doi:10.1158/0008-5472.CAN-03-1101

Simonnet H, Alazard N, Pfeiffer K, Gallou C, Beroud C, Demont J et al (2002) Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. Carcinogenesis 23:759–768. doi:10.1093/carcin/23.5.759

Tseng LM, Yin PH, Chi CW, Hsu CY, Wu CW, Lee LM et al (2006) Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. Genes Chromosomes Cancer 45:629–638. doi:10.1002/gcc.20326

Wang Y, Liu VW, Xue WC, Tsang PC, Cheung AN, Ngan HY (2005) The increase of mitochondrial DNA content in endometrial adenocarcinoma cells: a quantitative study using laser-captured microdissected tissues. Gynecol Oncol 98:104–110. doi:10.1016/j.ygyno.2005.04.015

Webb P (1986) 24-hour energy expenditure and the menstrual cycle. Am J Clin Nutr 44:614–619

Yager JD, Chen JQ (2007) Mitochondrial estrogen receptors—new insights into specific functions. Trends Endocrinol Metab 18:89–91. doi:10.1016/j.tem.2007.02.006

Ye C, Shu XO, Wen W, Pierce L, Courtney R, Gao YT et al (2008) Quantitative analysis of mitochondrial DNA 4977-bp deletion in sporadic breast cancer and benign breast diseases. Breast Cancer Res Treat 108:427–434. doi:10.1007/s10549-007-9613-9

Yin PH, Lee HC, Chau GY, Wu YT, Li SH, Lui WY et al (2004) Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. Br J Cancer 90:2390–2396

Yu MZY, Shi Y, Ning L, Yang Y, Wei X et al (2007) Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. IUBMB Life 59:450–457. doi:10.1080/15216540701509955

Zhang XN, Qi M (2008) Mitochondrion and its related disorders: making a comeback. J Zhejiang Univ Sci B 9:90–92. doi:10.1631/jzus.B0710621

Zhang SP, Song SJ, Li YX (2008) Association between mitochondrial DNA mutations and cancer in human. Yi Chuan 30:263–268. doi:10.3724/SP.J.1005.2008.00263

Paper 6.

High throughput multiplex assay for the simultaneous detection of 22 mutations of mtDNA in breast cancer tissues using microarray chip based MALDI-TOF MS

Alex Xiu-Cheng Fan[1], Corina Kohler[1], Ramin Radpour[1], Mahdi Montazer Haghighi[2], R. Zanetti-Dällenbach[3], Edward Wight[3], Wolfgang Holzgreve[4], Xiao Yan Zhong[1]

[1] Laboratory for Prenatal Medicine and Gynecologic Oncology, Women's Hospital / Department of Biomedicine, University of Basel, Switzerland

[2] Azad University, East of Tehran Branch, Iran

[3] Department of Gynaecology and Obstetrics, Women's Hospital, University of Basel, Switzerland

.[4] University Medical Centre of Freiburg, Germany

Switzerland

Correspondence to:

Prof. Dr. Xiao Yan Zhong

Laboratory for Prenatal Medicine and Gynecologic Oncology

Women's Hospital / Department Research

University of Basel

Hebelstrasse 20, Room Nr. 416

CH 4031 Basel, Switzerland

Tel: ++41 61 265 9224 / 9595

Fax: ++41 61 265 9399

e-mail:zhongx@uhbs.ch

Abstract

Mutations and up-/down-regulations of mitochondrial DNA (mtDNA) have been found in various cancers. The mtDNA alterations are suspected to be associated with carcinogenesis. In this study, we developed microarray chip based multiplex assay using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) for simultaneously detecting 22 mtDNA single nucleotide variants in 6 breast cancer cell lines and in the 102 cancerous and adjacent breast tissue samples from 51 patients with breast cancer. Totally, 164 mutations were identified in 66.67% of breast cancer cell lines and in 49.12% patients' tissue samples, in both the cancerous and adjacent parts. Most of the detected mtDNA variants in breast cancer samples were distributed in the regions of D-loop, 12S ribosomal RNA, 16S ribosomal RNA and tRNA. In our previous study, we found down-regulation of mtDNA in the breast cancerous tissues compared to the adjacent tissues. In this study, we investigated the relationship between the quantitative and qualitative alterations of mtDNA in breast cancer. No correlation was found between the down-regulation and the mutations of mtDNA in breast cancer tissues. Also there was no association was found between the mutations and clinic-pathological parameters including tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors. Our study suggests that down-regulation and mutations of mtDNA in breast cancer tissues may be resulted by different mechanism. Both the qualitative and quantitative alterations of mtDNA in breast cancer may serve as biomarkers independent of clinic-pathological parameters for breast cancer on early screening, diagnosis, monitoring treatment effects, prognosis, and follow up. MALDI-TOF MS based MicroARRAY multiplex assay is a high-throughput, and cost-efficient tool for large scale allelotyping of mtDNA informative mutations or single nucleotide mutation in life science.

Keywords: Quantitative alteration, qualitative alteration**,** mitochondrial DNA (mtDNA), breast cancer, MALDI-TOF MS Based MicroARRAY Assay, allelotyping

Introduction

Mitochondria as cellular power sources play fundamental roles in energy metabolism, generation of oxygen species (ROS), aging and initiation of apoptosis. These cellular organelles have their own independent genome -mitochondrial DNA (mtDNA), which is a double stranded circular DNA of 16.6 kb encoding 13 respiratory chain protein subunits, 22 tRNAs, and 2 rRNAs (Anderson et al, 1981). Due to the close proximity to reactive oxygen species (ROS), lack of the protective histone proteins and less efficient DNA repair mechanisms, compared to nuclear DNA (nDNA), mtDNA is more vulnerable to oxidative injury and has a higher level of mutation rate (Croteau et al. 1997; Jackson et al.1998; Zienolddiny et al. 2000), implying their importance in pathogenesis of disorders. It was hypothesized that cancer might be caused by the irreversible injury to the mitochondrial respiratory machinery (Warburg et al.1924, 1956). Many studies have shown that quantitative and qualitative mitochondrial aberrations are seen in various cancers of the bladder, breast, head and neck, thyroid, kidney, liver, lung, esophagean, stomach, colorectual, overian (Ebner et al. 1991; Horton et al.1996; Mambo et al. 2005; Tseng et al. 2006; Yu et al. 2007; Simonne et al. 2002; Capuano et al. 1996; Bianchi et al. 1995; Parrella et al. 2001; Zhu et al. 2005; Fliss et al. 2000; Kumimoto et al. 2004; Tamura et al. 1999; Polyak et al.1998; Liu et al. 2001).

Using temporal temperature gradient gel electrophoresis (TTGE) and 32 pairs of overlapping primers, followed by direct DNA sequencing, Tan et al (2002) screened the entire mitochondrial genome for somatic mutations of mtDNA in breast cancer. Multi (27 mutations) somatic mtDNA

mutations were identified in most of breast cancer tissue samples. Using 9 overlapping primer sets, Zhu et al (2005) sequenced the entire mitochondrial genome in breast cancer samples. The generated fragments in the study were 1886–2075 base pair in length for the sequencing. Again, multi (45 mutations) somatic mutations were discovered in breast cancer tissues in the study. The two studies showed the feasibility of screening the entire mitochondrial genome in breast cancer tissue; however, the clinical relevance of the finding remains unknown and multiplexed assays for simultaneously detecting multiple mutations of mtDNA in a single reaction are currently not available.

In the present study, we used a novel approach combining of MicroARRAY chip based MALDI-TOF MS and multiplex assay based iPLEX protocol developed by Sequenom (Sequenom, San Diego, USA) for simultaneous detection of up to 22 mutations of mtDNA in a single reaction. The 22 single nucleotide variants found in breast cancer are distributed on D-loop, 12S ribosomal RNA, 16S ribosomal RNA, tRNA, NADH dehydrogenase subunits, ATPase subunit 8, Cytochrome C oxidase subunite III, and cytochrome b regions (Zhu et al, 2005). The technique meets the requirements of high-throughput assays as a highly automated process and enables high-resolution analyses of single nucleotide changes at high multiplex levels. The feasibility of 22-plex assay for detection of 22 mtDNA variants simultaneously was explored by testing 51 breast cancerous tissues and 51 corresponding adjacent tissue samples from the same patients, as well as by examining 6 of breast cancer cell lines. The relationship between mutations and quantitative changes of mtDNA in breast cancer tissues, as well as the correlation between mutations of mtDNA and clinic-pathological parameters was analysed.

Method and Materials

Tissue samples

The local ethical committee approved the use of the archived tissues samples. Fifty-one paired cancerous and adjacent, formalin-fixed, paraffin-embedded breast tissue samples from patients with breast cancer were examined by two experienced pathologists. DNA was extracted from five sections of each 10 µm thick paraffin-embedded sample (around 20 mg of tissue) using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol and eluted into 150 µl of elution buffer. The eluted DNA was stored at -20°C until further use.

Cell culture

MDA-MB-231, MCF-7, and HS578T cells were grown in Dulbecco's modified Eagle medium (DMEM) (4.5g/l glucose L-glutamine); BT549 and T47D cells were grown in RPMI 1640 (L-glutamine); SKBR3 cells were grown in McCoy's 5A medium. All media purchased from Gibco/Invitrogen (Carlsbad CA, USA) were supplemented 10% FCS and 1% penicillin-streptomycin. The cells were maintained in a humified incubator at 37°C 5% CO2, and harvested at 80% confluency. Before DNA extraction the cells were washed 1xPBS. DNA was extracted from 1 X 10^6 cells by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and eluted in 100 µl of elution buffer and stored at -20°C until further use.

Quantitative analysis of mtDNA using real-time PCR

The amounts of nDNA and mtDNA were quantified by multiplex TaqMan real-time PCR for both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene of nDNA and mitochondrial

ATPase subunit 8 (MtATP 8) gene of mtDNA starting at locus 8446. The GAPDH and MtATP 8 primer and probe sequences are shown as follows: GAPDH (forward):5' CCC CAC ACA CAT GCA CTT ACC3'; (reverse): 5'CCT AGT CCC AGG GCT TTG ATT 3'; probe5' (MGB) TAG GAA GGA CAG GCA AC (VIC) 3'. MtATP 8 (forward): 5' AAT ATT AAA CAC AAA CTA CCA CCT ACC 3'; (reverse): 5' TGG TTC TCA GGG TTT GTT ATA 3'; probe: 5' (MGB) CCT CAC CAA AGC CCA TA (FAM) 3'.The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI). 2.5 µl DNA were used as template for the PCR analysis. The real-time PCR was carried out in 25 µl of total reaction volume using a 2 minute incubation at 50°C, followed by an initial denaturation step at 95°C for 10 minutes and 40 cycles of one minute at 60°C and 15 seconds at 95°C.

The theory, practice and the amplification efficiency of PCR for both GAPDH and MtATP 8 has been demonstrated in our previous study (Xia et al. 2009). According to the comparable amplification efficiency of two assays, in our study, the average threshold cycle number (Ct) values of the nDNA and mtDNA were obtained from each case for quantitative assessments. The content of mtDNA was calculated by using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt = CtnDNA-CtmtDNA) in the same well as an exponent of 2 ($2^{\Delta Ct}$).

Detection of mtDNA mutations using microarray chip based MALDI-TOF MS

The PCR and extension primers used to analyze the mtDNA mutations were designed using MassARRAY Assay Design v.3.1 software (Sequenom, San Diego, USA). Based on Cambridge Reference Sequence (CRS) of human mitochondrial genome and the publication of Zhu et al (2005), we summarized total 45 mutations at 35 nucleotide positions to 37 types of mutant input format for the assay design. Due to the compromise of mutation capture amplicon length, extend

primer length and the mass range, and to avoid weak extend dimmer potential, false priming potential, and extend self-dimmer potential, 22 single nucleotide variants (Table 1) were selected by the software randomly available for the analysis at a multiple level in a single reaction. Of these 22 somatic mutations, 3 are located in D-loop, 1 in 12S ribosomal RNA, 1 in 16S ribosomal RNA, 2 in tRNA, 11 in NADH dehydrogenase subunits, 1 in ATPase subunit 8, 1 in Cytochrome C oxidase subunite III, and 1 in cytochrome b region. Fifteen mutations lead to codon changes, and 8 lead to amino acids changes (Table 1, Figure 1). For this study, two primers for amplifying each variant (capture primers) and one more primer for extending each mutation (extension primer) were designed using the software. The 66 primers for the examination of 22 single nucleotide variants in total are listed in table 2 (Table 2). A 10-mer tag of 5'-ACGTTGGATG-3' to the 5'end of the each primer for PCR amplification was used to avoid chaos in mass spectrum and to improve PCR sufficiency according to the manufacture's protocol.

The capture PCR reactions for amplifying 22 fragments containing the 22 sequence variants in a single well were carried out in 10 µl PCR cocktail mixes comprising 1µl DNA, 1.626mM MgCl2, 500uM dNTP mix, 0.5U HotStarTaq DNA polymerase and primer pairs. The amplification was performed under the following conditions: incubation at 94°C for 15 minutes, followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 5 min. To remove the non-incorporated dNTPs, a shrimp alkaline phosphatase (SAP) treatment was performed after the PCR reaction under the following conditions: 37°C for 20 minutes, 85°C for 5 minutes and cooling to 4°C. The iPLEX extension reaction was performed using the iPLEX gold cocktail mix (Sequeonom, San Diego, USA), which contains buffer, termination mix, enzyme and extension primers, under the following conditions: 94° C for 30 seconds, followed by 40 cycles of 94°C for 5 seconds, plus 5 sub-cycles of 52°C for 5 seconds and 80°C for 5 seconds; one cycle of final extension at 72°C for 5 min,

then cooling to 4°C. The iPLEX reaction products were desalted using clean resin to optimise mass spectrometric analysis and dispensed onto a 384-element SpectroCHIP bioarray by using a nanodispenser (Sequeonom, San Diego, USA) for MALDI-TOF MS analysis. The MassARRAY Typer 4.0 software (Sequeonom, San Diego, USA) was used to process and analyze iPLEX SpectroCHIP bioarrays. The call rate, extension rate and peak area for all allele-specific analyses in any given assay were calculated using the software, Typer 4.0, provided by the manufacturer. DNA from a mitochondria negative cell line, which was obtained by sub-culturing that cell line in the presence of low concentration of ethidium bromide until the cells were devoid any of mitochondria, was used as negative control. The negative control was run at each step and on each chip.

Statistical Analysis

The data were analyzed using SPSS software (Statistical Software Package for Windows v. 15.0). Content of mtDNA is given as the median, the range and the fold difference. The Shapiro-Wilk test was used for the analysis of the distribution of data. The Mann–Whitney U test and Kruskal–Wallis test was applied to compare the mtDNA content between study groups. Pearson chi-square test was applied to analyze the association between mtDNA variants and clinicopathological parameters.

Results

Quantitative analysis of mtDNA in 6 cell lines and the tissues samples

Nuclear DNA and mtDNA were co-extracted from the 10^7 cultured cells from each cell line. The average Ct values for the GAPDH sequence, representing total nDNA, were 26.65, 26.92, 27.01, 27.40, 24.21, and 17.83 for BT549, HS578T, T47D, SKBR3, MCF-7, and MDA-DB231 cells

respectively, while the average Ct values for the MTATP 8 gene sequence, representing total mtDNA, were 16.12, 17.26, 15.3, 15.83, 14.38, and 16.3 respectively. The average Ct values of mtDNA were less than those of nDNA, and in all cases, represented higher amounts of mtDNA than those of nDNA in these cell lines. The ΔCT values between GAPDH gene amplification and MTATP 8 gene amplification show 10.53, 9.66, 11.71, 11.57, 9.83 and 1.53 cycles difference respectively. The mtDNA content was calculated by the formula of $2^{\Delta CT}$. After normalization to GAPDH, mtDNA contents were 1478.58, 809.00, 3350.13, 3040.30, 910.18, and 2.89-fold high in BT549, HS578T, T47D, SKBR3, MCF-7, and MDA-DB231 cells, respectively. The content of the mtDNA in both cancerous and normal breast cancer tissues from the 51 patients with breast cancer has been shown in our previous study (Fan et al, 2009).

22plex MALDI-TOF MS Based MicroARRAY assay for somatic mtDNA mutations

The advantage of having as high plex level as possible per well is to make the screening of somatic mitochondrial DNA mutations more effective. The efficiency of 22-plex assay was assessed by call-rate and call-possibility software Typer 4.0 (Sequenom Inc, San Diego, Germany) automatically, and revaluated by visual examination of each mass spectrum. Excluding the mtDNA negative cells, each of these 22 variants could be identified successfully with a call-rate range of 89.04% to 100% in 108 samples (102 breast tissue samples and 6 cell lines) (Table 1). The average of call possibilities is 95.03%.

Using the 22-plex assay, we analysed the 22 mtDNA variants in 51 cancerous and paired normal tissue samples, as well as in 6 cell lines. In total, 164 mutations were found in our examined samples. The distribution of the mtDNA variants identified in this study is listed in the table 3. MtDNA mutations 709 G>A and 2709 A>G were found in the 4 (MDA-MB 231, MCF-7, BT 549

and SKBR 3) out of the 6 cell lines (66.67%), and mtDNA mutations 709 G>A on 12S ribosomal RNA (Figure 3) , 2706 A>G on 16S ribosomal RNA (Figure 4), 15924 A>G on tRNA (Figure 5) and 16145 A>G on D-loop (Figure 6 and Figure 7) were identified in 25 out of 51 patients samples (49.12%). Twenty-two out of 25 patients (88.00%) showed one mutation in their tissue samples, while 3 patients had 2. The mutations were distributed in 22 out of 51 cancerous tissues (43.14%) and in 24 out of 51 adjacent tissues (47.06%) respectively. The mutation prevalence between breast cancer cell lines and breast cancer tissues, as well as between the cancerous tissues and adjacent tissues were comparable ($p = 0.408$ and 1.000 respectively). Most of the paired cancerous and adjacent tissues (22/25 = 88 %) showed the identical mutations (Table 3). While the cases 807 and 828 showed a completely 16145 A>G mutation in cancerous tissues, a mixture of a wild-type form G and a mutant form A was found in the adjacent tissue. For the cases 824, 829 and 846, we found mutations 709 G>A or 16145 A>G in adjacent tissues, but not in cancerous tissues. In all of the samples, a 311-315 insertion C was found, in which 49 out of 51 cases showed a homoplasmic form (Figure 8) and two cases (822 and 841) revealed a heteroplasmic alteration (Figure 9). We assume that the 311-315 insertion C might be a polymorphism in the study cohort and is not listed in the table 3.

In the cancerous tissues, we found 164 mutations at 5 nucleotide positions in all 6 cell lines and 51 paired tissue samples. Ten out of these mutations were only present in 6 cell lines and 154 out of those were found in tissue samples. Of 56.86% (29/51) cancerous tissue samples, 50.98 % (26/51) normal tissue samples and 33.33% (2/6) cell lines, each had one mutation detected. In 41.18 % (21/51) cancerous samples, 47.06 (24/51) normal samples and 66.67% (4/6) cell lines, 2 mutations were identified in each sample, while in one paired samples 3 sequence variants were found in both normal and cancerous samples (Table 3). The sequence variants at np 311-315 were found in all the samples. One C insertion at np 311-315 (Figure 3) was the most frequent

sequence variant identified in 49 pairs of tissue samples and 6 cell lines. 709A and 2706 A were also identified in both tissue samples and cell lines (Figure 5, 6). 15924 G, 16145 A and 16145 AG were found only in tissue samples (Figure 7, 8, 9). In paired tissue samples, 8 (5.2%) of the sequence variant were heteroplasmic, and 146 (94.8%) were homoplasmic. 4 heteroplasmic C ins/- at np311-315 (Figure 4) were found both in 2 paired cancerous and normal samples, while the other 4 heteroplasmic AG at np 16145 (Figure 8) were found in 4 different normal tissue samples. 12 sequence variants found in either cancerous or normal tissue samples were different from the paired corresponding samples. All the detected sequence variants were distributed at 5 np in a hotspot region around the D-loop; two were in D-loop, one in 12S RNA, one in 16S RNA, and one in tRNA- threonine.

Due to the extremely high frequency of sequence variant at np 311-315, a single-plex MALDI-TOF MS based MassARRAY assay was performed to confirm the detection, and the coherent results were identified by single-plex assay in comparison with the results by 22 plex assay. The sequence variant at np 311-315 present in all samples was regarded as a physiological variation and excluded from the further analysis in this study.

In this study we investigated the relationship between reduced mtDNA content and mtDNA mutations in breast cancer. For the 6 breast cancer cell lines, it doesn't seem the mtDNA content associates with mtDNA mutations conditions.

For the analysis of the 51 paired tissue samples, we divided the cases into different subgroups according to sequence variants on mtDNA and sample types. In both of cancerous and normal tissue samples the samples containing mtDNA sequence variants showed significant reduced mtDNA contents (P=0.039) compared to the samples without detectable mtDNA variants.

However, the quantitative alteration of mtDNA in the tissues were not found having any association with any single certain variation at nucleotide positions of 709, 2706, 15924, and 16145 mutations respectively (Table 4). Likewise, no significant difference was found in the comparison of mtDNA content between the groups with and without sequence variants within either normal or cancerous tissue groups, therefore most likely in this Iran cohort the difference of mtDNA content between with and without variant groups was caused only by the different tissue types (Table 4).

Furthermore we analyzed the relationship between somatic mtDNA mutation status and the traditional clinicopathological factors in breast cancer patients. In this study we did not find somatic mtDNA mutations was associated with age, tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors (Table 5). As well in breast cancer patients with mtDNA variants we did not find any association between tissue types and clinicopathological parameters (Table 6).

Discussions

Many mtDNA mutations have been observed in many cancers; however, there were no common mutations could be found in a certain cancer type for subsequent investigations, suggesting that a high-throughput assay for individually detecting multiple mutations simultaneously is requested. In our study, we developed a MALDI-TOF MS based MicroARRAY multiplex assay to detect 22 mtDNA sequence variants in a single reaction. We found mtDNA sequence variants in all 6 cell lines, cancerous and normal tissue samples. Except the sequence variant at 311-315 np, the frequency of a sequence variant at each nucleotide position was identified in tissue samples at a

variant range from 7.41% to 14.71 %. In the study by Zhu et al (Zhu et al. 2005) the parentages of mutation at each np were ranged from 3.33% to 10 % of 15 paired breast cancerous tissue samples. In the other two studies on breast cancer this rate is from 2.63 to 10.53% in 19 paired tissue samples (Tan et al. 2002) and from 0.83% to 10% in 60 paired tissue samples (Yu et al. 2007) respectively.

In this study the sequence variants were detected at 5 np in D-loop and the neighbourhood tRNA threonine, 12S and16S RNA genes, none in any coding region. The similar regions have been reported as hot spots for high mutation frequency of the mitochondrial genome (Fliss et al. 2000; Liu et al. 2001; Tan et al. 2002; and Tseng et al. 2006). It has also been reported that somatic mitochondrial mutations in the D-loop, the replication control region, are associated with a reduction of mtDNA content (Lee et al. 2004; Tseng et al. 2005).

In breast cancer, besides the alterations in the nucleotide sequence, the alterations in mtDNA content (Mambo et al 2005; Tseng et al 2006; Yu et al. 2007) had also been reported. It has been suspected that both mtDNA content alteration and mtDNA mutations were involved in the development and progression of malignant neoplasm (Shay et al. 1987). In our previous study we found mtDNA content decreased in cancerous breast tissue samples compared with the corresponding normal tissue samples (Fan et al. 2009). In this study we identify the mtDNA sequence variants happened at np 311-315 identified in all the samples, which is most likely a physiological polymorphism. Even firstly it has been reported a simple repeat of five cytosine residues at np 311−315 as rare polymorphic alleles in CRS, whereas it was also stated in the reanalysis and revision by the investigators there are six residues at the same np in most other human mtDNAs (Richard et al. 1999). Similarly as indicated in Global human mtDNA phylogenetic tree - Build 3 (1 Mar 2009) analysis, the highly recurrent sequence variation 315.

1C were not regarded as phylogenetic reconstruction subsequently excluding from mtDNA phylogenetic tree (van Oven et al. 2009). Therefore we do exclude 1C insertion at 315 from the further association analysis.

The method we used for this study has been shown a good sensitivity to detect as a low amount as 5% variant mtDNA submerged in a wild type background (Fan et al. 2008), and compared with sequencing an accuracy of more than 99.86% concordance could be achieved (Garritsen et al. 2008). However, it could be undetectable if a mutation potential was lower than 5%, when some rare variants alleles were submerged among vast predominance of normal sequences. In addition, the mutations outside of this combination of 22 np can not be detected by this assay, which need to be further investigated.

To the best of our knowledge, this is the first report of highest multiplex assays for simultaneous detecting mutations of mtDNA. Compared to the conventional methods, the MALDI-TOF MS based MicroARRAY multiplex assay is a high-throughput and cost-efficient method, which could be a sufficient tool for large scale somatic mtDNA mutation analysis, and might have far-reaching potential on routine clinical application.

.

Acknowledgement

This work was supported in part by Swiss National Science Foundation (320000-119722/1), Swiss Cancer League, Krebsliga Beider Basel and Dr Hans Altschueler Stiftung. We thank Mrs Vivian Kiefer and Ms Nicole Chiodetti for their help.

Reference

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet.Oct; 23(2):147.

Anderson S, Bankier AT, Barrell BG, de-Bruijn MHL, Coulson AR, et al. (1981). Sequence and organization of the human mitochondrial genome. Nature 290: 427–465.

Attardi G, Schatz G. Biogenesis of mitochondria. Annul Rev Cell Biol 1988; 4: 289-333.

Bianchi MS, Bianchi NO, Bailliet G. (1995) Mitochondrial DNA mutations in normal and tumor tissues from breast cancer patients. Cytogenet Cell Genet. 1995;71(1):99-103.

Capuano F, Varone D, D'Eri N, Russo E, Tommasi S, Montemurro S, Prete F, Papa S. Oxidative phosphorylation and F(O)F(1) ATP synthase activity of human hepatocellular carcinoma. Biochem Mol Biol Int 1996; 38: 1013-22.

Cavalli LR, Liang BC. (1998) Mutagenesis, tumorigenicity and apoptosis: are the mitochondria involved? Mutat. Res., 398, 19--26.

Copeland WC, Wachsman JT, Johnson FM, Penta JS. Mitochondrial DNA alterations in cancer. Cancer Invest 2002; 20: 557-69.

Croteau DL, Bohr VA. 1997. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem 272: 25409–25412.

Ebner D, Rodel G, Pavenstaedt I, Haferkamp O. Functional and molecular analysis of mitochondria in thyroid oncocytoma. Virchows Arch B Cell Pathol Incl Mol Pathol 1991; 60: 139-44.

Fan AX, Garritsen HSP, Tarhouny S, Morris M, Hahn S, Holzgreve W, Zhong XY. A rapid and accurate approach to identify single nucleotide mutations of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med. 2008; 46(3):299-305.

Fan AX, Radpour R, Haghighi MM, Kohler C, Xia P, Hahn S, Holzgreve W, Zhong XY. (2009) Mitochondrial DNA content in paired normal and cancerous breast tissue samples from patients with breast cancer. J Cancer Res Clin Oncol. 2009 Jan 6. [Epub ahead of print]

Fliss,M.S., Usadel,H., Caballero,O.L., Wu,L., Buta,M.R., Eleff,S.M., Jen,J. and Sidransky,D. (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science, 287, 2017--2019.

Garritsen HSP, Fan AX, Hannig H, Holzgreve W, Zhong XY. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for Genotyping Human Platelet Specific Antigens (HPAs) Transfusion. 2008 Oct 29. [Epub ahead of print]

Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, Ghelli A, Moretti M, Betts CM, Martinelli GN, Ceroni AR, Curcio F, Carelli V, Rugolo M, Tallini G, Romeo G.(2007) Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic. Proc Natl Acad Sci U S A. 104(21):9001-6.

Garritsen HS, Fan AX, Bosse N, and etc. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for genotyping of human platelet-specific antigens. Transfusion. 2008 Oct 29. [Epub ahead of print]

G. Tamura, S. Nishizuka, C. Maesawa, et al., Mutations in mitochondrial control region DNA in gastric tumours of Japanese patients, Eur. J. Cancer 35 (1999) 316–319.

Heddi A, Faure-Vigny H, Wallace DC, Stepien G. (1996) Coordinate expression of nuclear and mitochondrial genes involved in energy production in carcinoma and oncocytoma. Biochim Biophys Acta, 1316: 203-9.

Jackson,A.L., Chen,R. and Loeb,L.A. (1998) Induction of microsatellite instability by oxidative DNA damage. Proc. Natl Acad. Sci. USA, 95,12468--12473.

K. Polyak, Y. LI, H. Zhu, et al., Somatic mutations of the mitochondrial genome in human colorectal tumours, Nature Genet. 20 (1998) 291–293.

Kumimoto H, Yamane Y, Nishimoto Y, Fukami H, Shinoda M, Hatooka S, Ishizaki K. Frequent somatic mutations of mitochondrial DNA in esophageal squamous cell carcinoma. Int J Cancer 2004; 108: 228-31.

LaBiche RA, Demars M, Nicolson GL. (1992). Transcripts of the mitochondrial gene ND5 are overexpressed in highly metastatic murine large cell lymphoma cells.In Vivo 6: 317–324.

LaBiche RA, Yoshida M, Gallick GE, Irimura T, Robberson DL, Klostergaard J et al. (1988). Gene expression and tumor cell escape from host effector mechanisms in murine large cell lymphoma. J Cellular Biochem 36: 393–403.

Lee HC, Li SH, Lin JC, Wu CC, Yeh DC, Wei YH. Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. Mutation Research. 2004;547:71–78.

Liu VW, Shi HH, Cheung AN, Chiu PM, Leung TW, Nagley P, Wong LC, Ngan HY. (2001) High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas. Cancer Res 61: 5998-6001.

Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung SC, Sukumar S, Sidransky D. (2005) Tumor-specific changes in mtDNA content in human cancer. Int J Cancer.116 (6):920-4.

Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, Nicol T, Gabrielson E, Cuomo C, Cohen D, Pandit S, Spencer M, Rabitti C, Fazio VM, Sidransky D. (2001)Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res. 61(20):7623-6.

Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH. (2002). Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. Biochim Biophys Acta 1555: 14–20.

Peng Xia, Ramin Radpour, Rebecca Zachariah, Alex Xiu Cheng Fan, Corina Kohler, Sinuhe Hahn, Wolfgang Holzgreve and Xiao Yan Zhong (2009) Simultaneous quantitative assessment of circulating cell-free mitochondrial and nuclear DNA by multiplex real-time PCR. Genetics and Molecular Biology, 32, 1, 20-24.

Penta JS, Johnson FM, Wachsman JT, and Copeland WC. (2001) Mitochondrial DNA in human malignancy. Mutat Res, 488: 119-33.

Polyak,K., Li,Y., Zhu,H., Lengauer,C., Willson,J.K., Markowitz,S.D., Trush,M.A., Kinzler,K.W. and Vogelstein,B. (1998) Somatic mutations of the mitochondrial genome in human colorectal tumours. Nature Genet., 20, 291--293.

Richard M. Andrews, Iwona Kubacka, Patrick F. Chinnery, Robert N. Lightowlers, Douglass M. Turnbull and Neil Howell. (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature Genetics 23, 147

Racker E, Spector M. Warburg effect revisited: merger of biochemistry and molecular biology. Science 1981; 213: 303-7.

Shay JW, Werbin H. Are mitochondrial DNA mutations involved in the carcinogenic process? Mutat Res 1987; 186: 149-60.

Simonnet H, Alazard N, Pfeiffer K, Gallou C, Beroud C, Demont J, Bouvier R, Schagger H, Godinot C. (2002) Low mitochondrial respiratory chain content correlates tumor aggressiveness in renal cell carcinoma. Carcinogenesis; 23: 759-68.

Tallini G, Ladanyi M, Rosai J, Jhanwar SC. (1994) Analysis of nuclear and mitochondrial DNA alterations in thyroid and renal oncocytic tumors. Cytogenet Cell Genet 66: 253-9.

Tamura,G., Nishizuka,S., Maesawa,C., Suzuki,Y., Iwaya,T., Sakata,K., Endoh,Y. and Motoyama,T. (1999) Mutations in mitochondrial control region DNA in gastric tumours of Japanese patients. Eur. J. Cancer, 35, 316--319.

T.H. Horton, J.A. Petros, A. Heddi, et al., Novel mitochondrial DNA deletion found in a renal cell carcinoma, Genes, Chromosomes Cancer 15 (1996) 95–101.

Torroni A, Stepien G, Hodge JA, Wallace DC. Neoplastic transformation is associated coordinate induction of nuclear and cytoplasmic oxidative phosphorylation genes. J Biol Chem 1990; 265: 20589-93.

Tseng LM, Yin PH, Chi CW, Hsu CY, Wu CW, Lee LM, Wei YH, Lee HC. (2006) Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. Genes Chromosomes Cancer. 45(7):629-38.

van Oven M, Kayser M. 2009. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat 30(2):E386-E394.

Valenta LJ, Michel-Bechet M, Warshaw JB, Maloof F. (1974) Human thyroid tumors composed of mitochondrion-rich cells: electron microscopic and biochemical findings. J Clin Endocrinol Metab, 39: 719-33.

Wallace DC. (2005). Annu Rev Genet. 39: 359–407.

Wang X. (2001) The expanding role of mitochondria in apoptosis. Genes Dev, 15: 2922-33.

Warburg O, Posener K, Negelein E. (1924) Ueber den Stoffwechsel der Tumoren; Biochemische Zeitschrift, 152: 319-344.

Warburg O. (1956). On the origin of cancer cells. Science 123: 309–314.

Xiu-Cheng Fan A**,** Garritsen HS**,** and etc**.** (2008) A rapid and accurate approach to identify single nucleotide mutations of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med. 46(3):299-305.

Yu M, Zhou Y, Shi Y, Ning L, Yang Y, Wei X, Zhang N, Hao X, Niu R. (2007) Reduced mitochondrial DNA copy number is correlated tumor progression and prognosis in Chinese breast cancer patients.IUBMB Life.59(7):450-7.

Zachariah RR, Schmid S, Buerki N, Radpour R, Holzgreve W, Zhong X. (2008) Levels of circulating cell-free nuclear and mitochondrial DNA in benign and malignant ovarian tumors. Obstet Gynecol;112(4):843-50.

Zienolddiny,S., Ryberg,D. and Haugen,A. (2000) Induction of microsatellite mutations by oxidative agents in human lung cancer cell lines.Carcinogenesis, 21, 1521--1526.

Nucleotide position (np)	Reference sequence	Nucleotide change	Gene	Codon change	Amino acid change	Success of Assay (%)
207	G	G>A	D-Loop	NCL		95.18
311-315	CCCCC	C ins	D-Loop	NCL		100
709	G	G>A	12S	NCL		99.12
2706	A	A>G	16S	NCL		97.81
3849	G	G>A	ND1	TTG-TTA	NO	99.12
4323	T	T> C	tRNA-I	NCL		97.37
4499	C	C>T	ND2	TAC-TAT	NO	99.12
4665	G	G>A	ND2	GCA-ACA	A60T	99.12
5240	A	A>T	ND2	CTA-CTT	NO	98.24
8498	A	A>G	ATP8	AAA-GAA	K45E	92.98
9885	T	T>A	COXIII	TTT-ATT	F227I	100
11768	A	A>G	ND4	ACT-GCT	T337A	97.37
12642	A	A>G	ND5	GAG-GAA	NO	98.68
12852	C	C>T	ND5	ATC-ATT	NO	96.93
13263	A	G>A	ND5	CAG-CAA	NO	89.04
13398	A	A>T	ND5	CAA-CAT	Q354H	96.93
13674	T	T>G	ND5	AAT-AAG	N446K	96.93
15700	C	C>T	ND5	CGC-CGT	NO	99.12
15783	C	C>T	CYTB	CTT-CCT	L346P	99.12
15824	A	A>G	CYTB	ACA-GCA	T360A	99.56
15924	A	A>G	tRNA-T	NCL		97.37
16145	G	G>A	D-Loop	NCL		97.81

Table 1. mtDNA sequence variants in breast cancer were selected for 22plex MS-ARRAY assay. NCL, no coding locus; 12S, 12S ribosomal RNA; 16S, 16S ribosomal RNA; ND 1, 2, 4, 5, NADH dehydrogenase subunit 1, 2, 4, 5; tRNA-I, tRNA isoleucine; ATP8, ATP synthase subunit 8; COXIII, cytochrome c oxidase subunit III; tRNA-T, tRNA threonine; ins, insertion.

Mutation	Mutation capture Primer	Extension primer
Mt311-315	ACGTTGGATGAAATTTCCACCAAACCCCCC ACGTTGGATGGCTGGTGTTAGGGTTCTTTG	CAAACCCCCCCTCCCCC
mt4499	ACGTTGGATGTGTTGGTTATACCCTTCCCG ACGTTGGATGTGATGAGTGTGCCTGCAAAG	GCCCAACCCGTCATCTA
mt4665	ACGTTGGATGCATCAAGTATTTCCTCACGC ACGTTGGATGATGGTTCATTGTCCGGAGAG	TCCTCACGCAAGCAACC
mt709	ACGTTGGATGAGGTTTGGTCCTAGCCTTTC ACGTTGGATGTCGTGGTGATTTAGAGGGTG	GGGTGAACTCACTGGAA
mt15783	ACGTTGGATGGCAGACCTCCTCATTCTAAC ACGTTGGATGCGGATGCTACTTGTCCAATG	GACAACCAGTAAGCTACC
mt15700	ACGTTGGATGTCCCCATCCTCCATATATCC ACGTTGGATGGCGGCTAGGAGTCAATAAAG	gAGTGATTGGCTTAGTGG
mt3849	ACGTTGGATGACACCTCTGATTACTCCTGC ACGTTGGATGTCGGTTGGTCTCTGCTAGTG	TCCTGCCATCATGACCCTT
mt13398	ACGTTGGATGCTATTTATGTGCTCCGGGTC ACGTTGGATGGGTTGAAGTGAGAGGTATGG	ACAACCTTAACAATGAACA
mt9885	ACGTTGGATGTATCTGCTTCATCCGCCAAC ACGTTGGATGAAAATGCCAGTATCAGGCGG	TCCGCCAACTAATATTTCAC
mt5240	ACGTTGGATGCTTAATTCCATCCACCCTCC ACGTTGGATGCTTCGATAATGGCCCATTTG	gATTTGGGCAAAAAGCCGGT
mt2706	ACGTTGGATGAGGGTTCAGCTGTCTCTTAC ACGTTGGATGCATAGGGTCTTCTCGTCTTG	gttaTTCTCGTCTTGCTGTGT
mt11768	ACGTTGGATGCGGGCTTACATCCTCATTAC ACGTTGGATGTGAGAGAGGATTATGATGCG	gggcACTCAAACTACGAACGC
mt4323	ACGTTGGATGCCCTCAAACCTAAGAAATATG ACGTTGGATGGGTTCGATTCTCATAGTCCT	ccccATAGGAGCTTAAACCCCC
mt12642	ACGTTGGATGCATCCCTGTAGCATTGTTCG ACGTTGGATGGATTAATGTTTGGGTCTGAG	gaGTTACATGGTCCATCATAGA
mt15824	ACGTTGGATGCATTGGACAAGTAGCATCCG ACGTTGGATGGGGAGATAGTTGGTATTAGG	cctcAGCATCCGTACTATACTTC
mt12852	ACGTTGGATGATCAGTTGATGATACGCCCG ACGTTGGATGGAAACCGATATCGCCGATAC	ttctCAGCAGCCATTCAAGCAAT
mt15924	ACGTTGGATGAAATGGGCCTGTCCTTGTAG ACGTTGGATGTTTCTCTGATTTGTCCTTGG	acACTAATACACCAGTCTTGTAA
mt16145	ACGTTGGATGGCCAGCCACCATGAATATTG ACGTTGGATGGGGTTTTGATGTGGATTGGG	TATTGTACGGTACCATAAATACTT
mt13674	ACGTTGGATGGCTTCCCCACCCTTACTAAC ACGTTGGATGAATCCTGCGAATAGGCTTCC	cccCCCTTACTAACATTAACGAAAA
mt13263	ACGTTGGATGATCGTAGCCTTCTCCACTTC ACGTTGGATGAGGAATGCTAGGTGTGGTTG	ttATTGTAACTATTATGAGTCCTAG
mt8498	ACGTTGGATGCACAAACTACCACCTACCTC ACGTTGGATGCGTTCATTTTGGTTCTCAGG	GGTTCTCAGGGTTTGTTATAATTTT
mt207	ACGTTGGATGTTACAGGCGAACATACTTAC ACGTTGGATGAAGTGGCTGTGCAGACATTC	tacatGCGAACATACTTACTAAAGTGT

Table 2. Sequences of 22plex mtDNA variants capture and extension primers.

Sample counting	Sample ID	characteristics	Nucleotide position (np)	Adjacent	Cancer (Cell line)
1	MDA-MB 231		709		A
2	MCF-7		2706		A
3	BT 549		2706		A
4	SKBR 3		2706		A
1	802	IDC	709	A	A
2	803		2706	A	A
3	804		16145	A	A
4	807		16145	AG	A
5	812		16145	AG	
	812		709		A
6	815		2706	A	A
7	817		16145	A	A
8	819		709	A	A
9	821		16145	A	A
10	822		311-315	C ins/-	C ins/-
11	823		709	A	A
	823		2706	A	A
12	824		709	A	
13	825		16145	A	A
14	827		15924	G	G
15	828		16145	AG	A
16	829		709	A	
17	830		15924		G
18	838		15924	G	G
19	840		709	A	A
20	841		311-315	C ins/-	C ins/-
21	843		2706	A	A
22	844		16145	AG	
	844		15924		G
23	846		16145	A	
24	851		15924	G	G
25	853		709	A	A

Table 3. mtDNA sequence variants were found in tissue samples. *Besides 822n/p and 841n/p paired samples only have one of C ins/- heteroplasmic mutations, each of the rest samples and cell lines have one mutation of C ins. (IDC = invasive ductal carcinoma)

Group	Case (percentage)	mtDNA content	P value
709-	89 (87.25)	15.35 (1.17-69272.73)	0.098*
709+	13 (12.75)	56.69 (1.56-2730.60)	
2706-	94 (92.16)	15.19 (1.17-69272.73)	0.700*
2706+	8 (7.84)	39.98 (1.49-2730.60)	
15924-	94 (92.16)	15.19 (1.17-69272.73)	0.429*
15924+	8 (7.84)	63.60 (2.17-13587.57)	
16145-	86 (84.31)	21.05 (1.17-69272.73)	0.800*
16145+	16 (14.71)	13.27 (1.35-749.61)	
Var –	60 (58.82)	9.73 (1.17-69272.73)	0.039*
Var+	42 (41.18)	37.42 (1.35-13587.57)	
[1]N & Var –	28 (27.45)	60.38 (3.05-9877.98)	1vs2: 0.130*; 3vs4: 0.070*
[2]N & Var+	23 (22.55)	216.02 (1.35-3888.51)	1vs3: 0.000*; 2vs4: 0.013*
[3]C & Var –	32 (31.37)	3.89 (1.17-69272.73)	0.421**
[4]C & Var+	19 (18.63)	15.03 (1.49-13587.57)	

Table 4. Sample profile of mtDNA content and variant groups (excluding 311-315). N: normal; C: cancerous; Var: Sequence Variant; "-": without; "+": with. * Mann-Whitney U test in normal tissue; ** Chi-square test was applied to analyze the association between tissue type and mutation.

Variables	Group (percentage of mutation:%)		mtDNA content		P value
			Without variant Median (range) cases	With variant Median (range) cases	
Age	<50	(35.48)	89.33 (3.05-9877.98) N=20	216.02 (1.35-2460.95) N=11	0.500
	≥50	(26.32)	51.58 (4.21-3888.51) N=14	346.09 (3.42-2730.60) N=5	
Histological type	Ductal	(31.71)	45.35 (3.05-9877.98) N=28	346.09 (1.35-2730.60) N=13	0.618
	Lobular	(40.00)	352.46 (15.35-823.14) N=6	201.39 (7.31-584.07) N=4	
Primary tumor	T1	(43.48)	106.15 (4.21-982.29) N=13	201.39 (1.35-749.61) N=10	0.328
	T2	(20.00)	34.94 (3.05-3888.51) N=12	554.48 (30.91-2730.60) N=3	
	T3	(30.00)	37.67 (5.31-2304.12) N=6	346.09 (22.86-2460.95) N=3	
Lymph node involment	Negative	(22.22)	72.50 (3.50-9877.98) N=7	108.68 (1.35-216.02) N=2	0.410[a],
	Positive	(36.59)	60.38 (4.21-3888.51) N=26	346.09 (3.42-2730.60) N=15	
Metastasis	M0	(30.00)	89.33 (3.05-9877.98) N=28	201.39 (1.35-2730.60) N=12	0.410[a],
	M1	(50.00)	49.35 (6.43-560.28) N=5	420.22 (3.42-584.07) N=5	
Stage	I	(40.00)	9.48 (3.05-106.15) N=3	108.68 (1.35-216.02) N=2	0.232[a],
	II	(25.93)	69.73 (4.21-3888.51) N=20	186.75 (7.31-2730.60) N=7	
	III	(50.00)	823.14 (25.99-2304.12) N=3	346.09 (22.86-2460.95) N=3	
	IV	(44.44)	49.35 (6.43-560.28) N=5	487.35 (7.31-584.07) N=4	
Histological grading	G1	(37.50)	9.48 (3.05-2304.12) N=5	689.78 (30.91-2460.95) N=3	0.957[a],
	G2	(31.58)	72.50 (5.31-982.29) N=13	450.29 (22.86-749.61) N=6	
	G3	(30.00)	33.67 (4.21-3888.51) N=10	149.09 (7.31-2730.60) N=5	
Nuclear grading	1	(0.00)	9.48 (5.13-106.15) N=3	N=0	0.249[a],
	2	(35.71)	41.36 (3.05-2304.12) N=9	346.09 (30.91-689.78) N=5	
	3	(50.00)	70.21(4.21-861.08) N=8	85.97 (3.42-2460.95) N=8	
ER	Negative	(38.89)	49.35(6.70-560.28) N=11	186.75 (3.42-749.61) N=7	0.854[a],
	Positive	(35.71)	823.14 (6.43-3888.51) N=9	689.78 (7.31-2730.60) N=5	
PR	Negative	(42.86)	36.67 (6.43-982.29) N=8	78.20 (3.42-554.48) N=6	0.581[a],
	Positive	(30.00)	160.58 (9.48-3888.51) N=12	719.70 (30.91-2730.60) N=6	

Her2	Negative(30.00)	210.67 (6.43-3888.51) N=10	584.07 (30.91-2730.60) N=5	0.647[a],
	Positive (41.18)	51.58 (6.70-982.29) N= 10	149.09 (3.42-2460.95) N=7	
P53	Negative(57.89)	89.81(6.43-3888.51) N=18	186.75 (3.42-2460.95) N=11	0.876[a],
	Positive (30.00)	1205.14 (106.15-2304.12) N=2	2730.60 (2730.6-2730.60) N=1	
PS2	Negative(40.00)	89.81 (6.70-3888.51) N=12	370.62 (7.31-2460.95) N=8	0.479[a],
	Positive (27.27)	195.10 (6.43-2304.12) N=8	689.78 (7.31-2730.60) N=3	
Ki62	Negative(35.48)	115.98 (6.43-3888.51) N=20	554.48 (7.31-2730.60) N=11	
	Positive	N=0	N=0	

Table 5. Pearson chi-square test was used to analyze association between mtDNA variants and clinicopathological parameters in 51 breast cancer patients.

Variable	Group	Normal	Cancerous	P-value
		Mut +	Mut +	
Age	< 50	14 (56.00)	11 (44.00)	0.743
	≥ 50	8 (61.54)	5 (38.46)	
Histological type	Ductal	18 (58.06)	13 (41.94)	0.893
	Lobular	5 (55.56)	4 (44.44)	
Primary tumor	T1	10 (50.00)	10 (50.00)	0.657
	T2	6 (60.00)	3 (30.00)	
	T3	5 (62.50)	3 (37.50)	
Lymph node involment	Negative	2 (13.33)	13 (86.67)	1.796
	Positive	20 (83.33)	4 (16.67)	
Metastasis	M0	17 (58.62)	12 (41.38)	0.635
	M1	5 (50.00)	5 (50.00)	
Stage	I	2 (50.00)	2 (50.00)	0.948
	II	10 (58.82)	7 (41.18)	
	III	5 (62.50)	3 (37.50)	
	IV	4 (50.00)	4 (50.00)	
Histological type	G1	4 (57.14)	3 (42.86)	0.998
	G2	8 (57.14)	6 (42.86)	
	G3	7 (58.33)	5 (41.67)	
Nuclear grading	1	0	0	0.340*
	2	9 (64.29)	5 (35.71)	
	3	7 (46.67)	8 (53.33)	
ER	Negative	9 (56.25)	7 (43.75)	0.774
	Positive	8 (61.54)	5 (38.46)	
PR	Negative	7 (53.85)	6 (46.15)	0.638
	Positive	10 (62.50)	6 (37.50)	
Her2	Negative	9 (64.29)	5 (35.71)	0.550
	Positive	8 (53.33)	7 (46.67)	
P53	Negative	15 (57.69)	11 (42.31)	0.765
	Positive	2 (66.67)	1 (33.33)	
PS2	Negative	11 (57.89)	8 (42.11)	0.824
	Positive	5 (62.50)	3 (37.50)	
Ki62	Negative	16 (59.26)	11 (41.74)	
	Positive	0	0	

Table 6. Pearson chi-square test was applied to the analyze association between tissue types and clinicopathological parameters in breast cancer patients with mtDNA variants. *P-value from 2x2 contingency table.

.

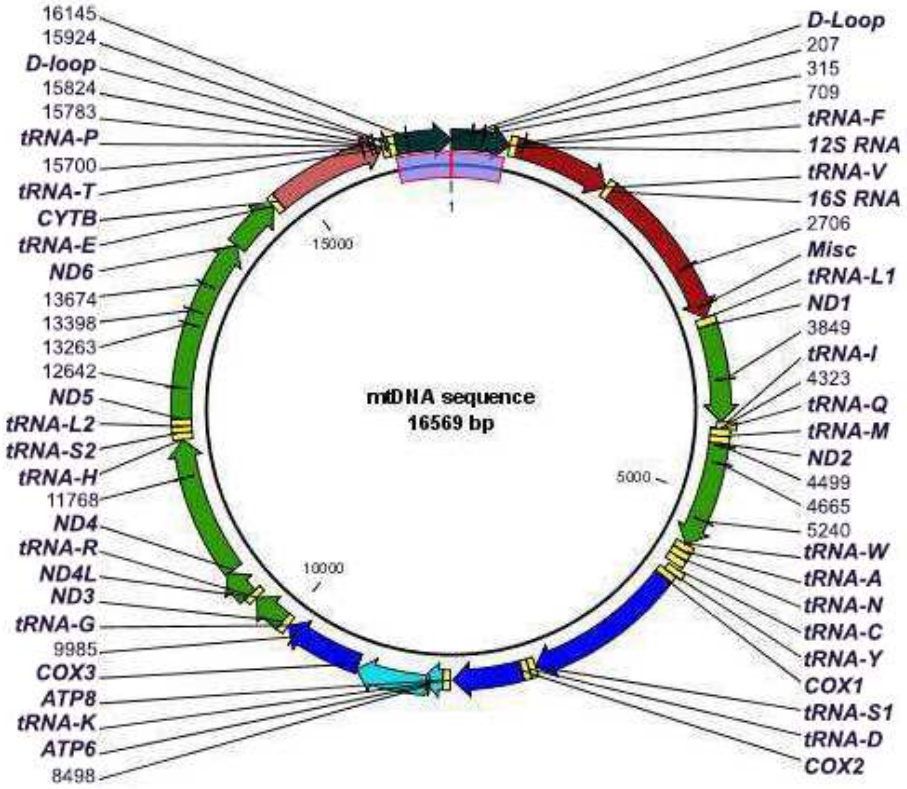


Figure1. mtDNA encodes 37 genes including 22 tRNA (were labelled in yellow) and 2 rRNA (were labelled in red) genes and 13 polypeptide genes (ND1, 2, 3, 4L, 4, 5, and 6 were labelled in green, COX1, 2, and 3 were labelled in blue, ATP 6 and 8 were labelled in light blue, and D-loop were labelled in black) being written in bigger bold italic letter. 22 mitochondrial variants were labelled black bar and nucleotide positions were written in smaller regular numbers.

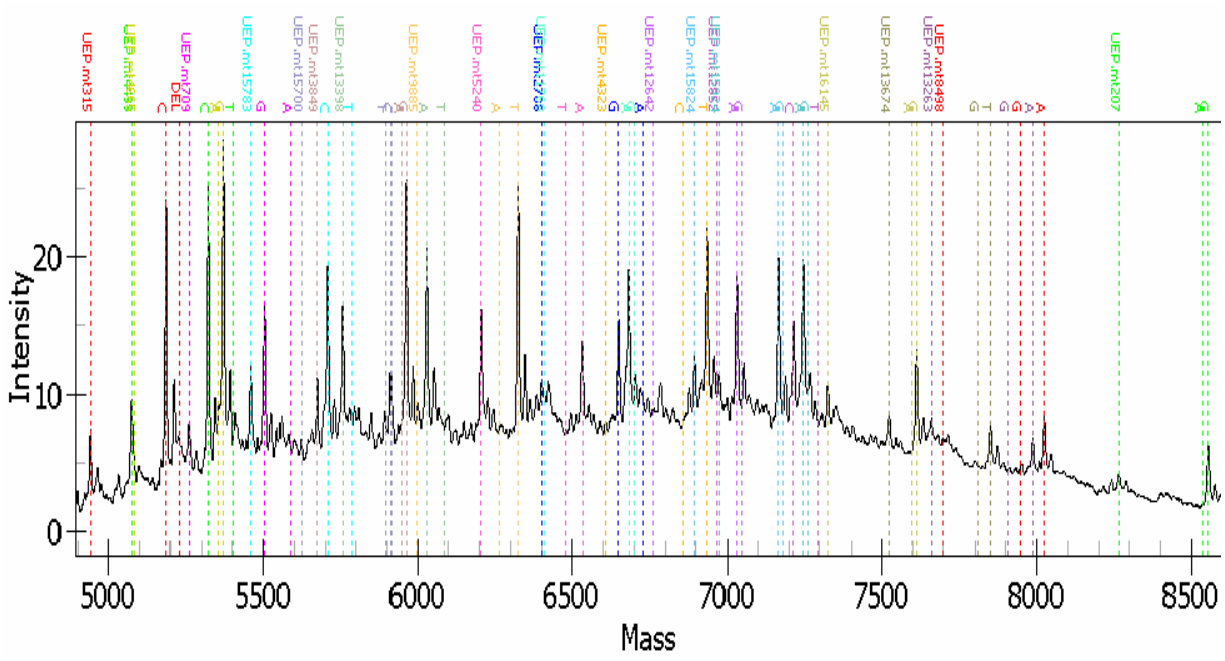


Figure 2. Mass spectra of mtDNA 22plex assay. Mass peaks of unextended extension primers and different alleles were labelled in different colours.

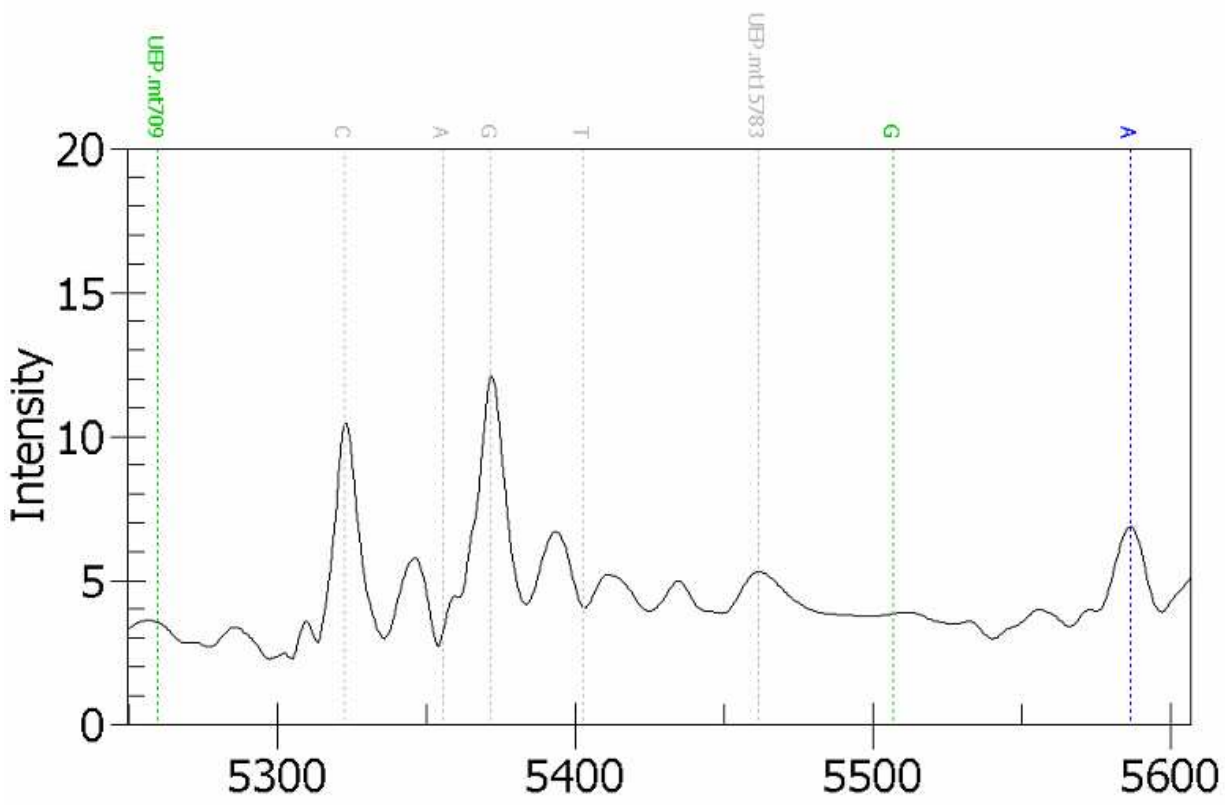


Figure 3. 709A was found in 14 tissue samples and 1 cell line.

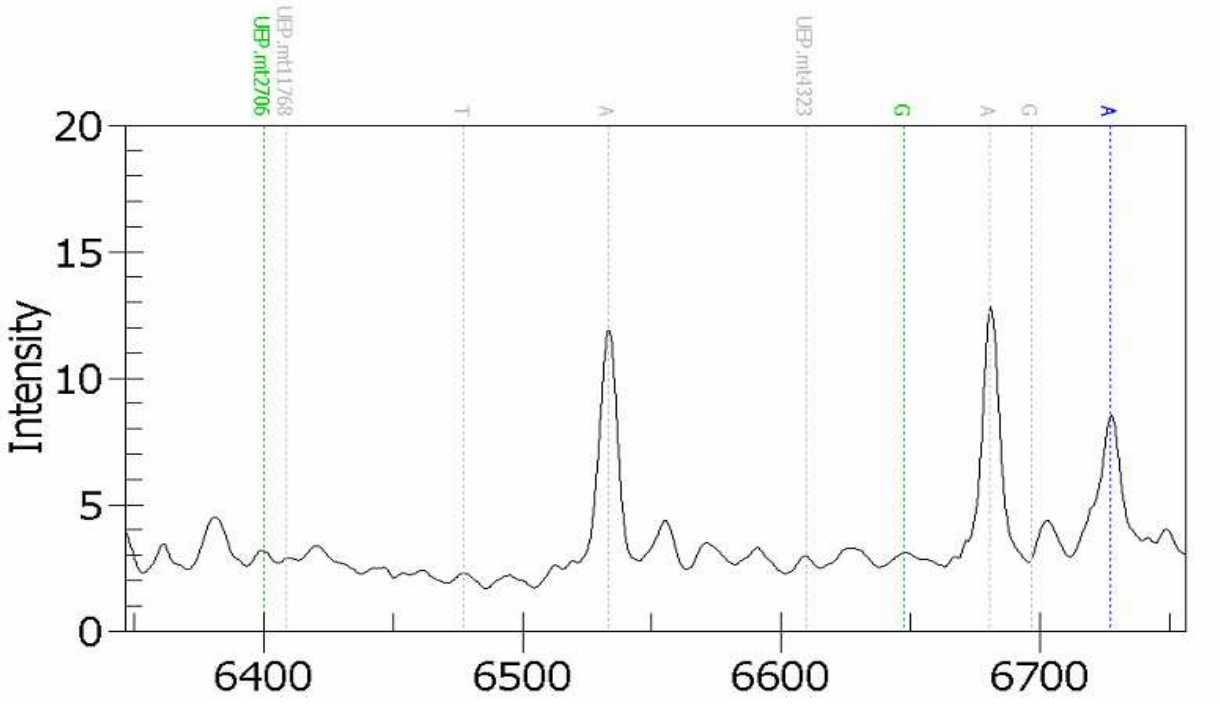


Figure 4. 2706 A was found in 8 tissue samples and 3 cell lines.

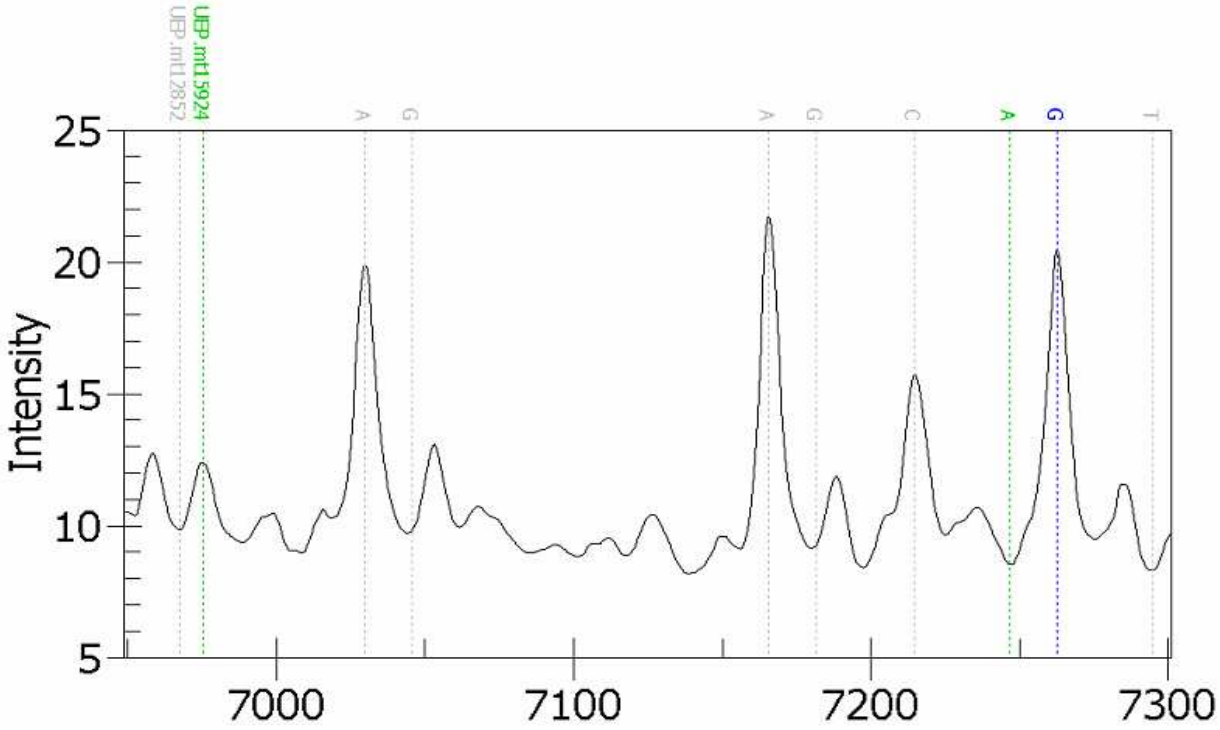


Figure 5. 15924 G was found in 8 tissue samples.

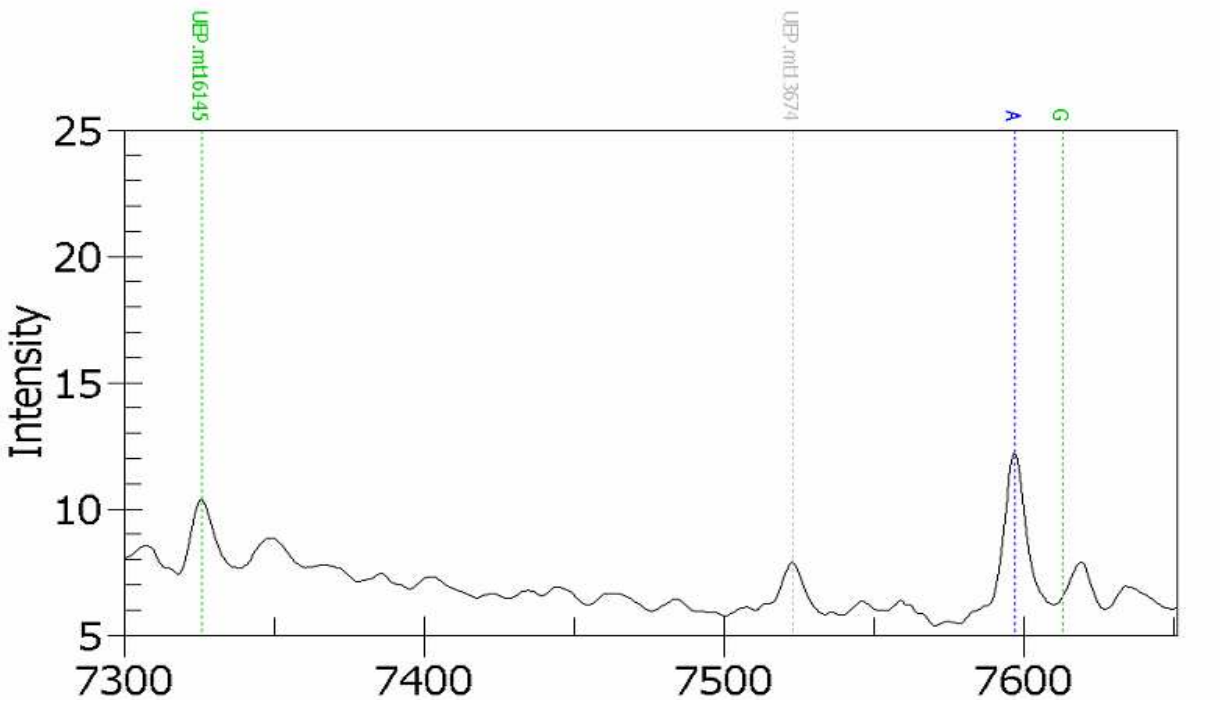


Figure 6. 16145 A was found in 11 tissue samples.

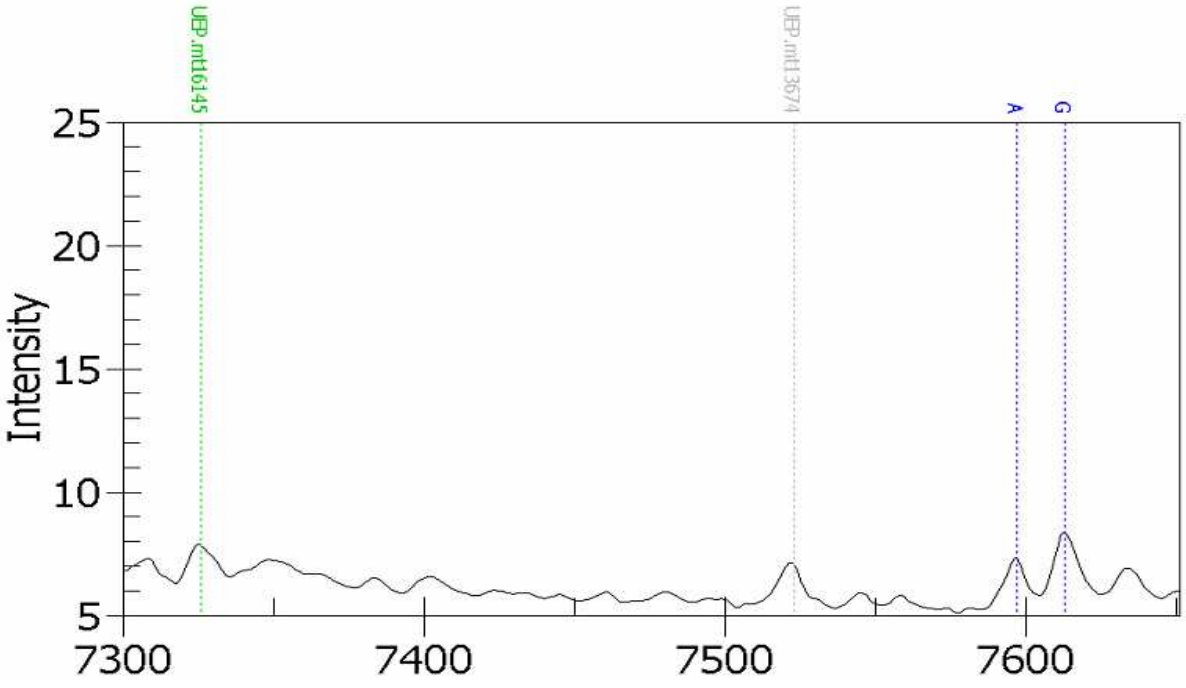
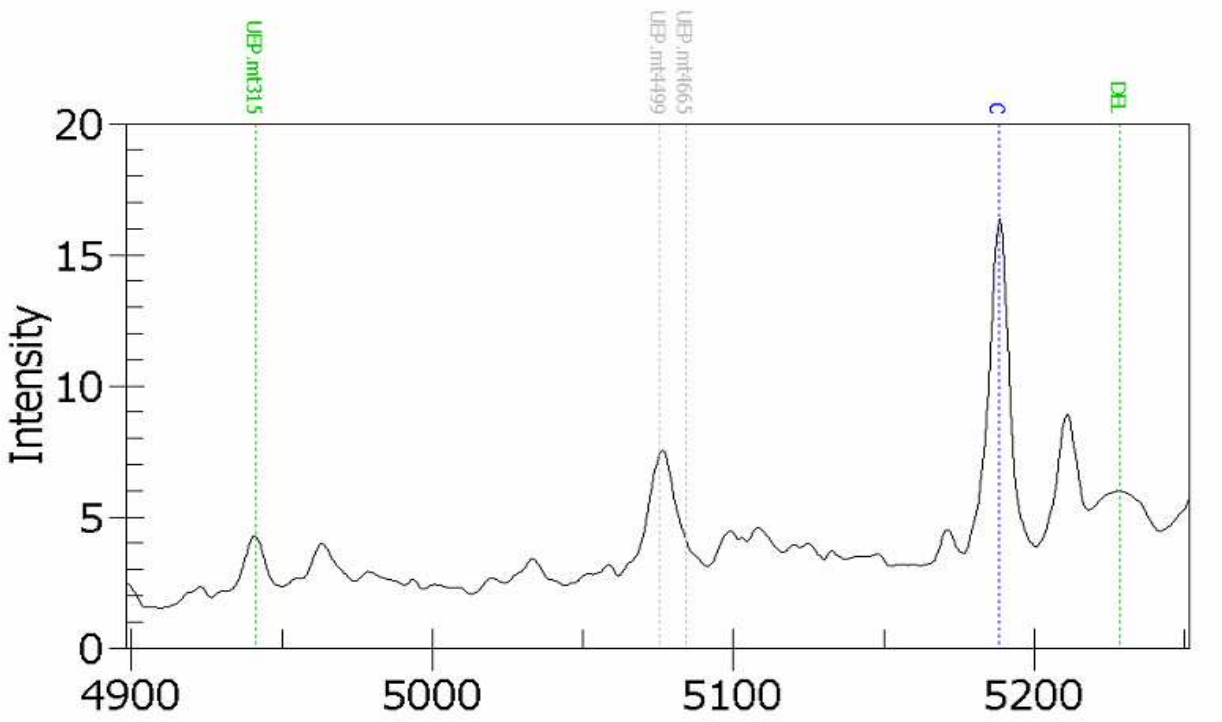


Figure 7. 16145 AG was found in 4 tissue samples.



.

Figure 8. C insertion at np 311-315 was found in 49 pairs of tissue samples and 6 cell lines.

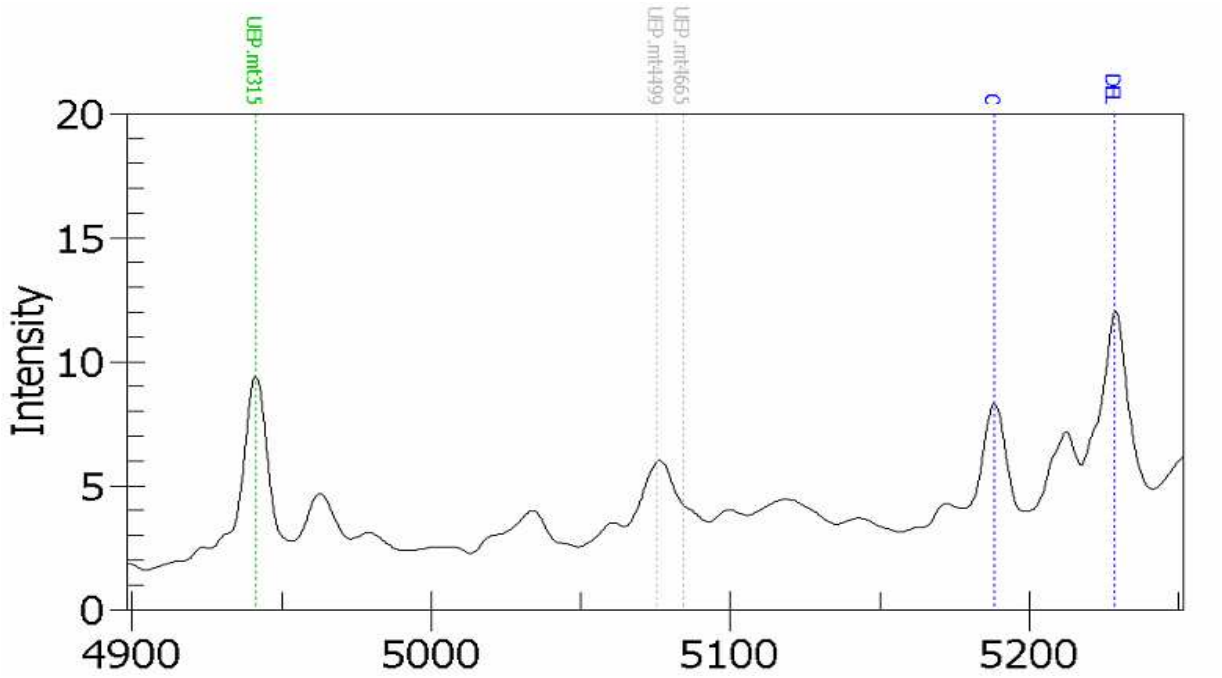


Figure 9. C ins/- at np 311-315 was found in 2 pairs of tissue samples.

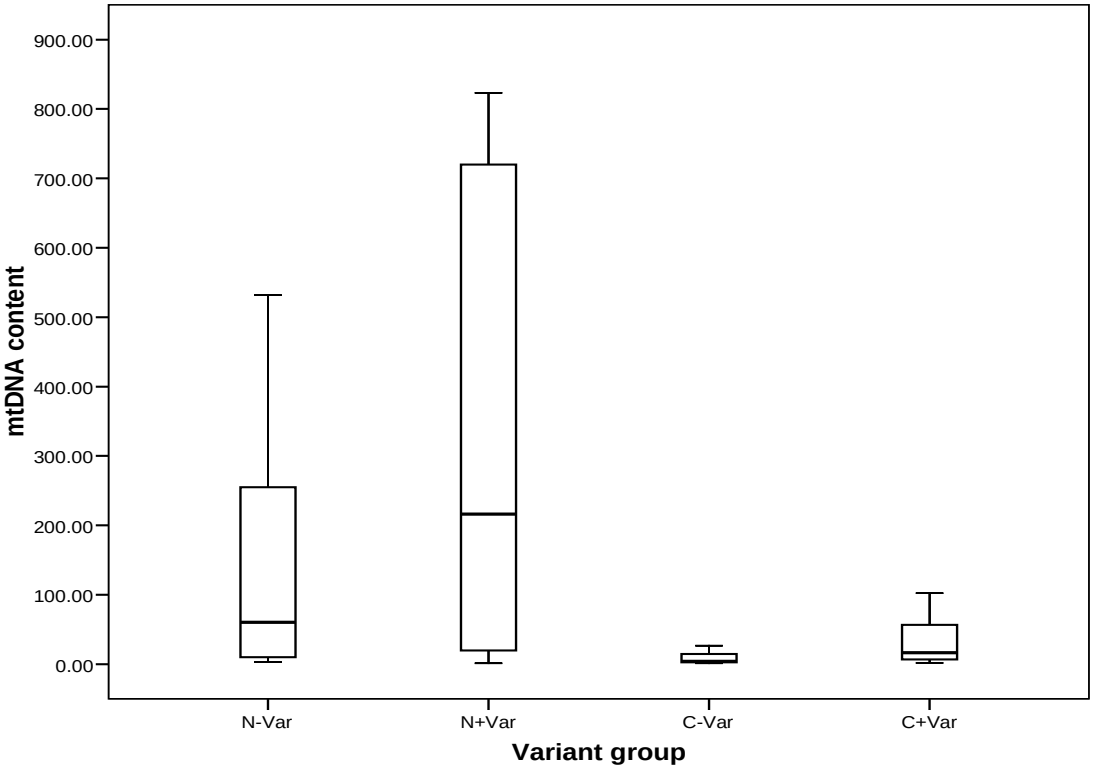


Figure 10. mtDNA in variant positive and variant negative normal and cancerous tissues. N: normal; C: cancer; Var: Variant; "-": without; "+": with.

Curriculum Vitae

Name**:** Alex Xiu-Cheng Fan (Mr.)
Day of birth: March 13, 1973
Nationality: Chinese
Johanniterstrasse 15, 4056 Basel, Switzerland
Tel: +41 61 2659248/ Fax: +41 61 2659399
Mobil: +41 78 7168333
Email: xfan@uhbs.ch
xiuchengfan@hotmail.com

Education:

2006--2009 PhD of Biochemistry on Experimental Oncology, University of Basel, Switzerland

Project: Investigation on Quantitative and Qualitative Alteration of MtDNA as potential Biomarker for Breast Cancer
Place: Lab of Prenatal Medicine and Gynecological Oncology, Department of Biomedicine, University Hospital Basel, Switzerland
Suprvisor: Prof. Xiao Yan Zhong

2004--2006 MSc Program of Physiology and Nutritional Genomics, Wageningen University and Research Center, the Netherlands

MSc Post Training: May 2006—Sep 2006
Project: Production of Bio-Pharmaceuticals in plants
Subtopic 1: Construction of rec-LTB in *Escherichia coli*
Place: Plant Research Institute B.V., Wageningen, the Netherlands
Supervisor: Dr.Ir. D.E.A. (Dion) Florack, Senior Scientist Protein and Glyco Technology Bioscience.

MSc Internship**:** Nov 2005—May 2006
Topic: Whether Elevated Prothymosin Alpha Expression Leads to Inhibition of Apoptosis in BEAS-2B Cells.
Place: Rikilt-Institute for Food Safety, Wageningen, Netherlands
Supervisor: Nicole LW Franssen-van Hal, Senior Scientist Food Bioactives

MSc Thesis: May 2005—Nov 2005
Topic: Effect of Vitamin K and Vitamin B12 on Osteoblast Differentiation with Human Mesenchymal Stem Cells.
Place: Department of Nutrition, Metabolism and Genomics, Division of Human Nutrition, Wageningen University and Research Center, the Netherlands
Supervisor: Wilma Steegenga, Assistant Professor.

1991--1996 BSc & MD Program of Clinical Medicine in Shenyang Medical College, China

Working Experience:

2003-2004 Coordinator of Quality Control Committee of the Shenyang Gongan Hospital.
2004 Coordinator of Community Bird Flu Prevention Group.
2003 Coordinator of Community SARS Prevention Group.
2002 Manager and main investigator of project “Healthy Community and Healthy Life”.
2001 Manager and main investigator of “project, General Health Investigation”.
2000-2004 Physician in charge of Emergency Department, Prevention Official of Epidemics, Investigator of Gastroscope and Colonoscopy Diagnosis and Therapy Group.
Sep 2000 Entitled Attending physician of Internal Medicine by Shenyang Bureau of Sanitation
1997-2000 Resident physician of Shenyang Gongan Hospital, a group member of the project, Main Investigator on project “Clinical Study on Integrated Therapy of Cerebral Infarction”.

Sep 1997 Entitled Doctor of Internal Medicine by Shenyang Bureau of Sanitation
1996--1997 Assistant physician and Physician of Emergency Department in Shenhe No.5 Hospital, Shenyang, China

Publications:

Fan XC and etc. Clinical analysis of treating acute cerebral infarction with defibrase, Chinese Journal of Practical Internal Medicine, 2001 Vol.21 No.3 P.165. (in Chinese)

1. **Fan AX**, Garritsen HSP, Tarhouny S, Morris M, Hahn S, Holzgreve W, Zhong XY. A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry. Clin Chem Lab Med. 2008; 46(3):299-305.

2. Seefeld M, Tarhouny S, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Parallel assessment of circulatory cell-free DNA and nucleosomes in patients with benign and malignant breast tumours. Int J Biol Markers. 2008 Apr-Jun; 23(2):69-73.

3. Zanetti-Dällenbach RA, Wight E, **Fan AX**, Lapaire O, Holzgreve H, Zhong XY. Positive Correlation of cell-free DNA in plasma/serum in patients with malignant and benign breast disease. Anticancer Res. 2008 Mar-Apr;28 (2A):921-5.

4. Tarhouny S, Seefeld M, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Comparison of Serum VEGF and its Soluble Receptor sVEGFR1 with Serum Cell Free DNA in Patient with Breast Tumour. 2008 Cytokine.

5. Xia P, Radpour R, Zachariah R, **Fan AX**, Kohler C, Hahn S, Holzgreve W, ZHong XY. Simultaneous Quantitative Assessment of Circulating Cell-Free Mitochondrial and Nuclear DNA by Multiplex Real-Time PCR. Genetics and Molecular Biology, 2009, 32, 1, 20-24.

6. Radpour R, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Current Understanding of Mitochondrial DNA in Breast Cancer. The Breast Journal, review (accepted).

7. **Fan AX**, Garritsen HSP, Hannig H, Holzgreve W, Zhong XY. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) for Genotyping Human Platelet Specific Antigens (HPAs) Transfusion. 2008 Oct 29. [Epub ahead of print] (Co-first author)

8. Zachariah R., Schmid S, Nicole Buerki, Radpour R, **Fan AX**, Hahn S, Holzgreve W, Zhong XY. Is Circulating Cell Free DNA a Potential Biomarker for Developing Non-invasive Diagnostic Test in Minimal and Mild Endometriosis? Reprod Biomed Online. 2009 Mar;18(3):407-11.

9. Radpour R, Haghighi MM, **Fan AX**, Torbati PM, Hahn S, Holzgreve W, Zhong XY. High-throughput hacking the methylation patterns in breast cancer by in vitro transcription and thymidine-specific cleavage mass array on MALDI-TOF silico-chip. Mol Cancer Res. 2008 Nov;6 (11):1702-9.

10. Xia P, Radpour R, Kohler C, Dang CX, **Fan AX**, Holzgreve W, Zhong XY. A selected pre-amplification strategy for genetic analysis using limited DNA targets. Clin Chem Lab Med. 2009 Feb 4. [Epub ahead of print]

11. Radpour R, Haghighi MM, **Fan AX**, Kohler C, Holzgreve W, Zhong XY. Methylation profile in breast cancer by MALDI-TOF Mass Array. (Revised version submmited)

12. **Fan AX**, Radpour R, Hahn S, Holzgreve W, Zhong XY. Mitochondrial DNA Content in Paired Adjacent Normal and Cancerous Breast Tissue Samples from Patients with Breast Cancer. J Cancer Res Clin Oncol. 2009 Jan 6. [Epub ahead of print]

13. Kohler C**,** Radpour R, **Fan AX**, Barekati Z, Asadollahi R, Bitzer J, Holzgreve W, Zhong XY. Levels of Plasma Circulating Cell Free Nuclear and Mitochondrial DNA as Potential Biomarkers for Breast Tumors. (Submitted)

14. **Fan AX**, Kohler C, Zanetti-Dällenbach R, Wight E, Radpour R, Holzgreve W, Zhong XY. High throughput multiplex assay for the simultaneous detection of 22 mutations of mtDNA in breast cancer tissues using microarray chip based MALDI-TOF MS. (Manuscript in preparation)

15. **Fan AX**, Kohler C, Garritsen HSP, Zhang J, Legath N, Hannig H, Holzgreve W, Zhong XY. MtDNA Polymorphism in Hypervariable Regions and MtDNA Quantitative Alteration in Breast Cancer. (Manuscript in preparation)

Search for “Fan AX OR Alex Xiu-Cheng Fan” on PubMed.