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Methylation profile of *TP53* regulatory pathway and mtDNA alterations in breast cancer patients lacking *TP53* mutations

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The present study investigated promoter hypermethylation of *TP53* regulatory pathways providing a potential link between epigenetic changes and mitochondrial DNA (mtDNA) alterations in breast cancer patients lacking a *TP53* mutation. The possibility of using the cancer-specific alterations in serum samples as a blood-based test was also explored. Triple-matched samples (cancerous tissues, matched adjacent normal tissues and serum samples) from breast cancer patients were screened for *TP53* mutations, and the promoter methylation profile of $P14^{ARF}$, *MDM2*, *TP53* and *PTEN* genes was analyzed as well as mtDNA alterations, including D-loop mutations and mtDNA content. In the studied cohort, no mutation was found in *TP53* (DNA-binding domain). Comparison of $P14^{ARF}$ and *PTEN* methylation patterns showed significant hypermethylation levels in tumor tissues ($P < 0.05$ and <0.01, respectively) whereas the *TP53* tumor suppressor gene was not hypermethylated ($P < 0.511$). The proportion of *PTEN* methylation was significantly higher in serum than in the normal tissues and it has a significant correlation to tumor tissues ($P < 0.05$). mtDNA analysis revealed 36.36% somatic and 90.91% germline mutations in the D-loop region and also significant mtDNA depletion in tumor tissues ($P < 0.01$). In addition, the mtDNA content in matched serum was significantly lower than in the normal tissues ($P < 0.05$). These data can provide an insight into the management of a therapeutic approach based on the reversal of epigenetic silencing of the crucial genes involved in regulatory pathways of the tumor suppressor *TP53*. Additionally, release of significant aberrant methylated *PTEN* in matched serum samples might represent a promising biomarker for breast cancer.

INTRODUCTION

Breast cancer is the most common malignancy among females worldwide. A large number of reports have discussed the important role of *TP53* alterations in breast cancer. The *TP53* tumor suppressor gene has a central role in cell cycle regulation (1), DNA repair (2,3), senescence and apoptosis (4), differentiation and development, and prevention of cancer (5).

Recent studies showed that the *TP53* gene contributes to transcription or replication regulatory mechanisms of mitochondria and also boosts mitochondrial genomic stability via stimulation of base excision repair (6–8). Human mitochondrial DNA

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(mtDNA) mutation can be induced by generation of reactive oxygen species (ROS). An increase of mtDNA sensitivity to DNA-damaging oxidative stress such as ROS has been shown to result via deregulation of p53 protein in cancer cells (8,9). Most of the mtDNA mutations occur in the D-loop region, the major replication and transcription site of both the heavy and light strands of mtDNA (10–12). Previous studies in this area showed that somatic mutation in the DNA-binding domain of the *TP53* gene contributes to mtDNA mutation and mtDNA depletion that indicates a possible role in tumorigenesis of breast cancer patients (8,13,14).

Deregulation of the tumor suppressor *TP53* gene during pathogenesis of most human cancers has been reported via mutation or through alteration in encoding regulators of *TP53* (15–17). The frequency of *TP53* mutations detected in breast cancer is ~20% that were mostly determined by sequence analysis of the DNA-binding domain (exons 5–8) (18,19). This frequency is lower than that of many other cancers. Other than *TP53* mutation, alteration of upstream and/or downstream *TP53* regulators could be another plausible mechanism for inactivation and suppression of this gene in breast cancer. Recently, association of promoter hypermethylation with mtDNA alteration and nuclear DNA crosstalk has been reported in cancer (20). Taken together, this can provide new insights into epigenetic silencing of some of the regulatory pathways, leading to the deregulation of p53 protein which most probably influences the mtDNA vulnerability in breast cancers lacking *TP53* mutation. The *P14^{ARF}/MDM2/TP53* and *PTEN* pathways are the two critical *TP53* control pathways, and alteration of either pathway can induce a variety of cancers and they are thus the focus of many breast cancer investigations (5,21–25).

Regarding the *P14^{ARF}/MDM2/TP53* pathway, Mdm2 normally inactivates the tumor suppressor *TP53* and, in response to stress, regulates the duration and activity of p53 protein. Mdm2 protein binds directly to *TP53* to inhibit transcription and export p53 from the nucleolus to the cytoplasm, resulting in the degradation of p53 by ubiquitination (26,27). The role of p14^{ARF} in the *TP53* pathway is to indirectly regulate the level of p53 protein. p14^{ARF} protein inhibits degradation of p53 protein by keeping Mdm2 in the nucleolus and also restricting E3 ubiquitin protein (26).

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is an essential component of the *TP53* gene response upon DNA damage. Pten protein indirectly regulates p53 function through keeping Akt inactive and making Mdm2 incapable of translocating into the cell nucleus for degradation of p53 (28,29). Indeed, Pten protects p53 even in the presence of Mdm2 by increasing p53-mediated transcription (28) as well as binding to p53, leading to an increase in the half-life of p53 (30).

The aim of the present study was to investigate the promoter methylation profile of the regulatory pathways of *TP53* (*P14^{ARF}/MDM2/TP53*, as well as *PTEN*), providing the potential link between epigenetic changes and mtDNA alteration in tumorigenesis in breast cancer patients lacking *TP53* mutation on the DNA-binding domain. The pathway analysis was performed to verify which of the selected genes were most relevant in breast cancer. Triple-matched samples from breast cancer patients (cancerous tissues, matched normal tissues and serum samples) were subjected to mutation analysis of the DNA-binding domain of *TP53*, assessment of the promoter methylation profile of four candidate genes (*P14^{ARF}/MDM2/TP53/PTEN*) and mtDNA alteration, including D-loop region integrity and mtDNA content to measure the correlation of the aforementioned subjects in the studied cohort. This approach presented the cancer-specific alterations in tissues followed by exploration of the possibility of using the alterations in corresponding serum samples as a blood-based test.

RESULTS

P14^{ARF}/MDM2/TP53 and *PTEN* pathway analysis

First, the network of the candidate genes was analyzed as a function of cellular interactions and breast cancer development. The direct interaction pathway analysis displayed specific upstream and downstream of four candidate genes that identify common targets and regulators for these genes. Additionally, it showed the binding partner of these genes and behaving role in breast cancer (Fig. 1). Signaling analysis including hierarchical and direct force analysis are shown in Supplementary Material 1. This analysis provided an approach to compare different kinds of interactions, including protein–protein interactions as well as genetic interaction.

TP53 mutation screening

The analysis of the results after direct sequencing of exons 5–8 of the *TP53* gene revealed no mutation in the 44 normal and cancerous breast tissue samples from 22 patients compared with the *TP53* GenBank sequence (NC_000017 [gi:224589808]).

Quantitative methylation profiles of *P14^{ARF}/MDM2/TP53* and the *PTEN* promoter using MALDI-TOF MS

In this study, we analyzed the methylation patterns of four breast cancer candidate genes in 66 triple-matched samples (cancerous tissues, matched normal tissues and serum samples) from 22 breast cancer patients. For all the genes, 1 amplicon per gene and 117 CpG sites in total per sample (total of 7020 sites in 66 analyzed samples) were analyzed (Table 1; Supplementary Material 3). In the present study, a low degree of methylation with mean methylation levels $<30\%$ was considered as hypomethylation and a high degree of methylation with mean methylation levels $>70\%$ was considered as hypermethylation.

Using two-way hierarchical cluster analysis, we found different levels of methylation between tumor tissue, the matched serum and the normal tissue samples. The methylation quantification data for each studied gene are illustrated in Supplementary Material 3. The methylation alteration level of the individual CpG sites or clusters per gene showed a variable signature in the studied cases (Supplementary Material 3); the mean methylation quantity of the informative CpG sites that represent valuable differences in each case was used to figure out the promoter alterations in the candidate genes. Hierarchical cluster analysis profiling of the promoter alterations of the four studied genes is shown in Figure 2A.

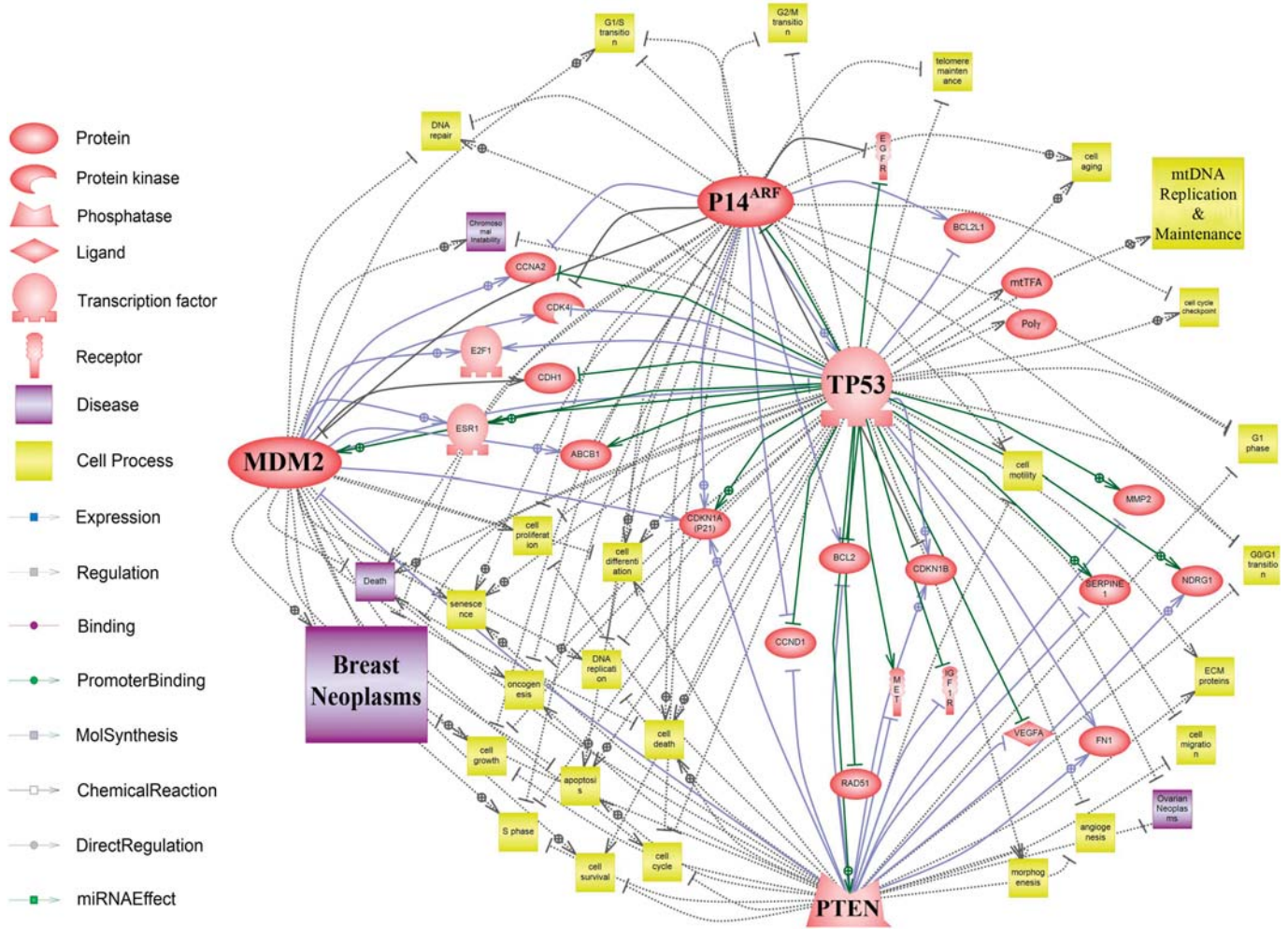

Figure 1. Direct interaction pathway analysis of four candidate genes related to breast cancer.

Table 1. High-throughput methylation analysis of CpG sites in the amplicons of the studied genes in breast cancer

Gene	Amplicon size (bp)	Total no. of CpG sites in amplicon	No. of analyzed CpG sites in amplicon	No. of analyzed CpG sites per amplicon	
				Single sites	Composite sites
MDM2	303	39	27	7	20
P14^{ARF}	425	36	26	6	20
PTEN	451	34	29	10	19
TP53	449	39	35	17	18

The *in silico* digestion was performed for the T-cleavage assay. The percentage of total CpG sites in the amplicon is divided into single sites (single CpG sites) and composite sites (two or more adjacent CpG sites fall within one fragment, or when fragment masses are overlapping).

The *MDM2* oncogene for both the tumor and normal tissues showed a relatively comparable hypomethylation pattern whereas matched serum was significantly different compared with normal tissue ($P < 0.05$). Evaluation of the methylation proportion of *P14^{ARF}* presented significant hypermethylation in tumor tissue versus normal tissue and in matched serum versus tumor tissue ($P < 0.05$ and <0.01, respectively). Comparison of the *PTEN* methylation pattern showed significantly higher hypermethylation levels in tumor tissue and matched serum than the normal tissue ($P < 0.01$ and <0.05, respectively). Our data showed significant concordance between the tumor and serum *PTEN* methylation profile (Spearman's rho test; $P < 0.05$). Methylation analysis of *TP53* showed no significant differences between tumor tissue and matched serum versus normal tissue whereas matched serum and tumor tissue revealed significant difference ($P = 0.01$; Fig. 2B).

Schematic comparison of methylation rate of analyzed genes with upstream regulatory sequence

The methylation rate and localization of each CpG site were compared with upstream sites for regulatory enhancers, CAAT box, GC box, transcription factor IIB recognition elements, TATA box, initiation site of transcription and downstream promoter elements. Figure 2C schematically compares the upstream regulatory sequences (in the range of -1000 to $+100$) with the methylation value of informative CpG sites of

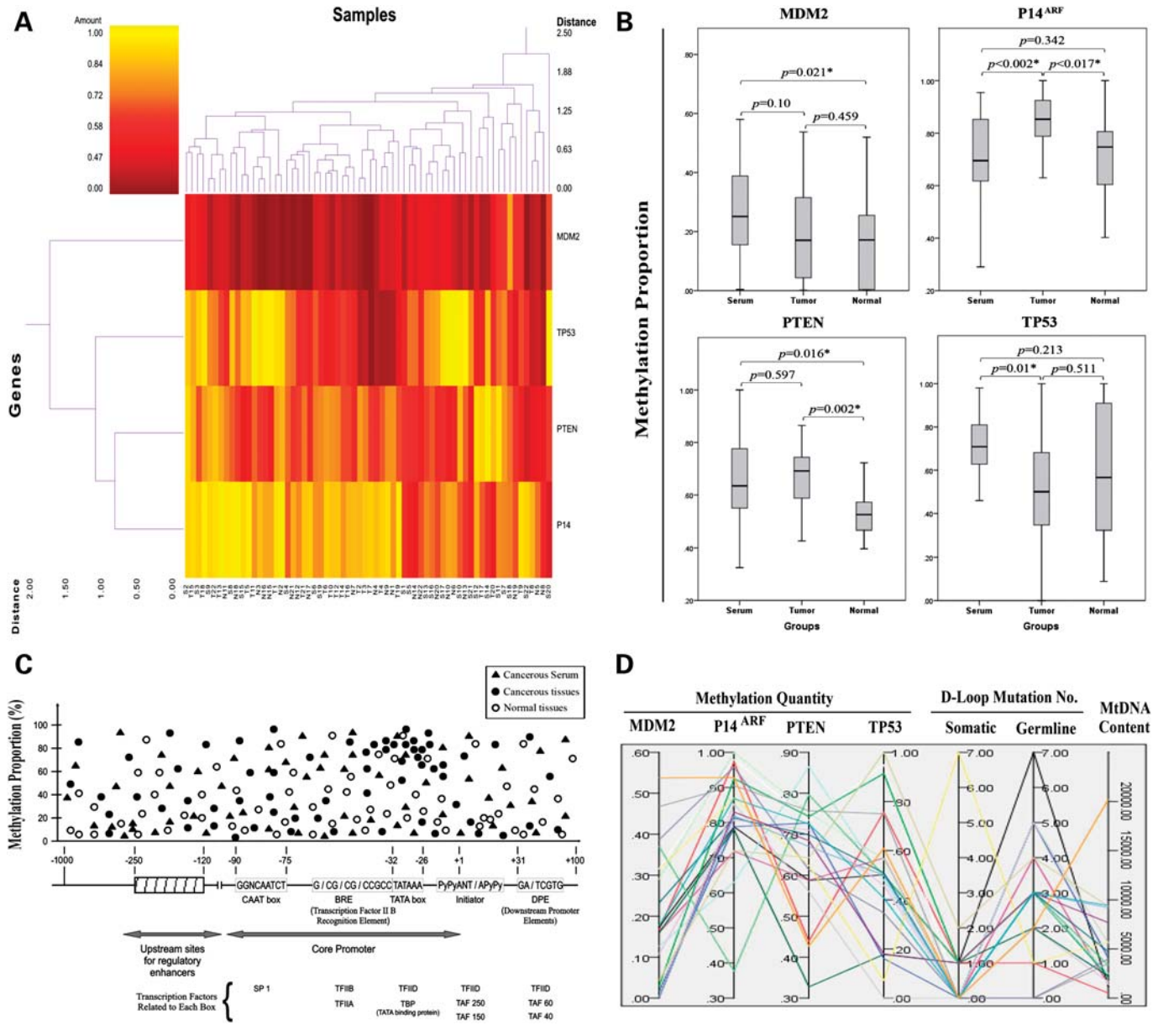

Figure 2. (**A**) Double dendrogram presents the methylation profiles of the four studied genes in triple-matched samples from 22 breast cancer patients (red clusters indicate 0% methylated, yellow clusters indicate 100% methylated, color gradient between red and yellow indicates methylation ranging from 0 to 100). (**B**) Comparison between quantitative analysis of methylation for the studied genes in triple-matched samples (*significant correlation; Mann–Whitney *U*-test). (**C**) Comparison of the methylation ratio and approximate position of informative CpG sites of the four studied genes according to the recognition sites of the transcription factors (in the range of −1000 to +100) in the triple-matched samples. (Each dot in the map corresponds to the mean amount of CpG site methylation in all cases of each studied group.) (**D**) Correlation study between methylation quantity, D-loop mutations and mtDNA content.

the four studied genes together. Accumulation of some hypermethylated CpG sites around the TATA box region was observed for tumor-derived samples. In serum and normal samples, the CpG sites were differentially methylated and located randomly in the 5′-UTRs of the studied genes (Fig. 2C).

D-loop region mutation screening

The results of the D-loop region after direct sequencing in a pair study of normal tissue versus tumor were analyzed and compared with the human mitochondrial sequence (GenBank sequence NC_012920 [gi:251831106]). D-loop region mutation screening showed somatic mutations in 36.36% (8 of 22) of studied cancerous samples. Six of the eight cases showed a single somatic mutation, whereas of the remainder one had 2 and the other had 7 somatic mutations. The prevalence of detected germline mutations in the D-loop region was markedly higher at 90.91% (20 of 22) than somatic mutations and with mostly multiple germline mutations in each case. A total of 82 mutations were detected in the D-loop region of the studied samples, including 15

Table 2. Summary of the D-loop mutations found in breast cancer patients

D-loop alteration	Mutation name	Mutation frequency (%)
Somatic	64 C > C/T (H)	1 (4.54)
	146 T > C	1 (4.54)
	152 T > C	1 (4.54)
	199 T > C	1 (4.54)
	235 A > G	1 (4.54)
	256 C > G	1 (4.54)
	257 A > G	1 (4.54)
	16290 C > T	1 (4.54)
	16291 C > T	1 (4.54)
	16311 T > C	2 (9.09)
	16319 G > A	2 (9.09)
	16389 G > G/C (H)	1 (4.54)
	16519 T > C	1 (4.54)
Germline	41 C > C/T (H)	1 (4.54)
	83 A > A/C (H)	1 (4.54)
	84 A > A/C (H)	1 (4.54)
	146 T > C	3 (13.64)
	150 C > T	3 (13.64)
	152 T > C	1 (4.54)
	199 T > C	1 (4.54)
	204 T > C	2 (9.09)
	207 G > A	3 (13.64)
	210 A > G	1 (4.54)
	235 A > G	2 (9.09)
	248 Del A	1 (4.54)
	297 A > C/A (H)	2 (9.09)
	390 A > G	1 (4.54)
	398 C > T	1 (4.54)
	489 T > C	4 (18.18)
	16278 C > T	2 (9.09)
	16290 C > T	3 (13.64)
	16291 C > T	1 (4.54)
	16304 T > C	2 (9.09)
	16311 T > C	4 (18.18)
	16317 A > G	1 (4.54)
	16319 G > A	3 (13.64)
	16357 T > C	1 (4.54)
	16362 T > C	11 (50)
	16438 G > A	1 (4.54)
	16497 A > G	1 (4.54)
	16519 T > C	8 (36.36)

H, heterozygosity; Del, nucleotide deletion.

somatic mutations and 67 germline mutations. The details of the prevalence of germline and somatic mutations are provided in Table 2, and the spectrum of the mutation along the length of the D-loop is illustrated in Figure 3.

Mitochondrial content in triple-matched samples

The mtDNA contents of the studied, cancerous and normal breast tissue, as well as serum samples were measured using multiplex quantitative real-time PCR. The mtDNA content results obtained from cancer tissues were significantly lower than that of the normal tissues, with 11.88-fold changes ($P < 0.01$). Nineteen of 22 cancer tissues compared with normal tissues had lower mtDNA content (86.36%). The content of mtDNA in matched serum was lower than in normal and cancer tissues. The mtDNA content of matched serum was significantly lower than that of normal tissues (Spearman's rho test; $P<0.05$; Fig. 4).

Relationship of *TP53*/*P14*/*MDM2* and *PTEN* methylation profile, mtDNA alteration and clinicopathological parameters

The correlation of the methylation proportion of the studied genes to mtDNA alteration (D-loop mutations and mtDNA content) is summarized in Figure 2D. A negative correlation between germline mutation number and *MDM2*, as well as $P14^{ARF}$ methylation profile in tumor tissues has been found (Spearman's rho test; $P < 0.05$).

The mtDNA content showed no difference between patients with and without D-loop region mutations, suggesting that the mtDNA depletion may not be a result of the mutations (Fig. 4).

Clinicopathological parameters with our findings were analyzed (Supplementary Material 4). Breast cancer patients with the ILC histological type showed significant association with somatic mutations of the D-loop region ($P < 0.05$). In matched serum samples, patients with histological grade II had a higher proportion of hypermethylation of the *PTEN* gene and lower mtDNA content than histological grade III ($P < 0.05$). Serum samples of patients with C-ERB2 expression showed a greater proportion of hypermentylation of $P14^{ARF}$ than patients lacking C-ERB2 expression ($P < 0.05$).

DISCUSSION

p53 dysfunction can be compromised by *TP53* mutation, methylation or gene alteration of crucial p53 regulators. The majority of *TP53* mutation occur in the DNA-binding domain (exons 5–8); this domain has a critical role for the biological activity of p53 (31). The *TP53* mutation occurs in approximately 20% of breast cancer cases (19), and this frequency is lower than that of the other types of cancer. In the present study, the results showed no mutation in the *TP53* gene (DNA-binding domain) of the studied cohort, thus these samples were subjected to further epigenetic analysis.

Apart from *TP53* mutation, alteration of upstream and/or downstream regulatory factors could be another plausible mechanism for *TP53* inactivation and suppression in breast cancer. To find the molecular mechanism involved in tumor suppressor p53 protein deregulation of breast cancer patients lacking *TP53* mutation, we assessed epigenetic alteration of $P14^{ARF}$/*MDM2*/*TP53* and *PTEN*, important regulatory pathways of p53. Aberrant methylation of CpG islands is the most common alteration in human cancer (33). In the present study, the promoter methylation profile of four candidate genes, $P14^{ARF}$, *MDM2*, *TP53* and *PTEN*, was analyzed in 66 samples (breast tumors, matched normal tissue and serum samples). In tumor tissue, the *MDM2* oncogene showed hypermethylation patterns comparable with normal tissues. Comparison of $P14^{ARF}$ and *PTEN* methylation patterns showed significant hypermethylation levels in tumor tissues when compared with the normal tissues ($P < 0.05$ and <0.01, respectively) while the *TP53* tumor suppressor gene was not hypermethylated. Additionally, significant accumulation of hypermethylated CpG sites in the promoter region was observed for tumor tissues, which might suggest prevention of RNA polymerase action at transcriptional promoter

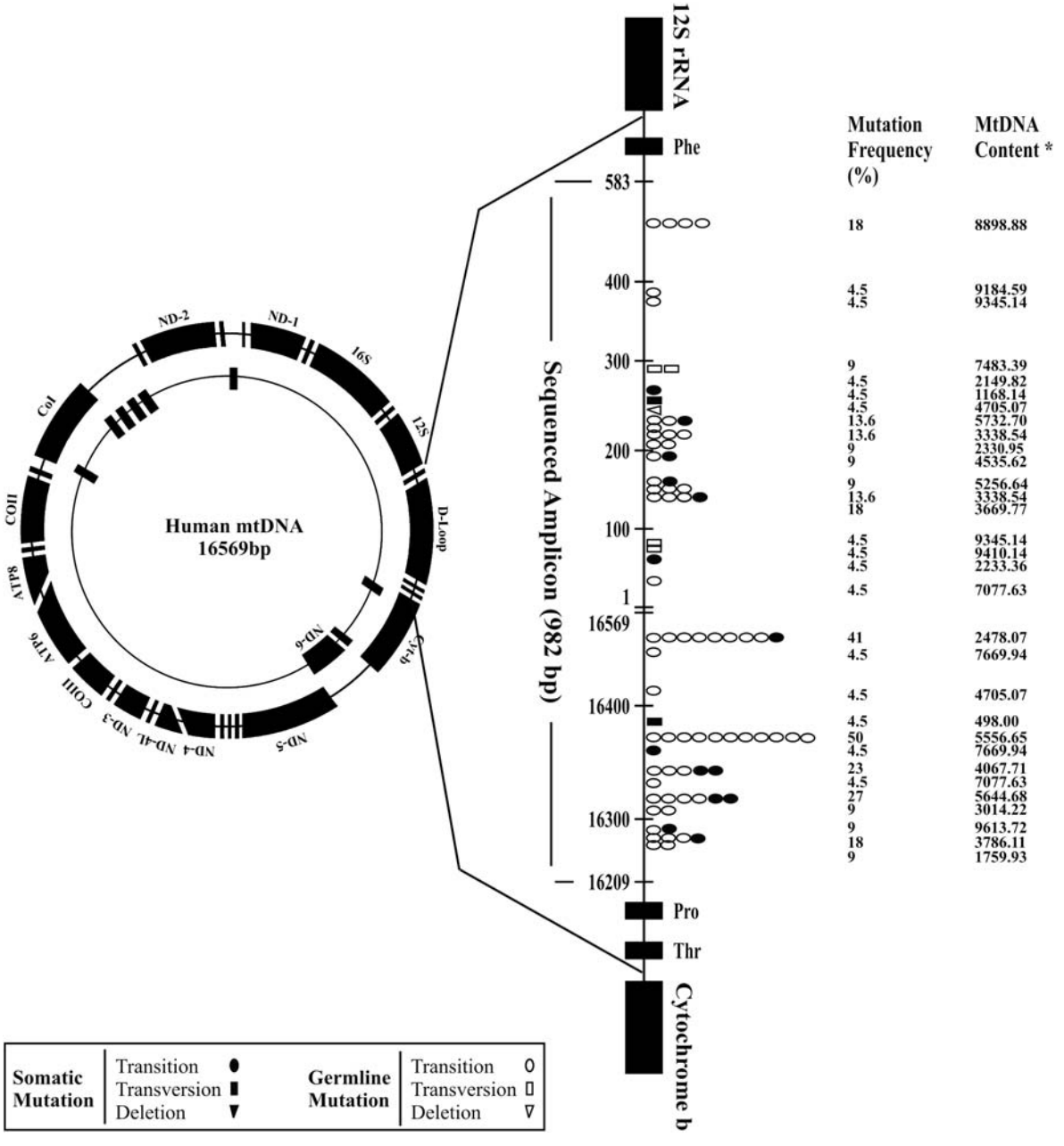

Figure 3. Distribution of the mutations in the D-loop region and mtDNA content. The asterisk indicates the median of mtDNA content of the patients who have the mutation in the same corresponding locus.

regions of *P14*^{ARF} and *PTEN* tumor suppressor genes leading to gene silencing. These results are in line with recent reports based upon methylation status and aberrant expression. Previous studies have investigated frequent aberrant methylation patterns of *P14*^{ARF} and a down-regulation of this tumor suppressor gene in breast tumors (21,34). Hypermethylation of the promoter of *PTEN* has also been found in breast carcinoma that led to *PTEN* inactivation in a subset of breast cancers (35–37).

Pathway analysis served as the important step toward an improved understanding of the relationship of studied genes in breast cancer. This investigation suggests the possibility of p53 deregulation probably by loss of *P14*^{ARF} and *PTEN* or by overexpression of *MDM2*. Mdm2 is responsible for inactivation of tumor suppressor *TP53* by inhibition of transcription and export of p53 from the nucleolus to the cytoplasm, resulting in the degradation of p53 by ubiquitination (26,27). Overexpression of the *MDM2* gene can result in an excessive inactivation of p53 and has been reported in 73% of breast carcinomas (22,23). p14^{ARF} protein, by keeping Mdm2 in the nucleolus and also restricting E3 ubiquitin protein, inhibits degradation of p53 (26). Indirect activation of p53 by p14^{ARF} inhibits growth of abnormal cells and prevents cancer (38). Indeed, p53 by binding to the *PTEN* promoter

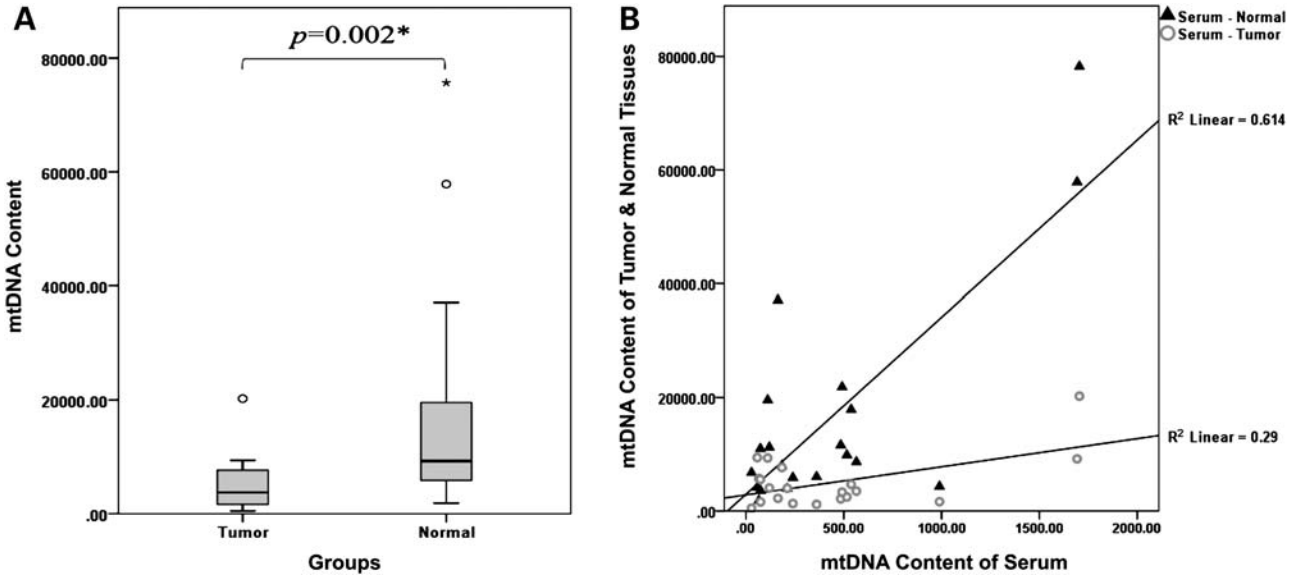

Figure 4. MtDNA content. (**A**) Comparison between mtDNA content in tumor and adjacent normal tissues. (**B**) Correlation of mtDNA content in serum versus tumor and normal samples. (* significant correlation; Mann–Whitney *U*-test).

leads to a positive feedback loop that decreases AKT activity by Pten and as a result inactivates Mdm2 and protects p53 from deregulation (39). Furthermore, Pten protein protects p53, even in the presence of Mdm2, by increasing *TP53*-mediated transcription (28) and also by binding to p53, leading to an increased p53 half-life (30). These findings emphasize the importance of an epigenetic alteration-mediated silencing of the tumor suppressor candidate genes and suggest that alteration of these genes could be a cause of p53 deregulation. These pathways might be targets for therapeutic strategies based on reversal of epigenetic silencing in breast cancer.

To determine the mechanistic link between inactivated tumor suppressor p53 through epigenetic silencing regulatory pathways and mtDNA alteration in cancer kinetics, we assessed mtDNA damage including D-loop region integrity and mtDNA content in breast cancer patients lacking *TP53* mutation. mtDNA has a higher mutation rate than nuclear DNA, due to its closeness to the respiratory chain, lack of histone protein and imitated DNA repair capacity. Most of the mutations occur in the D-loop, a major control site of replication and transcription for both the heavy and light strands of mtDNA (10–12). Association of *TP53* alteration with mtDNA damage has been reported in a breast cancer patient (13). Our data present a high rate of D-loop region mutations (36.36% somatic and 90.91% germline mutations) in breast cancer patients.

Depletion of mtDNA content has been investigated in breast cancer and it has been attempted to correlate this to mtDNA somatic mutation (18,40–43). The present data showed a significant mtDNA depletion in tumor tissues compared with normal tissues ($P < 0.01$). It has been demonstrated that the depletion of mtDNA resulted from a loss of p53 function (8). The mtDNA damage in the studied cohort, D-loop mutations and mtDNA depletion, might be explained by the indirect effect of p53 inactivation on mtDNA vulnerability. Additionally, we found an inverse correlation between germline mutations and *MDM2* and $P14^{ARF}$ hypermethylation in tumor tissues which might suggest a susceptibility to p53 inactivation in breast cancer patients with a background of D-loop mutations. Furthermore, breast cancer patients with the ILC histological type showed a significant association with somatic mutations of the D-loop region ($P < 0.05$).

Comparison of clinicopathological parameters with tumor-specific methylation changes and mtDNA content did not reveal additional information, and this remains to be determined in a larger cohort.

In the present study, in order to find cancer-specific markers in tissues for the development of a blood-based test, we assessed epigenetic alterations and mtDNA changes in matched serum samples of the studied cohort. Epigenetic alterations in serum of different cancers have been reported (44–49). DNA from solid tumors are released into the systemic circulation most probably through cellular necrosis or apoptosis (50). The present study investigated the proportion of methylation of four candidate genes in serum samples. The $P14^{ARF}$ tumor suppressor genes showed higher hypermethylation levels than the normal tissues, though this failed to reach the statistical significance. The hypermethylation level of $P14^{ARF}$ in serum samples was lower than in tumor tissues; this trend has also been reported previously (34). The proportion of *PTEN* methylation was significantly higher in matched serum than in the normal tissues, with a significant concordance between the tumor and serum *PTEN* methylation profile ($P < 0.05$), suggesting a possible use of the marker for the development of a blood-based test with the goal of patient management. Assessment of mtDNA content revealed depletion of mtDNA in matched serum versus control tissues ($P < 0.05$). Using mtDNA depletion as a blood-based test, a biomarker for breast cancer patients was suggested in a previous publication (42). Release of significant aberrant methylated *PTEN* in blood might represent

a promising biomarker for breast cancer with ease of access to methylation analysis.

In conclusion, the obtained results from breast cancer patients suggest hypermethylation of *P14^{ARF}* and *PTEN* could break down the *P14^{ARF}*/*MDM2*/*TP53* and *PTEN* regulatory pathways, resulting in p53 inactivation in breast cancer patients lacking *TP53* mutation in the DNA-binding domain. Moreover, mtDNA damage can be affected by p53 deregulated through epigenetic silencing of the studied pathways. These data can provide an insight into the management of a therapeutic approach based on reversal of epigenetic silencing of the crucial genes involved in regulatory pathways of the *TP53* tumor suppressor gene. Additionally, release of significant aberrant methylated *PTEN* in matched serum samples might represent a promising biomarker for breast cancer with ease of access to analysis.

MATERIALS AND METHODS

P14^{ARF}/*MDM2*/*TP53* and *PTEN* pathway analysis

Pathway analysis was accessed by Pathway Studio® software program version 7 and the ResNet® 7 (Mammal) database (Ariadne Genomics, Inc., Rockville, USA). Four candidate genes (*P14^{ARF}*, *MDM2*, *TP53* and *PTEN*) were analyzed in depth by this software to increase the biological perspective of this study and facilitate the understanding beyond their functional link to breast neoplasm (Fig. 1, Supplementary Material 1).

Sampling and pathological classification

DNA was isolated from 66 triple-matched samples (cancerous tissues, matched adjacent normal tissues and serum samples) from 22 breast cancer patients according to the standardized protocol as described previously (51,52). The study was approved by the local institutional review board (Ethic commission beider Basel and Sichuan University China). Written consent forms were collected from all patients who were involved in this study. All the blood samples were collected before delivering any therapeutic treatments.

Staging and grading was evaluated according to the WHO histological classification. According to pathological tumor type and immunohistochemistry staining, we separated our patient samples into two subgroups: invasive ductal carcinoma (IDC) and Infiltrating Lobular Carcinoma (ILC). Breast cancer characteristics, such as staging, histological grading and hormone receptor expression, of the patients are listed in Table 3.

Mutation analysis of *TP53*

Screening of *TP53* mutation has been performed on the DNA-binding domain (exons 5–8). Information on the primer sequences and PCR conditions used for sequence analysis are listed in Supplementary Material 2. Direct DNA sequencing was performed by using a Big Dye terminator v3.1 cycle sequencing kit and an automated sequencer (ABI 3130, Applied Biosystem). The results of DNA sequence analysis were compared with the reference sequences of

Table 3. Clinical characteristics of triple-matched patient samples

Breast cancer tumor type	Total no. of patients	Age mean ± S (range)	Pathologic stage		No. of patients with lymph node involvement	No. of patients with metastasis	Histological grade			ER-positive patients	PR-positive patients	C-ERB2-positive patients
			Early	Late			1	2	3			
Invasive ductal carcinoma (IDC)	16	51 ± 12.6 (32–77)	9	7	12	0	0	3	13	13	10	5
Infiltrating Lobular Carcinoma (ILC)	6	48 ± 10.4 (37–61)	2	4	3	0	0	3	3	5	1	3

GenBank (www.ncbi.nlm.mih.gov/nucleotide) using DNASTAR sequence alignment software (DNASTAR Lasergene 8, Inc., Madison, USA). All sequencing reactions were performed in both directions and confirmed for concordance.

Methylation analysis of *P14^{ARF}*/*MDM2*/*TP53* and *PTEN* pathways using thymidine-specific cleavage mass array on MALDI-TOF MS

The SEQUENOM's EpiTYPER^{TM} assay is a methylation quantification method which relies on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (53). In the current report, this assay was used to quantify the methylation of *TP53*, *P14^{ARF}*, *MDM2* and *PTEN* promoter regions.

The robustness of this approach for DNA methylation quantification has been confirmed by previous studies (32,52).

Bisulfite treatment. Bisulfite conversion of the target sequences was performed according to the instructions of a commercial Epitect® Bisulfite kit (QIAGEN AG, Basel, Switzerland).

Primer designing and PCR tagging for EpiTYPER^{TM} assay. To design PCR primer for the candidate genes, CpG density and CpG sites of four target sequences were analyzed. Primer pairs have been designed to cover the promoter regions with the most CpG sites using MethPrimer (54). In PCR amplification, a T7 promoter tag was added to the reverse primer, and a 10mer tag sequence was added to the forward primer to balance the PCR primer length. Ensuring that *PTEN* primers discriminate between the gene and pseudogene (*psiPTEN*), the reverse primer was designed in a promoter region consisting of different nucleotides compared with the *psiPTEN* sequence (AL356489 and AF029308) especially at the 3′ end of the primer. The primer sequences, annealing temperatures (T_a) and PCR conditions are described in Supplementary Material 2.

In vitro transcription, T-cleavage assay and mass spectrometry. Unincorporated dNTPs were dephosphorylated by adding 1.7 μl of H_2O and 0.3 U of shrimp alkaline phosphatase (SAP; SEQUENOM, Inc., San Diego, CA, USA). The reaction mixture was incubated at 37°C for 20 min, and the SAP was then heat inactivated for 10 min at 85°C. Typically, 2 μl of the PCR were directly used as a template in a 5 μl transcription reaction. Twenty units of T7 RNA polymerase and DNA polymerase (Epicentre, Madison, WI, USA) were used to incorporate dTTP in to the transcripts. Ribonucleotides were used at 1 mmol/l and the dNTP substrate at 2.5 mmol/l. In the same step, the *in vitro* transcription RNase A (SEQUENOM) was added to cleave the *in vitro* transcript (T-cleavage assay). The mixture was further diluted with H_2O to a final volume of 27 μl. Twenty-two nanoliters of cleavage reaction were robotically dispensed (nanodispenser) onto silicon chips preloaded with matrix (SpectroCHIP; SEQUENOM). Mass spectra were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM) and the spectra's methylation proportions were generated by the Epityper software v1.0 (SEQUENOM).

mtDNA alteration analysis

Mutation analysis of the mtDNA D-loop region. Mutation in the D-loop region of mtDNA was analyzed according to a previously published method (43). Information on primers and PCR conditions used for sequence analysis are listed in Supplementary Material 2. The results of DNA sequencing were analyzed as described before. Nucleotide numbering was based on the current mutation nomenclature recommendations (www.hgvs.org/mutnomen). Somatic mutations were scored as any difference between tumor and normal tissues. We considered this to be germline variation when it was found in both the tumor and normal tissues but different from the GenBank data base.

Quantity assessment of mtDNA and data collection. The mtDNA content was measured by multiplex quantitative PCR (q-PCR) using the mtDNA-encoded ATPase 8 (MTATP 8, starting at locus 8446) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) reference gene. The efficiency of the multiplex synchronized quantification of both *GAPDH* and *MTATP 8* was measured in the previous study of our group using standard curves obtained by serial dilution (40). The sequence and information on the primers and probe for the *GAPDH* and *MTATP 8* reference genes and the q-PCR conditions are summarized in Supplementary Material 2.

Q-PCR was carried out using the ABI PRISM 7000 sequence detection system in 25 μl of total reaction volume containing 7 μl of H_2O, 12.5 μl of PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 0.75 μl of each of the above-mentioned 10 μM primers (Microsynth, Balagach, Switzerland), 1 μl of a 5 μM FAM-labeled MTATP 8 probe and 0.5 μl of a 5 μM VIC-labeled GAPDH probe (both probes from Applied Biosystems) and for each reaction 1 μl of DNA was added.

Each sample was measured in duplicate and one negative control was included in every run.

Standard curves with known genomic DNA concentrations ranging from 3.125×10^4 to 10 pg/μl with six serial dilutions (i.e. 31 250, 6250, 1250, 250, 50 and 10 pg/μl) were performed. The threshold cycle (Ct) values of *GAPDH* and *MTATP* 8 were obtained by ABI Prism 7000 SDS software. The standard curves had average slopes at approximately −3.3 (∼100% efficiency) and R^2 was >0.98. The content of mtDNA was calculated using the delta Ct (ΔCt) of an average Ct of mtDNA and nDNA ($\Delta Ct = CtnDNA - CtmtDNA$) in the same well as an exponent of 2 ($2^{\Delta Ct}$) (55).

Statistical methods

Data analysis was performed using the SPSS software (Statistical Software Package for Windows, version 17). Distribution of data was analyzed by Kolmogorov–Smirnov test that demonstrated our data set was not normally distributed ($P < 0.001$). The quantitative methylation profile of the four genes was compared between cancerous tissues, matched normal tissues and serum samples using two-way hierarchical cluster analysis. The most variable CpG sites for each gene were clustered based on pair-wise Euclidean distances and a linkage algorithm for all studied samples according to the

previously developed method by Gene Expression Statistical System (GESS) version 7.1.19 (NCSS, Kaysville, UT, USA) (32,52,56) followed by Mann–Whitney *U*-test.

The Mann–Whitney *U*-test and non-parametric Spearman's rho test were performed to determine the significance and correlation of mtDNA content analysis, D-loop mutations and clinical–pathological parameters.

Additional information

Cell signaling and pathway analysis data are available in Supplementary Material 1. The sequence of PCR primers and PCR conditions are available in Supplementary Material 2. The complete data for high-throughput methylation analysis of informative CpG sites in four breast cancer-related genes, including gene location, amplicon size and two-way hierarchical cluster analysis, are shown in Supplementary Material 3. Correlation between promoter methylation, D-loop mutations and mtDNA content with clinical parameters is given in Supplementary Material 4.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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