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Aberrant promoter methylation in *p53* and *ATM* genes was not associated with sporadic breast carcinogenesis in Bulgarian patients

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ABSTRACT

Epigenetic silencing of gene expression has become an intensively studied topic regarding breast cancer during the last years. Previous investigations have shown that hypermethylation in gene promoters is an alternative to mutations cancer-related mechanism for inactivation of tumor suppressor gene function. The present study was designed for evaluation of promoter methylation in *p53* and *ATM* tumor suppressor genes in Bulgarian patients with sporadic breast cancer. Sodium bisulfite conversion and methylation-specific PCR were performed on tumor DNA isolated from 55 patients with sporadic breast cancer. The data showed normal profile of methylation in the promoter region of *p53* and *ATM* genes in all studied patients. Cases with hypermethylation were not observed. Our results did not support the involvement of *p53* and *ATM* promoter hypermethylation in the neoplastic pathways of sporadic breast cancer. The lack of promoter hypermethylation in *p53* and *ATM* is suggestive of a greater share of genetic aberrations in loss of tumor suppressor function of these genes and presumes the involvement of other epigenetic cancer mechanisms. To our knowledge this study provides the first data on promoter hypermethylation status of *p53* and *ATM* genes in Bulgarian patients.

Key words: *p53, ATM*, breast cancer, DNA hypermethylation, epigenetics

Introduction

Breast cancer (BC) is a major cause of cancer death in women worldwide. Recently established data showed 1384155 newly diagnosed BC cases globally a year, estimating to 22.9% of all cancers in women (http://globocan.iarc.fr). In Bulgaria, the newly diagnosed cases are 3655, amounting to 25.8% (Dimitrova et al., 2012).

The disease has a complex multifactorial nature. Carcinogenesis is a multi-step process due to genetic and/or epigenetic disorders in a number of tumor suppressor genes and proto-oncogenes. Such genes are involved in the normal processes of cell growth and division, DNA repair, chromosome segregation, control of cell cycle and cellular responses.

Recent accumulated data support the fact that different in nature epigenetic processes can act as an alternative to mutations in the process of carcinogenesis. Several mechanisms for epigenetic alterations in cancer are recognized, such as DNA methylation, histone modifications, alterations in chromatin condensation, RNA interference. The epigenetic changes disrupt gene function without changing DNA sequence and can be inherited. Aberrant methylation of CpG islands in the promoter area of genes is one of the most common epigenetic alterations in human cancers. The CpG islands often carry a modified 5-methyl-cytosine, which can be maintained in the genome through DNA replication.

DNA methylation/demethylation is associated with decreasing/increasing of transcriptional activity of genes. The maintenance of normal methylation profile is crucial for

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genomic integrity. Aberrations in methylation pattern lead to changes in gene expression and could be a cause for cancer development. Unique profiles of promoter hypermethylation exist for different tumor types (Esteller et al., 2001). Some genes are hypermethylated in various tumor types (*p16INK4a* and *MGMT*), whereas others - predominantly in specific type of tumor (*p14ARF* and *APC* in colorectal adenomas, *BRCA1* in breast and ovarian carcinomas). In addition to *BRCA1* (13%), hypermethylation in other genes was found in breast tumors (*CDH1* - 42%, *GSTP1* -31%, *TIMP3* - 27%, *p16INK4a* - 17%).

The human p53 is a key tumor suppressor. It acts as a transcription factor, which takes a major part in maintenance of genomic stability, differentiation and development. In contrast to *p53* mutations, which have been elaborately studied during the last few decades as a major cancer-related mechanism (IARC TP53 data base), the available data on the methylation status of *p53* are very limited. Recent publications reported aberrant methylation in *p53* promoter in epithelial ovarian cancer (Chmelarova et al., 2013), cervical cancer (Jha et al., 2012), oral cancer (Yeh et al., 2003).

ATM gene codes for a nuclear kinase involved in the control of cell cycle, cell death, DNA repair in response to double-strand breaks. The ATM kinase directly phosphorylates different downstream cellular substrates, including p53. Germline *ATM* mutations are the cause of Ataxia telangiectasia. Hypermethylation in *ATM* promoter was found in tumors of the colon (Bai et al., 2004), head and neck (Ai et al., 2004), lung (Safar et al., 2005), lymphoma (Huang et al., 2007).

To our knowledge, the epigenetic status of *p53* and *ATM* genes in Bulgarian patients with sporadic BC is not studied. In our previous study we investigated the mutational frequency and spectra of *p53* and *ATM* in a group of 145 Bulgarian patients with sporadic BC (Bozhanov et al., 2010). Here we further explored the promoter hypermethylation profile of these genes in 55 of the patients for whom sufficient amount of tumor DNA was available using methylation-specific PCR assay. Hypermethylation in *ATM* and *p53* promoters was not found.

Materials and Methods

Clinical data of patients

A group of 55 female patients with sporadic BC was studied. Patients were treated at the Specialized hospital for active treatment in oncology, Sofia between 2000 and 2003. Tumor samples of the patients were provided by Prof. Dr. Ivan G. Gavrilov. Clinical data and patients' informed consent for conducting the research was provided by Dr. Maria B. Nacheva. Clinical information was presented in a way preventing identification of patients. Blood samples of clinically healthy women were used as negative controls in each analysis.

Patients were staged according to the TNM classification of Union International Contre le Cancer (UICC) (Table 1). The average age was 53.7 years (SD ± 10,2). The tumor size of most patients was relatively small (T1 and T2). Regarding to the tumor grade, the majority of patients were diagnosed at G2. Almost all patients (52/55) had ductal type of BC. HER2 overexpression was present in 20% of the patients, *p53* mutations – in 18.2%, *ATM* mutations – in 5.5%, *PIK3CA* mutations – in 41.8%.

Table 1. *Patients' clinical and molecular characteristics*.

Characteristics	Classification	Total patients (n = 55)
Age, years	Median	53,7
	Range	35-78
	<50	20
	>50	35
Tumor size (T)	T1	27
	T2	19
	T3	3
	T4	6
Grade (G)	G1	2
	G2	40
	G3	13
Nodal status (N)	Positive	21
	Negative	34
Histological type	Ductal	52
	Lobular	3
Estrogen receptor (ER)	Positive	23
	Negative	32
Progesterone receptor (PR)	Positive	27
	Negative	28
HER2	Overexpressed	9
	Normal	36
	Unknown	10
p53	Mutant	10
	Wild-type	45
ATM	Mutant	3
	Wild-type	52
PIK3CA	Mutant	23
	Wild-type	32

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Patients were followed up for a 5-year period. Overall survival was estimated to 72.73% (40/55).

DNA isolation

Genomic DNA was isolated from fresh frozen breast tumor tissues by a standard Proteinase K/Phenol procedure, including tissue homogenization in SE lysis buffer (0.01 M Tris/HCl pH 8.3, 0.4 M NaCl, 0.002 M Na-EDTA, 0.14 mg/ml Proteinase K, 1% SDS) at 37ºC for 48 h, phenol/chloroform/isoamyl alcohol (25:24:1) purification and ethanol precipitation. DNA was dissolved into TE buffer (0.01 M Tris-HCl pH 7.4, 0.001 M EDTA pH 8.0). DNA was isolated from blood specimens of healthy controls using a standard procedure.

DNA purity and concentration was determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech).

Sodium bisulfite conversion

A total of 2 µg genomic DNA was used for conversion of unmethylated cytosine to uracil, according to the manufacturers' instructions of EZ DNA Methylation Kit™ (Zymo Research Corporation, USA).

Methylation-specific PCR (MSP)

An amount of 150 ng of the converted genomic DNA was used as a template in each MSP reaction. Primers used to distinguish methylated (M) and unmethylated (U) DNA (Yeh et al., 2003; Ai et al., 2004) are listed on Table 2 and amounted to 20 and 15 pmol of each primer per reaction for *p53* and *ATM* genes, respectively.

A hot-start Taq-Polymerase mix was used (QIAGEN) containing 10x PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$. PCR reactions were performed in a total volume of 20 µl. PCR cycling parameters for *p53* were as follows: 33 cycles of denaturation at 94°C for 1 min (first cycle – 5 min), annealing at 63ºC for 1.30 min and elongation at 72°C for 1.30 min, followed by a final 5 min extension at 72°C. PCR cycling parameters for *ATM* were: 35 cycles of denaturation at 95°C for 30 sec (first cycle – 5 min), annealing at 54ºC for 30 sec and elongation at 72°C for 1 min, followed by a final 5 min extension at 72°C. PCR assay was performed on Techne Cyclogen and Labnet Multi Gene II.

PCR products were analyzed on 3% agarose gel electrophoresis for 1.30 hours in 1xTBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA-pH 8.0) and 0.5 µg/ml ethidium bromide for imaging under UV.

Results

Analysis of promoter hypermethylation profile of p53 gene

The optimal MSP conditions were established experimentally. To avoid misinterpretation of results, unmethylated DNA obtained from clinically healthy persons was used as a negative control. Totally methylated *in vitro* DNA was used as a positive control in each PCR reaction.

We evaluated the level of methylation in the promoter region of *p53* in the studied group of patients with BC. The results showed normal profile of methylation in the promoter region of the gene in all studied patients. Cases with hypermethylation in *p53* were not observed. Only 310 bp amplification products were registered corresponding to unmethylated *p53* promoter status. The positive control produced 285 bp PCR products (Figure 1).

Analysis of promoter hypermethylation profile of ATM gene

The methylation status of *ATM* gene in the studied group of patients was assessed using experimentally optimized MSP conditions. The methylation profile was identical to that in the negative controls, demonstrating lack of *ATM* gene promoter hypermethylation.

Table 2. *Primer sequences for unmethylated and methylated DNA template*

Gene	Unmethylated		Methylated	
p53	5'-TTTAAAATGTTAGTATTTATGGTATTAGGTTGGT-3' 5'-CATCATAAAAAACACACTCCCAACCCAAACA-3'	310bp	5'-ATTTACGGTATTAGGTCGGC-3' 5'-ACACGCTCCCAACCCGAACG-3'	285bp
ATM	5'-GTTTTGGAGTTTGAGTTGAAGGGT-3' 5'-AACTACCTACTCCCACTTCCAA-3'	246bp	5'-GGAGTTCGAGTCGAAGGGC-3' 5'-CTACCTACTCCCGCTTCCGA-3'	239bp

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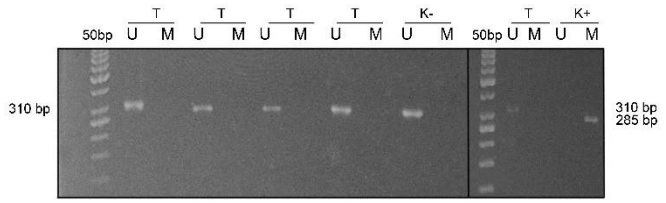

Figure 1. *MSP analysis of p53 using 3% agarose gel electrophoresis. U – unmethylated; M – methylated DNA, 50 bp molecular weight marker, T – tumor sample; K(-) – negative control; K(+) – positive control.*

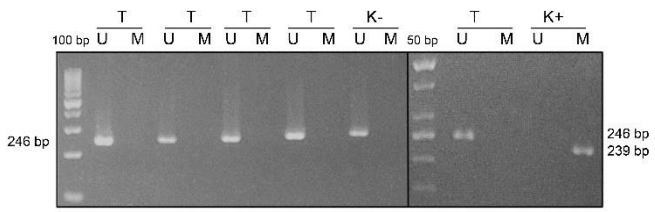

Figure 2. *MSP analysis of ATM using 3% agarose gel electrophoresis. U – unmethylated; M – methylated DNA, 50 bp and 100 bp molecular weight markers, T – tumor sample; K(-) – negative control; K(+) – positive control.*

Only PCR products with the size of 246 bp, corresponding to unmethylated status of the gene, were detected (Figure 2). The positive control produced 239 bp PCR products.

Discussion

Epigenetic processes may act as alternative to mutations through aberration of proto-oncogenes and tumor suppressor genes function, thus serving as a basis for carcinogenic development. The epigenetic alterations differ from genetic mainly in their higher frequency, partial reversibility and localization in fixed genome regions. They affect the early stages of carcinogenic process nominating them as promising markers in early diagnostics.

The tumor suppressor genes *p53* and *ATM* have been a subject of profound investigations in view of the genetic mechanisms, involved in their inactivation in human tumors. However, little is known about the epigenetic impact on this process. There are a limited number of studies on the methylation status of *p53* in breast tumors. Kang et al. first demonstrated presence of CpG methylation in the *p53* promoter in BC tissue (Kang et al., 2001). Hypermethylation in *p53* promoter correlated with decrease in transcriptional activity (Schroeder & Mass, 1997). Our analysis of *p53* promoter methylation status in a group of Bulgarian patients with BC did not lead to identification of hypermethylation cases. This lack of aberrant methylation is accompanied with a relatively high percentage of *p53* mutations found in the same patients (32/145 i.e. 22.07%, Bozhanov et al., 2010). The mutations spectrum was heterogeneous and specific for the Bulgarian patients. Patients with *p53* mutations had larger tumors, higher grade of malignancy, and poorer overall survival. Our results are in agreement to those of other authors who found that *p53* hypermethylation was not significantly associated in breast tumors (Barekati et al., 2010; Pal et al., 2010). However, Kang et al. (2001) found 11.5% (3 of 26 cases) of *p53* hypermethylation and concluded that methylation in the *p53* promoter region is an alternative pathway to neoplastic progression irrespective of the status of invasion.

A limited number of publications proposed aberrant *ATM* hypermethylation as a likely epigenetic factor for its inactivation. Currently, data on the *ATM* methylation status in breast tumors are very limited and contradictory. With the exception of a single study (Vo et al., 2004), the data for lack of aberrant *ATM* promoter methylation in sporadic BC are predominating (Soukupova et al., 2008; Dejeux et al., 2010). Our data also showed absence of aberrant *ATM* promoter hypermethylation, which was accompanied with 7.6% (11/145) frequency of *ATM* mutations found previously (Bozhanov et al., 2010). *ATM* mutational spectrum was homogenous. Patients with mutated *ATM* had predominantly tumors with lower grade of malignancy and lobular histological type.

In conclusion, our results did not support the involvement of *p53* and *ATM* promoter hypermethylation in the neoplastic pathways of sporadic BC. However, such an involvement could not be excluded. Other epigenetic mechanisms may affect the functional activity of *p53* and *ATM* in the process of tumorigenesis. With respect to these genes, the impact of genetic mechanisms in sporadic breast carcinogenesis seems predominating.

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