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Breast cancer patients with hypermethylation in the promoter of *BRCA1* gene exhibit favorable clinical status

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Promoter hypermethylation was shown to be involved in human cancerogenesis through silencing gene expression. Several studies were dedicated to explore the frequency and clinical significance of *BRCA1* hypermethylation in sporadic breast cancer to identify a specific molecular and clinico-pathological phenotype. However the available data are limited and rather too heterogeneous. In this study we investigated the level of methylation in the promoter region of *BRCA1* and its correlation with clinico-pathological and molecular characteristics in a group of 135 Bulgarian patients. Methylation specific PCR was applied to determine methylation status of tumor samples. Clinical impact of *BRCA1* hypermethylation was estimated using standard statistical methods including Fisher's exact and the Chi-squared tests, the Kaplan-Meier method, the univariate and multivariate Cox proportional hazards regression model. We found that hypermethylation was present in 17.04% of the cases (23/135). Patients with hypermethylation in *BRCA1* displayed favorable clinical status as their tumors were smaller in size ($P = 0.066$), lacked *p53* gene mutations ($P = 0.073$) and were of lobular type ($P = 0.046$). The presence of hypermethylation was weakly associated with better overall survival ($P = 0.2$) with a hazard ratio of 0.47 (95% CI 0.14-1.54, $P = 0.213$). Our study provides the first data on the *BRCA1* hypermethylation of Bulgarian patients and contributes to elucidation of its clinical significance in sporadic breast cancer.

Key words: Breast cancer, BRCA1, Hypermethylation, Clinicopathological characteristics, Overall survival

Breast cancer (BC) represents a major challenge to modern human oncology because of its high and constantly increasing frequency, and growing mortality and morbidity rate in women below the age of 45. Every year in Bulgaria, approximately 3600 women are diagnosed and about 1300 die of BC (1). The major risk factors are sex, age, family predisposition, as well as some reproductive and hormonal factors like early menarche and late menopause, late first childbirth, shorter breastfeeding period, use of oral contraceptives and hormone replacement therapy. Clinically BC is very heterogeneous, which is due to heterogeneity in disease mechanisms.

BC results from accumulation of genetic and epigenetic changes in tumor suppressor genes and proto-oncogenes. Some of these genes - *p53*, *PIK3CA*, *CHEK2*, *ATM*, *HER2*, are involved in the pathogenesis of different type of tumors. Others are specific only to breast/ovarian cancer - *BRCA1* and *BRCA2*. A large number of studies demonstrate a correlation between the genetic/epigenetic status of BC related genes and clinico-pathological characteristics of the patients. Such investigations could contribute to a more effective BC prevention and therapy, which will increase the survival rate and will significantly improve the quality of life of the patients. However, the results so far are contradictory and need further elucidation.

BRCA1 (BReast CAncer susceptibility gene 1) tumor suppressor gene maps to 17q12 – 21 (2). The gene is organized in 2 non-coding and 22 coding exons which span over > 80 kb of genomic DNA and encodes a 1,863 amino acids protein (3). Though its exact biological functions are not fully elucidated, it is known that BRCA1 is involved in maintaining genome integrity through participation in DNA damage repair (4), in the control of cell cycle checkpoints (5), in apoptosis (6), in preventing global DNA hypomethylation (7) and others. Mutations in *BRCA1* were proved to be the main genetic event in hereditary type of breast cancer. Between 20 and 45 % of hereditary cases are due to a germline mutation in the *BRCA1* gene. A *BRCA1* mutation carrier has an early age at onset, and lifetime risk for breast cancer 50 to 85%. However, no or limited somatic mutations in *BRCA1* have been found in the

more common sporadic form of the disease. Nevertheless, *BRCA1* mRNA level was found to be reduced or absent in invasive sporadic breast tumors (8), thus assigning a role of *BRCA1* in sporadic breast cancer as well. This suggests that alternative mechanisms for loss of BRCA1 function could be involved, including DNA hypermethylation, dysregulation of transcriptional activators and/or repressors binding to the *BRCA1* locus or post-transcriptional processes.

The concept that gene silencing by promoter hypermethylation is a common mechanism for tumor initiation or progression is recently gaining more support. Most frequently hypermethylation occurs on 5'-methylcytosine residues of 5'-CpG-3' dinucleotides. Unlike mutations which are often randomly spread over the genome, the CpG sites are clustered into islands, typically found in the promoter regions of genes. Hypermethylation leads to structural changes in chromatin, which prevent the binding of transcriptional factors to DNA, thus decreasing the transcriptional activity of the genes. The maintenance of the normal methylation pattern is critical for the proper gene expression. As aberrant methylation leads to alteration in gene expression, it may serve as a basis for cancer development. Hypermethylation in a tumor suppressor gene was first described for the *RB* gene in sporadic cases of retinoblastoma (9), followed by the *VHL* gene in clear cell renal carcinoma (10), the *APC* gene in colorectal carcinoma (11), the *ATM* gene in head and neck squamous cell carcinoma (12) and others. Hypermethylation of the *BRCA1* promoter in sporadic breast cancer samples was first detected by Dobrovic and Simpfendorfer (13). *BRCA1* hypermethylation was found to be significantly more common in breast and ovarian tumors (14) indicating that it is breast/ovarian cancer specific (15). Hypermethylation of the *BRCA1* promoter was proved to silence gene expression as it was associated with a decrease in the expression of *BRCA1* mRNA (16). The limited data available on the clinical impact of *BRCA1* hypermethylation failed thus far to outline a generally accepted clinico-pathological phenotype.

In this study we investigated the level of methylation in the promoter region of *BRCA1* in *BRCA1* mutation negative Bulgarian patients with sporadic breast cancer. Methylation status was determined using methylation specific PCR and clinical impact of *BRCA1* hypermethylation was estimated using standard statistical methods. The first data on *BRCA1* hypermethylation frequency in Bulgarian patients was provided and statistically significant correlations were found.

Patients and Methods

A group of 135 Bulgarian female patients with sporadic invasive primary breast carcinoma was included in the study. Patients were treated at the Thoracic Clinic of the Bulgarian National Oncological Centre Hospital, Sofia between 2000 and 2003. Staging was done according to the TNM classification of Union International Contre le Cancer (UICC). The average age of the patients was 54.11yrs, standard deviation (SD) ±11.5 yrs. Five patients had highly differentiated tumors (G1), 97 – moderately differentiated (G2) and 33 – poorly differentiated (G3). Eighty-five patients had negative nodal status and 50 – positive. Ductal type of carcinoma was found in 122 of the patients and lobular – in 13. Fifty-nine patients were ER-negative, 76 – ER-positive. PR-negative were 58 patients, PR-positive – 77. Adjuvant therapy was applied according to accepted practice guidelines at that time. Patients were followed for a five-year period. All patients were *BRCA1* mutation negative as confirmed by previous PCR-SSCP analysis (17). Written consent was taken from all participants in the study. Clinical information was obtained from the existing medical records and is presented in a way preventing patients' identification.

DNA isolation. Tumor DNA was isolated from fresh frozen breast tumor tissue by a standard Proteinase K/Phenol procedure including tissue homogenization in lysis buffer (10 mM Tris/HCl pH 8.3, 400 mM NaCl, 2 mM EDTA-Na, 0.14 mg/ml Proteinase K, 1% SDS) at 37°C for 48 h, phenol/chloroform/isoamyl alcohol purification and ethanol precipitation. DNA concentration and purity was determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech).

Methylation specific PCR (MSP). The methylation status was determined by MSP of sodium bisulfite-converted DNA. To convert DNA, EZ DNA Methylation KitTM was used following the manufacturer's recommendations (Zymo Research Corporation). One μg of DNA of each sample was subjected to conversion. The obtained pure modified DNA was further PCR analyzed using primers that distinguish methylated (M) and unmethylated (U) DNA. Primer sequences were as follows: U forward, ggt taa ttt aga gtt ttg aga gat g; U reverse, t caa caa act cac acc aca caa tca; M forward, ggt taa ttt aga gtt tcg aga gac g; and M reverse, tca acg aac tca cgc cgc gca atc g (18). Both U and M primers amplify a 182 bp product. All amplifications used a hot-start Taq- Polymerase mix (HotStarTaq DNA Polymerase, QIAGEN) and were performed in a total volume of 20 μl containing 20 pmol of each primer and 100 ng of modified template DNA. The PCR cycling parameters were as follows: for M primers - 94°C for 10 min; 40 cycles of 94°C for 15 s, 65°C for 30 s, and 72°C for 30 s (final extension for 7 min) and for U primers - 94°C for 10 min; 35 cycles of 94°C for 15 s, 61°C for 30 s, and 72°C for 30 s (final extension for 7 min). PCR products were electrophoresed on a 2% agarose gel in 1xTBE, stained with ethidium bromide, and visualized under UV illumination. We included in each assay both methylated and unmethylated DNA as controls to avoid misinterpretation of the results.

Statistical analysis. The relationship between *BRCA1* methylation status and clinico-pathological characteristics of the patients was evaluated using Fisher's exact test and the Chi-squared test. Overall survival (OS) was estimated by the Kaplan-Meier method, and differences between survival curves were assessed for statistical significance using the log-rank test. Cox proportional hazards regression model was used to calculate the hazard ratios (HR) and their 95% confidence intervals (95%CI) for each variable in the univariate and multivariate analyses. All *P*-values were two-sided, and results were considered statistically significant at *P* less than

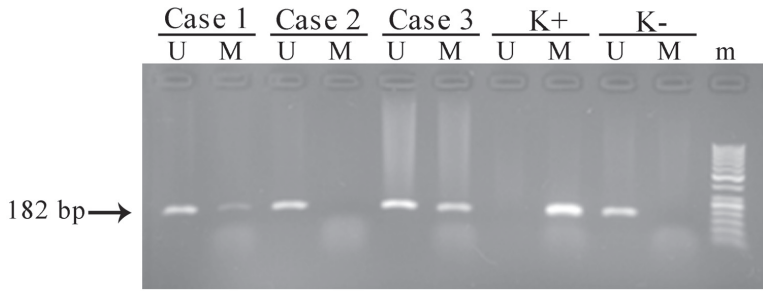

Figure 1. MSP-analysis of *BRCA1* in patients with sporadic breast cancer. 2%-agarose gel-electrophoresis of PCR products obtained with primers for unmethylated (U) and methylated (M) DNA; Case 1 and Case 3 – patients with methylated *BRCA1* and Case 2 – patient with unmethylated *BRCA1*; K+ is totally methylated positive control; K- is unmethylated negative control; m – 50 bp marker

0.05. Analyses were done with the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

***BRCA1* promoter hypermethylation in primary breast carcinomas. Correlation with clinico-pathological and molecular characteristics.** We evaluated the level of methylation in the promoter region of *BRCA1* in the studied group of patients with breast cancer. Methylation status was analyzed using methylation specific PCR. Hypermethylation was found in 17.04% of the cases (23/135) (Fig. 1). For a positive control, a totally methylated *in vitro* DNA (Zymo Research) was included in each amplification reaction. DNA isolated from blood samples of healthy persons was used as a negative control.

To analyze the clinical impact of *BRCA1* hypermethylation we compared the methylation status with standard prognostic factors including age of diagnosis, tumor size (T), nodal (N) status, grade of malignancy (G), histological type, and estrogen (ER) and progesterone receptor (PR) status (Table 1). We found

Table 1. *BRCA1* methylation and clinico-pathological variables of breast carcinoma

Variable		Total cases n = 135	Methylated n = 23	Non-methylated n = 112	*P*
Age	Years (mean±SD)	54.11±11.5	50.57±9.5	54.84±11.8	
	Range	29-88	32-69	29-88	
	<50	51	12	39	0.156
	>/=50	84	11	73	
Tumor size	T1	63	15	48	0.0664
	T2-T4	72	8	64	
N status	N0	85	13	72	0.487
	N+	50	10	40	
Grade	G1	5	0	5	0.832
	G2	97	17	80	
	G3	33	6	27	
Histological type	Lobular	13	5	8	**0.0464**
	Ductal	122	18	104	
ER expression	Positive	76	12	64	0.818
	Negative	59	11	48	
PgR expression	Positive	77	12	65	0.648
	Negative	58	11	47	
***p53* mutation**	Positive	16	0	16	0.073
	Negative	119	23	96	
***ATM* mutation**	Positive	11	0	11	0.210
	Negative	124	23	101	
***PIK3CA* mutation**	Positive	43	5	38	0.329
	Negative	92	18	74	
***HER2* overexpression**	Positive	19	3	16	0.367
	Negative	51	14	37	
	Unknown	65	6	59	

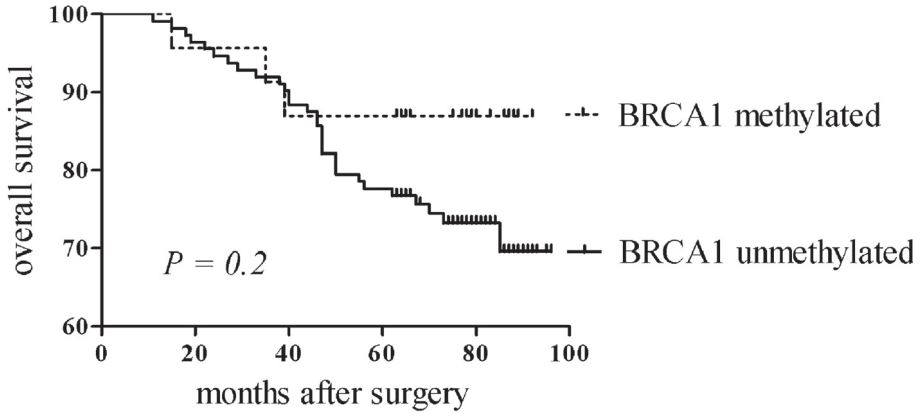

Figure 2. Kaplan-Meier survival curves. OS rates of patients with unmethylated and methylated *BRCA1* promoters.

that the disease had a four-year earlier manifest in patients with hypermethylation compared to patients with non-methylated tumors. Lobular carcinomas were significantly more frequent in patients with hypermethylation compared to patients with normally methylated tumors ($P = 0.046$). Tumors with abnormal *BRCA1* methylation tended to be smaller in size, predominantly at T1 ($P = 0.066$).

BRCA1 methylation status was further correlated with several tumor molecular characteristics found previously (17), including mutations in breast cancer related genes such as *p53*, *ATM* and *PIK3CA* genes, and overexpression of HER2 protein (Table 1). Interestingly, we found that all hypermethylated tumors were *p53* negative ($P = 0.073$).

***BRCA1* hypermethylation and overall survival.** The overall survival (OS) of the studied group of patients with BC was estimated as a five-year survival rate, as with BC it is considered that those who had survived the five-year period are quite likely to be cured of the disease. The OS in the studied group was estimated to 75.55% (103/135). Kaplan–Meier analysis showed that patients with hypermethylation in *BRCA1* had a better survival rate compared to patients with normally methylated *BRCA1* promoter though this finding did not reach statistical significance ($P = 0.2$) (Fig.2).

Univariate and multivariate Cox proportional hazards model was used to estimate the hazard ratio for carriers of *BRCA1* hypermethylation compared to non-carriers (Table 2). The analysis included traditional prognostic factors such as age of diagnosis, tumor size, nodal status, grade of malignancy, estrogen and progesterone receptor status, as well as the mutational status of *p53*, *PIK3CA* and *ATM* genes, and the expression profile of *HER2* proto-oncogene. Univariate analysis indicated a protective effect of *BRCA1* hypermethylation with a HR of 0.47 though not statistically significant (95% CI 0.14-1.54, $P = 0.213$). Similar was the influence of *PIK3CA* mutations (HR of 0.52, 95% CI 0.22-1.19, $P = 0.124$) and PR status (HR of 0.49, 95% CI 0.24-0.97, $P = 0.042$). The opposite effect had *p53* mutations and tumor size. Patients with *p53* mutations had 2.29-fold increased risk of dying from breast cancer (95% CI 0.99-5.27, $P = 0.052$), and those with T1 tumors – 1.93-fold (95% CI 0.94-3.99, $P = 0.074$). The only

Table 2. Univariate and multivariate survival analysis

Factor	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
BRCA1, methylated vs. unmethylated	0.47	0.14-1.54	0.213	0.91	0.24-3.41	0.891
p53, mutated vs. wild type	2.29	0.99-5.27	**0.052**	1.04	0.34-3.15	0.945
PIK3CA, mutated vs. wild type	0.52	0.22-1.19	0.124	0.48	0.17-1.34	0.160
ATM, mutated vs. wild type	1.07	0.33-3.51	0.912	1.71	0.35-8.41	0.508
HER2, overexpressed vs. normal	1.16	0.43-3.12	0.775	0.86	0.26-2.89	0.810
Age, <50 vs. >=50 years	0.99	0.97-1.02	0.707	0.98	0.95-1.02	0.400
G, G1 vs. G2 vs. G3	1.38	0.69-2.74	0.357	1.43	0.55-3.72	0.459
T, T1 vs. T2-T4	1.93	0.94-3.99	0.074	4.51	1.43-14.17	**0.010**
N, positive vs. negative	0.91	0.45-1.86	0.799	0.86	0.33-2.28	0.766
ER, positive vs. negative	1.28	0.64-2.57	0.493	0.86	0.33-2.26	0.764
PR, positive vs. negative	0.49	0.24-0.97	**0.042**	0.5	0.21-1.23	0.133

Abbreviations: HR, hazard ratio; CI, confidence interval; G, grade of malignancy; T, tumor size; N, nodal status; ER, estrogen receptor; PR, progesterone receptor
Significant *P* values are in bold

independent prognostic factor, as shown by the multivariate analysis, that contributed significantly to a decrease in OS was tumor size with a fourfold increased risk of death (HR = 4.51, 95% CI 1.43–14.17, $P = 0.010$).

Discussion

Ever since *BRCA1* hypermethylation was proved to be involved in sporadic breast carcinoma (13), several studies were dedicated to explore its frequency and correlation with disease characteristics in order to identify a subset of sporadic breast cancers with a specific molecular and clinicopathological phenotype. However, the efforts so far failed to outline such a phenotype, as the available data are rather too heterogeneous and contradictory. Heterogeneity is observed both with respect to frequency and clinical correlations. The reported frequency of *BRCA1* promoter hypermethylation in sporadic breast carcinomas is in the range of 9 to 59% (15,19-29). The frequency found here is the first reported in Bulgarian patients with BC and is a little below the average *BRCA1* hypermethylation frequency. Several factors may account for the differences in the frequency of hypermethylation: the applied methodological approaches and scale of studied groups, adjacent normal tissue contamination, partial hypermethylation, population differences due to exposure to specific environmental agents and others.

Though tumors with *BRCA1* promoter methylation display various cancer phenotypes, several most frequent features could be outlined. Thus, though not a rule, most studies demonstrated that *BRCA1* hypermethylation correlated with lack of estrogen and progesterone receptor expression (19,20,22,24,25,30) and is most frequently present in younger women, below the age of 50 (19,24,29). As this in some way resembles the familial *BRCA1* mutated tumors, it has been suggested that *BRCA1* hypermethylated tumors might phenocopy familial *BRCA1* tumors (31). The mechanisms underlying the correlation of *BRCA1* methylation with estrogen and progesterone receptor expression have not been fully elucidated. However, it is known that estrogens stimulate the expression of *BRCA1* (32), while BRCA1 was shown to directly interact with estrogen receptor thus inhibiting the cellular response to estrogens (33,34). Nevertheless, in our study we did not observe any association between *BRCA1* hypermethylation and ER/PR status. This is not an exception, as Xu X et al (29) also didn't find a statistically significant correlation with hormone receptor status. Interestingly, Matros et al. (21), found even the reverse association - a high frequency of *BRCA1* promoter methylation among ER positive tumors, suggesting a more complex phenotype association. Similar to other authors (19,24,29) we found that abnormal *BRCA1* methylation was more common in breast tumors from patients less than 50 years. Besides, the patients with hypermethylation were 4 years younger than patients with non-methylated tumors.

In our study the subset of patients with hypermethylated *BRCA1* displayed more favorable clinical status. Thus, we found that *BRCA1* hypermethylation most strongly correlated with lobular histological type, in contrast to other authors who found a correlation with ductal (19,27) and medullary and mucinous (15) types. Lobular breast carcinoma is considered to be more favorable histological type with better survival rates of the patients (35). Additional favorable marker was the finding that tumors with hypermethylation tended to be smaller than non-methylated tumors. The larger size of the tumor is proved to be one of the most significant indicators for a poor prognosis. Another favorable characteristic of the hypermethylated tumors was the observation that none of them had a *p53* mutation. Mutated *p53* was found to significantly correlate with a greater carcinogenic aggressiveness and worse OS of breast cancer patients (17, 36-38). Since *p53* and *BRCA1* are involved in a same cancer pathway, it could be speculated that the inactivation of the pathway does not require inactivation of both genes (inactivation of one gene may be sufficient).

In a recent study, Stefansson O. et al. found that CpG island hypermethylation of *BRCA1* significantly associates with the basal/triple-negative breast cancer (TNBC) (39). In general patients with TNBC are associated with poor prognosis including larger tumor size, younger age, high tumor grade, positive nodal involvement ect. However, this is not contradictory to our findings since TNBC is a subtype of BC, accounting for only 15% of all breast cancer patients. Furthermore, in their study Stefansson O. et al. defined a subgroup of TNBCs with *BRCA1* aberrations and favorable disease outcome.

We found a weak association between *BRCA1* hypermethylation and better OS of patients. Similar correlation was registered by Li S et al (27). However, Xu X et al. (29) on the basis of a large population-based study demonstrated decreased survival associated with *BRCA1* promoter methylation among women with BC. Similar were the findings of Karray-Chouayekh S et al. (28) and Chen Y et al. (23). The better survival rate found here by the Kaplan-Meier method was supported by a protective, though not statistically significant, effect of *BRCA1* hypermethylation as revealed by the univariate Cox proportional hazards model. The observed better OS of patients with *BRCA1* hypermethylation might be partly explained by the favorable clinical and molecular characteristic of *BRCA1*-hypermethylated tumors.

In conclusion, these are the first reported data on the involvement of *BRCA1* promoter hypermethylation in sporadic BC pathogenesis in Bulgarian patients. The patients with hypermethylation in *BRCA1* exhibited more favorable clinical status as their tumors were smaller; they lacked *p53* mutations and were of lobular type. The presence of *BRCA1* hypermethylation was weakly associated with better OS. A specific *BRCA1*-related phenotype can not be thus far outlined as the available data are rather too contradictory and heterogeneous.

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