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ORIGINAL ARTICLE

Promoter hypermethylation of *p16*, *BRCA1* and *RASSF1A* genes in triple-negative breast cancer patients from Serbia

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Summary

Purpose: *In order to investigate if aberrant promoter methylation of p16, BRCA1 and RASSF1A genes contributes to biological behavior of triple-negative breast cancer (TNBC), marked as the most aggressive phenotype of breast cancer, we compared the hypermethylation pattern between TNBC and ER+PR+Her2- breast cancer.*

Methods: *131 patients with histologically confirmed breast cancers were included - 61 TNBC and 70 ER+PR+Her2- cases. The patients were followed up for 1-87 months (median 78). DNA from tumor tissues was isolated by the salting out procedure. The methylation status was assessed by nested methylation-specific PCR after bisulfite modification of DNA.*

Results: *The frequency of p16 hypermethylated breast cancer cases was significantly higher in TNBC than in ER+PR+Her2- group (33; 54.1% vs. 20; 28.6%, p=0.00298). Co-methylated p16 and RASSF1A genes were more frequent in the TNBC than in ER+PR+Her2- group (20; 32.8% vs. 10; 14.3%, p=0.0225). The same result was observed when hypermethylated BRCA1 gene was added in the analysis: 12; 19.7% vs. 3; 4.3%, p=0.00791. Although there was significant difference in disease-free survival (DFS) and overall survival (OS) between TNBC and ER+PR+Her2- group, further analysis of co-methylation of p16 and RASSF1A (p16+RASSF1A+) showed that DFS was significantly shorter in the patients with both genes co-methylated in TNBC than in ER+PR+Her-2- group (8/20; 40% vs. 2/10; 20%, p=0.03272).*

Conclusions: *The obtained data indicate that hypermethylated p16 and RASSF1A cell-cycle inhibitor genes might be considered as biomarkers for bad prognosis in breast cancer. Hypermethylation of these genes may influence the clinical disease course, distinguishing a particular group of TNBC patients with even more aggressive phenotype.*

Key words: *breast cancer, BRCA1, epigenetic changes, hypermethylation, p16, RASSF1A*

Introduction

Breast carcinoma, the most frequent cancer in females worldwide, ranks first among malignant tumors in females in Serbia with about 4000 newly diagnosed cases per year [1]. Breast cancer is a very heterogeneous disease due to diverse histology, presence of biological markers of disease, the ability to form metastases, aggressiveness and potential for progression, as well as response to anticancer therapy. Besides classical prognostic parameters such as tumor size, lymph node metastasis and distant metastasis (TNM), biological markers such as steroid receptors describing estrogen-dependent tumor growth, Her-2 receptors describing ability for autonomous growth and Ki67 describing the rate of proliferation in tumor tissue are used for the estimation of prognosis as well as prediction of anticancer therapies including chemo, endocrine or targeted biological therapies.

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TNBC is characterized by the absence of the most prominent biological markers– steroid receptors: estrogen receptors (ER) and progesterone receptors (PR), as well as Her-2 receptor. About 15-20% of breast cancers belong to this type [2]. TNBC exhibits more aggressive phenotype with bad prognosis – relapse of disease occurs within 1-3 years after diagnosis and the majority of patients die within the first 5 years after diagnosis [3]. This is the consequence of the lack of biological targeted therapies as well as lack of markers of conventional anticancer therapy response. Molecular targets that can be used in therapy monitoring as well as for development of new anticancer therapies are intensively searched upon [4]. On the contrary, breast cancer with presence of steroid receptors and absence of Her-2 receptor (ER+PR+Her2-) is, at least in a short time after diagnosis, the best prognostic type of breast cancer [5]. The genes that play important role in carcinogenesis may be altered in their normal function by genetic and epigenetic mechanisms. Epigenetics refers to changes in the gene expression that occur independently of the changes in the DNA nucleotide sequence. The pattern of epigenetic changes in tumors is characterized by regional hypermethylation and global hypomethylation and these changes are tumor-specific with potential to be used as biomarkers [6]. In breast cancer, aberrant methylation can be related to silencing of different genes expression, including cell-cycle inhibitor genes (*p16*, *RASSF1A*) and DNA repair genes (*BRCA1*) [7]. Tumor suppressor gene *BRCA1* is characterized as a breast cancer susceptibility gene. *BRCA1* protein is involved in the mechanism of double stranded DNA repair by homologous recombination. About 20-30% of TNBC cases carry *BRCA1* germline mutation [8]. Other mechanisms of inactivation of *BRCA1* gene, such as aberrant methylation, can be present in TNBC [8]. These sporadic TNBC cancers manifest clinical features of *BRCA1* mutated cancers and their biological behavior can be considered as BRCAness [8]. *p16* tumor suppressor is one of the key growth regulatory genes acting as a cyclin-dependent kinase inhibitor, regulating cell promotion across G1 phase of the cell cycle. *RASSF1A* inhibits tumor growth in both *in vitro* and *in vivo* systems supporting its role as a tumor suppressor gene. The promoter methylation level of *RASSF1A* gene may be a potential biomarker for detecting field cancerization of tumor surrounding tissue in breast cancer patients [9].

In this study, by using candidate-gene approach, we compared the promoter methylation status of *p16*, *BRCA1* and *RASSF1A* genes in patients with TNBC and patients with ER+PR+Her2- breast cancer from Serbia. Also, we compared the clinical behavior of these two groups of patients – occurrence of disease relapse, DFS and OS.

Table 1. Patient and tumor characteristics of TNBC and ER+PR+Her-2- groups of patients

	TNBC		*ER+PR+Her-2-*	
	Number	*%*	*Number*	*%*
Patient characteristics				
Premenopausal	7	11.5	12	17.4
Postmenopausal	54	88.5	58	82.6
Age (median), years	60		59	
Tumor characteristics				
Size (pT)				
T1	19	31.1	22	31.4
T2	37	60.7	47	67.1
T3	5	8.2	1	1.5
Histological type				
IDC	38	62.3	31	44.3
Other (ILC, medullary, mixed)	23	37.7	39	55.7
Histological grade				
G1	0	0	6	8.5
G2+G3	61	100	64	91.5
Regional lymph node involvement (pN)				
N0	30	49.2	30	42.9
N1	30	49.2	39	55.7
NA	1	1.6	1	1.4

IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma

Methods

Patients

131 patients with histologically confirmed breast cancer were included in this study. Among them, 61 were TNBC and 70 were ER+PR+Her2- cases. All patients were surgically treated and classified according to the TNM (pTN) classification of the UICC. Tumor-host characteristics of both groups of patients are shown in Table 1.

Patients were followed up from 1 to 87 months (median 78). TNBC patients were followed from 1 to 85 months (median 43), while ER+PR+Her2- patients were followed up from 55 to 87 months (median 82). The clinical course of the disease was defined by DFS (time from surgery during which no signs of disease were found) and OS (time from surgery to death regardless of the cause of death or last contact).

DNA isolation

DNA was isolated from tumor tissues by the salting out procedure. The protocol involved initial cell disruption and digestion with SDS–proteinase K, followed by addition of high concentrations of salts (6 M sodium chloride). DNA was precipitated using ethanol and was further purified by adding 70% ethanol. DNA quantity and quality was measured by BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan).

MS PCR

After isolation, DNA was subjected to bisulfite modification using the EpiTec Kit, Qiagen, Hilden, Germany, as specified by the manufacturer. Briefly, treatment with sodium bisulfite deaminates unmethylated cytosines, which are converted to uracil upon hydrolic deamination and alkali desulfonation. In contrast, 5-methylcytosines are resistant to bisulphite-mediated conversion. Methylation status was assessed by nested methylation-specific PCR (MS-PCR) [10]. The nested, two-stage methylation specific PCR approach, increases the specificity and PCR yield, and allows the detection of 1 methylated in 50,000 unmethylated alleles [11].

DNA from the peripheral blood lymphocytes of a healthy individual was used as an unmethylated control. To create completely methylated DNA at all CpG dinucleotides, DNA from healthy volunteers was treated with *SssI* methyltransferase (New England Biolabs, Massachusetts, USA) and used as a positive, methylated control.

Statistics

Differences in the frequency of hypermethylated genes and occurrence of disease relapse in the examined groups of patients were tested by the chi-square test with Yates' continuity correction and Fisher's exact test. DFS and OS were calculated by the Kaplan-Meir method. Differences between the curves were tested by the log-rank test. The cut-off for significance was 0.05.

Results

When promoter hypermethylation of *p16*, *BRCA1* and *RASSF1A* genes were compared between TNBC and ER+PR+Her2- groups, the following results were found:

The number of *p16* hypermethylated breast cancer cases was significantly higher in the TNBC in comparison with ER+PR+Her2- group: 33 (54.1%) *vs.* 20 (28.6%), p=0.00298 (Figure 1).

Although the incidence of *BRCA1* hypermethylation between the examined groups of patients did not reach statistical significance, we observed a tendency towards higher occurrence of *BRCA1* hypermethylation in TNBC compared to ER+PR+Her2- cases: 33 (54.1%) *vs.* 27 (38.6%), p=0.0752.

There was no statistically significant difference between the incidence of *RASSF1A* promoter hypermethylation among the examined groups of patients: 31 (50.8%) in TNBC *vs.* 34 (47.9%) in ER+PR+Her2- group, p=0.7974.

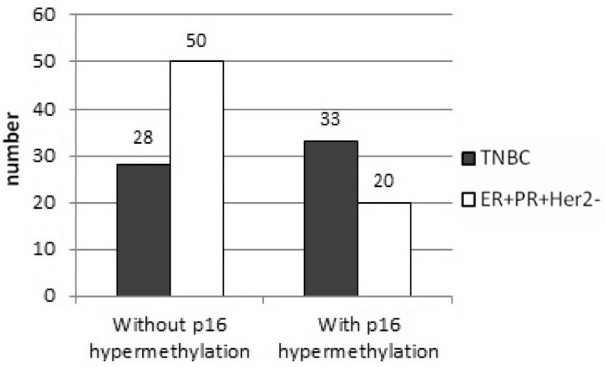

Figure 1. Comparison of *p16* gene promoter hypermethylation between TNBC (n=61) and ER+PR+Her2- (n=70) patients. p=0.00298 (x^2 test).

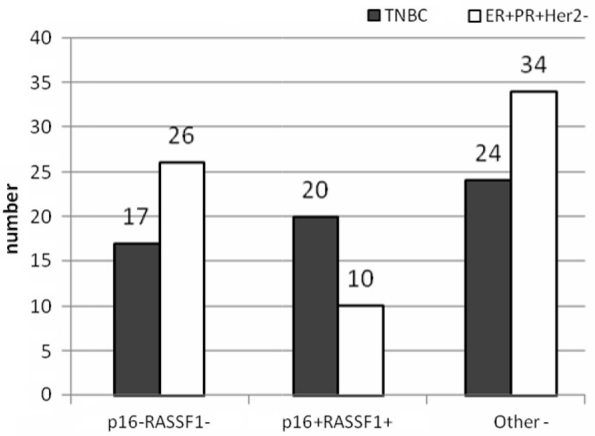

Figure 2. Comparison of both p16 and *RASSF1A* promoter hypermethylation between TNBC (n=61) and ER+PR+Her2- (n=70) patients. p=0.0225 (x^2 test). + (hypermethylated gene), - (nonmethylated gene).

When the frequencies of co-methylated (combined hypermethylation of the examined genes) *p16* and *RASSF1A* genes (*p16+RASSF1A+*) were compared between the breast cancer patients with TNBC and ER+PR+Her2- characteristics, we found that the number of patients with both hypermethylated genes was significantly higher in the TNBC than in ER+PR+Her2- group: 20 (32.8%) *vs.* 10 (14.3%), p=0.0225 (Figure 2). Figure 2 also shows the comparison between the groups in cases when both (*p16-RASSF1A-*) or one of the genes were not hypermethylated.

Comparison of the frequency of *p16, BRCA1* and *RASSF1A* co-methylated genes among the breast cancer patients with TNBC and ER+PR+Her2- characteristics, showed that there was statistically significant difference between the groups. The number of hypermethylated genes was significantly higher in the TNBC than in ER+PR+Her2- group: 12 (19.7%) *vs.* 3 (4.3%), p=0.00791 (Figure 3). Figure 3 also shows the group of other cases with at least one hypermethylated gene (*p16, BRCA1, RASSF1A*) and the group where neither of the genes was hypermethylated (*p16-BRCA1-RASSF1A-*).

We compared the clinical course of the disease between the groups. Although significant difference was not found in the incidence of disease relapse between the TNBC and ER+PR+ Her2- groups (18; 29.5% *vs.* 12; 17.1%, respectively), we observed a tendency towards higher disease relapse in the TNBC cohort (p=0.09294). However, when the occurrence of distant metastases in soft tissues was compared, significantly higher incidence of these metastases was detected in TNBC compared to ER+PR+ Her2- cohort (9 *vs.* 1; p=0.00589) (Figure 4). Furthermore, comparison between the TNBC and ER+PR+Her2- groups of the occurrence of visceral (10; 16.4% *vs.* 0; 0%) (Figure 5) and brain metastases (4; 6.6% *vs.* 0; 0%) (Figure 6) showed significantly higher incidence of both types of metastases in TNBC. Visceral as well as brain metastases were not detected in the ER+PR+Her2- group of patients. There was no statistically significant difference in the appearance of bone metastases between the examined groups.

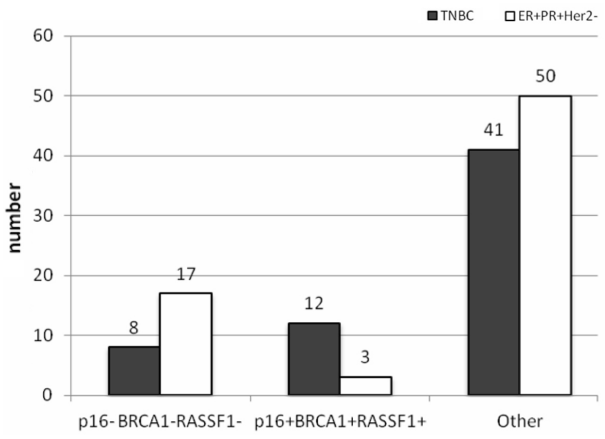

Figure 3. Comparison of p16, *BRCA1* and *RASSF1A* promoter co-methylation between TNBC (n=61) and ER+PR+Her2- (n=70) patients. p=0.0791 (x^2 test). + (hypermethylated gene), - (nonmethylated gene).

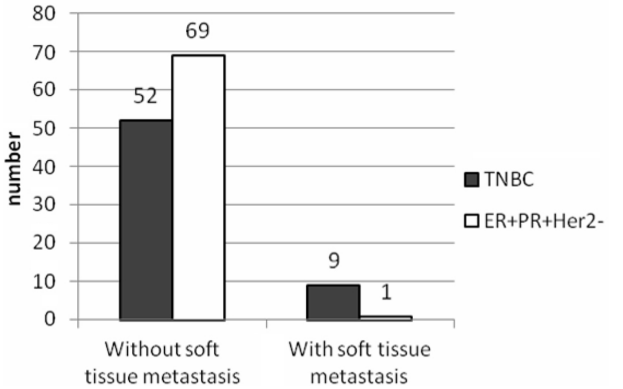

Figure 4. Comparison of occurrence of soft tissue metastasis between TNBC (n=61) and ER+PR+Her2- (n=70) patients. Fisher exact test, p=0.0059.

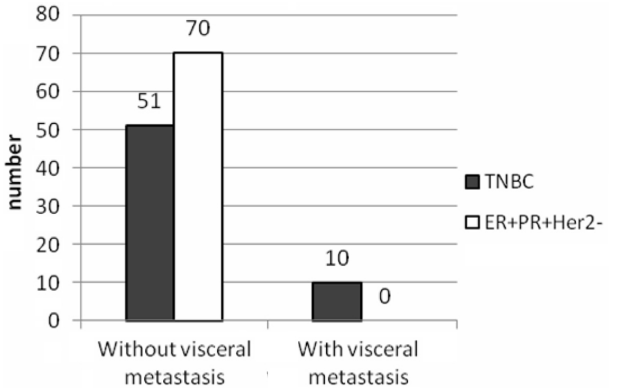

Figure 5. Comparison of occurrence of visceral metastasis between TNBC (n=61) and ER+PR+Her2- (n=70) patients. Fisher exact test, p=0.0484.

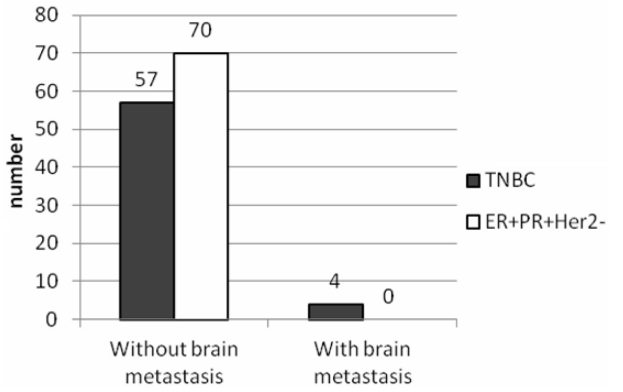

Figure 6. Comparison of occurrence of brain metastasis between TNBC (n=61) and ER+PR+Her2- (n=70) patients. Fisher exact test, p=0.0445.

When we compared DFS between the groups, statistically significant difference was found – DFS was significantly shorter in the TNBC group (median not reached) (p=0.00016). In the TNBC group we detected 18 (29.5%) relapses of disease *vs.* 12 (17.1%) in ER+PR+Her2- group (Figure 7). Also, when we compared OS between the examined groups, significantly shorter OS was found for TNBC in comparison with ER+PR+Her2- breast cancer (median not reached) (p=0.00034). Fourteen (22.9%) deaths were detected in TNBC group *vs.* 5 (7.1%) in ER+PR+Her2- group (Figure 8).

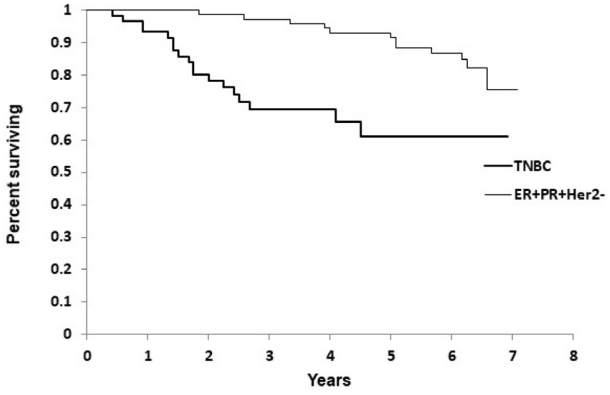

Figure 7. Comparison of DFS between TNBC (n=61) and ER+PR+Her2- breast cancer patients (n=70). p=1618e-4.

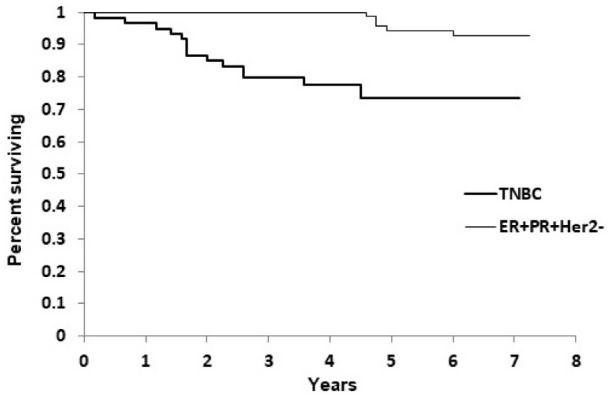

Figure 8. Comparison of OS between TNBC (n=61) and ER+PR+Her2- breast cancer patients (n=70). p=3.380e-4.

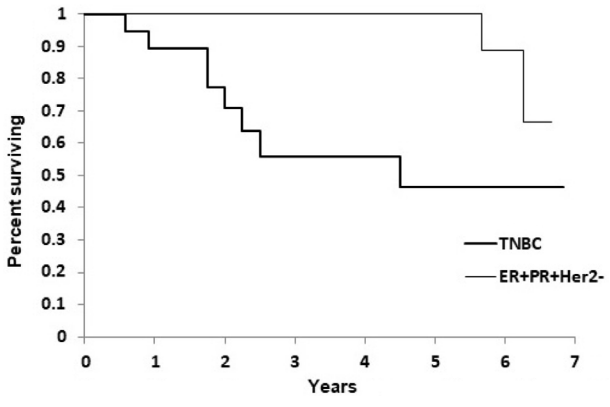

Figure 9. Comparison of DFS in regard to p16 and *RASSF1A* gene hypermethylation between TNBC (n=20) and ER+PR+Her2- breast cancer patients (n=10). p=0.0465.

Differences in DFS and OS between the two groups of patients regarding the methylation status of *p16*, *RASSF1A* and *BRCA1* as a single event were not detected. However, when we analyzed co-methylation of *p16* and *RASSF1A* (*p16+RASSF1A+*), we found that DFS was significantly shorter in the patients with both genes co-methylated in TNBC than with ER+PR+Her-2- group (8/20; 40% *vs.* 2/10; 20%, respectively ; median 54 months *vs.* median not reached, p=0.03272;Figure 9).

Among the patients with TNBC, no statistically significant difference in DFS was observed between *p16+RASSF1A+* (n=20) and *p16-RASSF1A-* (n=17) subgroups. The median value of DFS for *p16+RASSF1A+* TNBC patients was 54 (≥27 months). The median value of DFS in the group of *p16-RASSF1A-* was not reached. The number of relapsed patients was 8/20 (40%) *vs.* 4/17 (23.5%).

Concerning OS, no significant difference was found, but there was a trend towards shorter OS in TNBC patients with co-methylated *p16* and *RASSF1A* genes (p=0.062). When hypermethylated *BRCA1* gene was added (*p16+RASSF1A+BRCA1+*), no significantly different DFS among the patients with TNBC and ER+PR+Her-2- breast cancers was shown. Also, there was no statistically significant difference among the patients with or without hypermethylated *p16*, *BRCA1* and *RASSF1A* genes alone, or in combination within TNBC group, as well as within ER+PR+HER2- group.

Discussion

Epigenetic changes are mitotically inherited, stable, but potentially reversible in gene expression. The number of genes subject to epigenetic inactivation is even higher than the number of genes inactivated by mutations [12]. One of the major mechanisms of epigenetic regulation of gene activity is DNA methylation of the 5-cytosine in CpG dinucleotides within the CpG islands, located in promoter regions of various genes. In normal cells, genes involved in important cell processes are non-methylated which enables the appropriate function of such genes. Epigenetic changes, including promoter hypermethylation, cause aberrant expression of these genes that may promote cancer development. During carcinogenesis, DNA hypermethylation of tumor suppressor genes promoters leads to their transcriptional silencing and aberrant expression [13]. Epigenetic changes are early events in malignant transformation.

Furthermore, tumor progression causes progressive accumulation of epigenetic alterations, indicating their important role in multistage carcinogenesis [14]. In breast cancer cells, genes that are silenced by epigenetic mechanism are classified as proapoptotic genes, cell cycle-inhibitor genes and DNA repair genes [7].

In the present study, the methylation status of *BRCA1*, *p16*, and *RASSF1A* genes was determined in tumor tissues because of the tissue and tumor specificity of DNA methylation pattern. These genes are recognized as genes important for carcinogenesis as well as for breast cancer behavior.

The obtained results showed that *p16* promoter hypermethylation as a single event differs in frequency between TNBC and ER+PR+Her2- cases. We observed higher frequency of *p16* hypermethylation in the TNBC subtype, characterized with aggressive phenotype, compared to the ER+PR+Her2- subtype, characterized with the best prognosis. Tumor suppressor gene *p16* is commonly hypermethylated in breast cancer cell lines, as well as in breast carcinoma [15]. Since somatic mutations of the *p16* are rare in sporadic breast cancer [16], DNA methylation has been proposed as a major mechanism for *p16* inactivation. The high frequency of *p16* promoter hypermethylation in intraductal carcinomas indicate that the *p16* methylation play a significant role in the early stages of breast carcinogenesis [17]. Higher prevalence of *p16* hypermethylation was detected in late-stage tumors, compared to the early stage breast carcinoma and the ductal carcinoma *in situ* [18]. A meta-analysis indicated that *p16* hypermethylation was associated with increased breast cancer risk [19]. However, a recent meta-analysis revealed the lack of association between *p16* promoter hypermethylation and survival [20].

Although the prevalence of *BRCA1* hypermethylation between the examined groups of patients did not reach statistical significance, we observed a tendency towards higher occurrence of *BRCA1* hypermethylation in TNBC compared to ER+PR+Her2- cases. It was observed that *BRCA1* hypermethylation more frequently occurs in TNBC tumors, compared to other sporadic breast cancers [21]. A meta-analysis revealed that *BRCA1* hypermethylation was associated with poor survival in breast cancer patients [22].

In this study, no statistically significant difference was noted between the incidence of *RASSF1A* promoter hypermethylation between TNBC and ER+PR+Her2- cases. Hypermethylation of *RASSF1A* gene is one of the earliest epigenetic changes in breast cancer and was detected in more than 60% of primary breast cancers [23]. RASFF1A aberrant methylation could potentially be a valuable marker for discriminating malignant and non-malignant breast tissue lesions. *RASSF1A* promoter hypermethylation has been associated with a worse survival in patients with breast cancer [24]. *RASSF1A*, ABCB1 and *GSTP1* showed significantly higher methylation levels in late stage compared to the early stage breast carcinoma [18].

When multiple events were analyzed, the obtained results showed higher incidence of both *p16* and *RASSF1A* hypermethylation in TNBC (*p16*+*RASSF1A*+). The same result was obtained when hypermethylated *BRCA1* gene was added into the analysis. *p16*+*BRCA1*+*RASSF1A*+ methylation phenotype was significantly more frequent in the TNBC group, indicating that aberrant methylation of the examined cell-cycle inhibitor genes (*p16*, *RASSF1A*) and DNA repair gene (*BRCA1*) may influence TNBC biological behavior. Co-methylation of three genes indicates the presence of possible BRCaness phenotype among TNBC patients [25].

Differential methylation was investigated in regard to different molecular subtypes of breast cancer. Basal-like subtype, covering the majority of TNBC cases, expressed lower overall levels of methylation, while higher levels of methylation were detected among luminal B molecular subtype of breast cancer which can cover a portion of our ER+PR+Her2- group [26]. The levels of methylation were 27.6% for basal-like and 31.3% for luminal A and 35.1% for luminal B subtype (average 33.1% for luminal subtypes) [26] . Since our ER+PR+Her2- group probably consisted of both luminal types, we detected hypermethylation frequency of only 4.3% for all 3 examined genes (*p16*+*BRCA1*+*RASSF1A*+). Higher methylation frequency for both luminal types was reported in the study of Holm et al. (33.1%) [27]. In the same study, average methylation for basal-like molecular subtypes of 27.6% was reported, while we detected 19.7% of all 3 hypermethylated genes (*p16*, *BRCA1*, *RASSF1A*) in TNBC cases. RASFF1A aberrant methylation could play a significant role in the different molecular subtypes of breast cancer [27]. *RASSF1A* is hypermethylated in HER2 positive breast tumors (ERBB2 and luminal B tumors) [18,26]. Our study showed that single gene hypermethylation was higher in TNBC than in ER+PR+Her2- (*p16* 54.1% *vs.* 28.6% respectively for *BRCA1* 54.1% *vs* 38.6% respectively and for *RASSF1A* 50.8% *vs* 48.6% respectively). Contrary to the study of Holm et al. who analyzed methylation by array with about 163 genes, our data for hypermethylation was obtained in candidate gene approach and is limited to only 3 genes which can influence the observed disagree-

ment. Also, our histologically determined groups are not completely the same as the groups formed on the basis of molecular subtyping, since 20-30% of TNBC cases are not basal-like [23].

We compared the TNBC group with the ER+PR+Her2- group with regard to the frequency of disease relapse and appearance of distant metastases. Although no difference in the frequency of relapse was found, we noticed significantly higher frequency of soft tissue, visceral and brain metastasis in the TNBC than in the ER+PR+Her2- groups. Furthermore, survival analysis showed significantly shorter DFS as well as OS in the TNBC group. The obtained data is in agreement with literature data describing TNBC as the breast cancer subtype with aggressive phenotype, higher rate of visceral and central nervous system metastases and worse DFS and disease-specific survival [3].

We further focused on the possible impact of methylation status on the clinical course of disease of the two groups of breast cancer patients. In order to investigate if hypermethylation of the analyzed genes provokes worse prognosis of the TNBC cases, we compared DFS between TNBC and ER+PR+Her2- cases with co-methylation of *p16* and *RASSF1A* (*p16+RASSF1A+*) and found significant difference. Furthermore, we analyzed the TNBC group independently in terms of DFS comparison between *p16+RASSF1A+* and *p16-RASSF1A-* subgroups. Difference in DFS between *p16+RASSF1A+* and *p16-RASSF1A-* subgroups within the TNBC existed but was not significant. Also, there was a difference in the number of relapsed patients between these two subgroups favoring the *p16+RASSF1A+* (40% *vs.* 23.5%). Although we did not observe significantly different DFS within the TNBC group, the observed difference within that group as well as the significant difference that existed between the groups (TNBC *vs.* ER+PR+Her2-), led us to presume that co-methylation of *p16* and *RASSF1A* genes might be an additional factor to be considered for further characterization of TNBC. However, a larger number of patients need to be analyzed in order to clarify the exact role of these epigenetic events in the course of disease.

In conclusion, the pattern of DNA methylation of various tumor-associated genes could be a powerful future non-invasive screening marker. The obtained data indicate that hypermethylated *p16* and *RASSF1A* cell-cycle inhibitor genes might be considered as biomarkers for bad prognosis in breast cancer patients. Hypermethylation of these genes may influence the clinical course of disease, distinguishing a particular group of TNBC patients with even more aggressive phenotype.

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Conflict of interests

The authors declare no conflict of interests.

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