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ORIGINAL ARTICLE

Correlation of HER2/TOP2A Gene Aberrations with RASSF1A/APC Gene Methylation Status in High-Risk Breast Cancer

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OBJECTIVE

Breast cancer (BC) is a heterogeneous malignancy and differs widely among different patients. The aim of this study was to investigate the relationship between the HER2/TOP2A gene aberrations and promoter methylation in RASSF1A/APC genes in patients with high-risk BC.

METHODS

Formalin-fixed paraffin embedded (FFPE) tissue samples from primary breast tumors (n=60) were assessed. HER2/TOP2A aberrations was evaluated using FISH method. DNA was extracted from FFPE tumor tissues, and Methylation-sensitive high resolution melting (MS-HRM) analysis were performed for RASSF1A/APC genes methylation status.

RESULTS

HER2 amplification and TOP2A aberration were observed in 15/60 (25%) and 18/60 (30%) cases, respectively. According to the statistical analysis, HER2 amplification was associated with higher tumor grade (p=0.001), PR status (p=0.025), and TOP2A aberrations (p=0.004). RASSF1A and APC methylation were 58/60 (96.6%) and 26/60 (43.3%), respectively. There was a significant correlation between APC methylation and TOP2A aberration. APC gene methylation was significantly more frequent in tumors with TOP2A aberration (p=0.026).

CONCLUSION

Our results suggested that APC gene promoter hypermethylation was associated with TOP2A gene aberrations in patients with high-risk BC. This may be significant for targeted individual therapy. Additionally, it was confirmed that there was significant association of TOP2A gene aberrations with the HER2 gene amplification seen in BC.

Keywords: APC; Breast cancer; HER2; TOP2A; MS-HRM; RASSF1A.

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Introduction

Breast cancer (BC) is the most common cancer type that affects women in the world population. Approximately 1.3 million women are diagnosed with BC annually worldwide.[1] Clinicopathologic features, such as tumor size, lymph node (LN) status, hormone receptor status, and invasion, play important roles in prognosis. Similar to other types of cancers, BC tumorigenesis is characterized as a multi-step process in which each step is thought to correlate genetic and/or non-genetic factors.

Human epidermal growth factor receptor 2 (HER2; aka erbB2) and its relatives belong to the HER family of receptor tyrosine kinases. The HER2 protein is a transmembrane glycoprotein with a size of 185-kD and belongs to the HER family of growth factor receptors.[2] The HER2 protein is overexpressed and/or amplified approximately in 15-20% of the BC and has both prognostic and predictive implications.[3] Topoisomerase 2 alpha (TOP2A) gene encodes a DNA topoisomerase that controls topologic states of DNA at transcription and replication.[4] TOP2A gene is found on chromosome 17 q12-q21, adjacent to the HER2 gene, and its aberrations (amplification or deletion) have been shown usually in HER2-positive breast cancers.[5]

Epigenetic abnormalities in neoplastic cells, including hypermethylation and hypomethylation of DNA, modified patterns of histones, and remodeled chromatin arrangement, lead to the modified expression of numerous fundamental genes. A well-categorized epigenetic change is hypermethylation of tumor-suppressor promoters that result in improper transcription silencing of these genes.[6] The tumor suppressor gene RAS-association domain family member 1 (RASSF1A) encodes a member of the group of RAS effectors that modulates cell proliferation, apoptosis, and microtubule stability. Hypermethylation of RASSF1A was detected in a significant percentage of several primary tumors.[7] Epigenetic silencing of the RASSF1A is assumed to be an early cancer biomarker, but this process is extended from primary to metastatic tumors during tumor progression.[8] The adenomatous polyposis coli (APC) gene, located in chromosome 5q21, plays an essential role in the pathogenesis of colorectal cancer, both in the autosomal dominant inherited familial APC syndrome and in sporadic colorectal cancer.[9] It has been proposed that the impairment of the APC/β-catenin pathway may play a role in BC. Lack of APC expression and upregulation of β-catenin have been identified in human BC and BC cells.[10]

Although BC therapy differs by subtype, there are standard treatments that are currently administered based on subtype. The oncogenic issues and signalling pathways that drive these tumor subtypes are definite, showing that a better comprehension of their molecular basis will render possibilities for predicting response to chemotherapy and implementing novel treatment modalities, to finally improve patient outcomes. Therefore, in this study, we aimed to investigate the relationship between HER2/TOP2A aberrations which in predictive markers in BC and methylation status of RASSF1A/APC genes in high-risk patients with BC.

Materials and Methods

Case Selection

In this study, formalin-fixed paraffin-embedded (FFPE) sections of tissue from 60 high-risk BC patients were obtained in the Department of Pathology, Medical Faculty, Eskisehir Osmangazi University, Eskisehir, Turkey. The inclusion criteria of samples were applied to include the BC patients with (1) tumor size ≥2 cm and/or (2) lymphatic metastases and/or distant metastases and/or (3) patients under 40 years. Clinical parameters, such as tumor grade, histopathological type, the status of estrogen receptor (ER) and progesterone receptor (PR), were obtained from patient's case files. All patients in this series were treated using standard anthracycline-based adjuvant chemotherapy. Informed consent was obtained from the patients whose clinical data could be accessed. The use of FFPE samples for this research was approved by the clinical studies local Ethics Committee (Eskisehir Osmangazi University-Medical Research Ethics 2010/173). This study was conducted in accord with the Helsinki Declaration.

Fluorescencein Situ Hybridization (FISH) Analysis

Fluorescence insitu hybridization (FISH) analysis was performed on 4 μm thick sections of FFPE samples. Commercially available FISH assays of CEP17, HER2 and TOP2A were done according to the manufacturer's protocols (Zytovision, Germany). The kit consisted of a mixture of spectrum green-labeled HER2, spectrum red labeled TOP2A gene and spectrum aqua labeled centromere 17 (CEP17) specific probes. The three-color FISH analysis was performed on the slides of the FFPE tissue samples located in two separate, distinct microscopic areas. The tumors sections, containing at least 85% puretumor cells, were selected during the histopathological analyses of the lesions. In the evaluation of fluorescence spots specific to HER2/TOP2A/CEP17,

absolute and relative numbers (relative to chromosome 17 copy number) of the individual genes were scored in a hundred randomly selected nuclei per tumor using an Olympus bx61 fluorescence microscope (Olympus, Tokyo, Japan) and images were captured using image analysis system (applied imaging, Newcastle, UK). In each FISH experiment, known positive and negative controls were used. In the FISH assessments, HER2/CEP 17, TOP2A/CEP17 ratios were calculated. HER2/CEP17 ratio of ≥2 and TOP2A/CEP17 ratio of ≥1.5 were defined as positive for HER2/TOP2A amplification. TOP2A was considered deleted when TOP2A/CEP17 ratio <0.8.[11]

Methylation-Sensitive High-Resolution Melting (MS-HRM) Analysis

After deparaffinization, genomic DNA was extracted using MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche) according to the manufacturer's instructions. The quantity and purity of isolated DNA were evaluated by NanoDrop 1000 spectrophotometer (Thermo Scientific, DE, USA). Subsequently, genomic DNA modified using sodium bisulfite to deaminate selectively unmethylated cytosine residues to uracil, while 5-methyl cytosine residues were not modified. The bisulphite modification was performed using the "EpiTect® Bisulfite Kit" (Qiagen) according to the manufacturer's recommendations.

To determine the promoter methylation status of RASSF1A and APC genes, we used a real-time polymerase chain reaction (PCR) approach followed by high resolution melting curve analysis (HRM). PCR and HRM analysis were consecutively performed on a LightCycler® 480 (Roche Applied Science, Laval, PQ, Canada). PCR was performed in a 19.5 µl reaction volume, and 10 µl of BSC DNA templates were added to each well which contained 10 µlLightCycler 480 High Resolution Melting (HRM) Master Mix® (Roche), 2.5 µl MgCl2 and 3.0 µl of each primer. The primer sequences were based on the previous report [12] as follows:Methylated RASSF1A; F-5'-GTGTTAACGCGTTGCGTATC-3';R-5'-AACCCCGCGAACTAAAAACGA-3'. RASSF1A unmethylated; F- 5'-TTTGGTTGGAGTGTGTTAATGTG-3'; R- 5'-CAAACCCCACAAACTAAAAACAA-3'APC methylated; F-5'-TATTGCGGAGTGCGGGTC-3'; R-5'-TCGACGAACTCCCGACGA-3'; APC unmethylated; F-5'-GTGTTTTATTGTGGAGTGTGGGTT-3'; R- 5'-CCAATCACAAACTCCCAACAA-3'. The amplification consisted of 10 min at 95°C, followed by 50 cycles of 10 s at 95°C, 15 s at annealing temperature and 25 s at 72 °C.Fluorescence data were collected at 25 acquisitions per second. The LC480-HRM Master Mix® employed a saturating dye (ResolLight™, Roche), which facilitated the precise measurement of the melt curves of the amplicons. The Roche Gene Scanning software was employed for end-product analysis. This algorithm allowed the raw melt curves to be normalized for fluorescence intensity, and a temperature shift was applied to align the normalized melt curves, which facilitated the analysis of samples with varying crossing point values. A difference curve was then derived from the first derivative of the melt curves. Data for the difference melt curves were transmitted to Excel (Office 2010; Microsoft Corp., Redmond, WA, USA). Both peak-height and area-under-the-curve from the normalized, temperature-shifted, difference curves were used to create a standard curve and determine the degree of methylation of each DNA sample.

Statistical Analysis

All statistical analyses were performed using IBM SPSS for Windows version 20.0 (SPSS, Chicago, IL, USA). Comparisons of categorical variables between theclinicopathological parameters, HER2/TOP2A aberrations and RASSF1A/APC methylation status were performed using the Fisher exact test and the Monte Carlo chi-square test. A two-sided p-value <0.05 was considered statistically significant. Overall survival was estimated with the Kaplan-Meier method (Log-rank Test).

Results

A total of 60 cases were included in this study. All cases were female. The median age of patients was 59.23±1.40 years (range 36 to 81 years). There was no statistical association between histopathological type, grade and ER/PR status. The baseline clinicopathological features of the tumor samples are presented in Table 1. The characteristics of tumor samples according to HER2 gene status are summarized in Table 2.

HER2/TOP2 aberrations

HER2 gene amplification was observed in 15/60 samples (25%). All samples with HER2 amplificated had invasive ductal carcinoma. HER2 amplification was more frequent in higher-grade tumors (p=0.001) and PR negativity (p=0.025), and TOP2A aberrations (p=0.004) (Table 3). TOP2A aberration was found in 18/69 (30.0%) (6.6% deletion and 23.4% amplification). Although there was not any statistical difference, the majority of the patients with HER2 and TOP2A aberration were over the age of 45 years.

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Table 1 Baseline characteristics of BC patients

Baseline characteristics	n (%)
Age Mean	59.23 (±1.40)
Histopathological Type	
Invasive ductal carcinoma	49 (81.6)
Invasive lobular carcinoma	5 (8.3)
Other	6 (10)
Grade	
I	6 (10)
II	23 (38.4)
III	13 (21.6)
Unknown	18 (40.0)
ER status	
Negative	25 (41.6)
Positive	35 (58.4)
PR status	
Negative	29 (48.3)
Positive	31 (51.7)
HER2	
Normal	45 (75)
Amplified	15 (25)
TOP2A	
Deleted	4 (6.6)
Normal	42 (70)
Amplified	14 (23.4)
RASSF1A	
Unmethylated	2 (3.4)
Methylated	58 (96.6)
APC	
Unmethylated	34 (56.7)
Methylated	26 (43.3)

When the HER2 gene status was evaluated in 19 patients who were alive and under follow-up, 17 patients with normal gene copy number were found to have a 0.82 probability of survival at the 4th year of life and 2 cases with HER2 gene amplification had 0.50 probability of survival at the 4th year of life. The difference was not statistically significant (Log-rank=0.139, p=0.399). When the TOP2A gene status was assessed in 19 patients who were alive and under follow-up, 15 patients with normal TOP2A gene had a 0.80 probability of survival in the 3rd year and four patients with TOP2A gene amplification had 0.25 probability of survival in the 3rd year. There was no statistically significant difference (Log-rank=0.710 p=0.399).

RASSF1A/APC methylation

RASSF1A and APC promoter methylation were observed in 58/60 samples (96.6%) and 26/60 (43.3%), respectively. The findings showed that there was no significant difference between RASSF1A/APC methylation status and histopathological type, grade and ER/PR status (p>0.05). There was a significant relationship between APC methylation and TOP2A aberration (p=0.026). APC gene methylation was significantly higher in patients with TOP2A aberration (p=0.026) (Table 3).

When RASSF1A gene methylation of 19 patients who are alive and under with follow-up was examined, the findings showed that that 18 tumor samples were methylated, and 1 sample was unmethylated. As APC gene methylation was assessed in 19 patients who were alive and under follow up, samples from 11 patients were unmethylated and samples from 8 patients were methylated. There was not any statistically significant result in life analysis carried out with RASSF1A and APC gene methylation.

Discussion

BC, a heterogeneous disease representing a wide range of pathological entities and clinical behaviors, is an important health problem in all over the world as well as in Turkey.In the present study, we investigated the correlation between HER2/TOP2A gene aberrations and RASSF1A/APC promoter methylation status in tumors with high-risk BC.

Human epidermal growth factor family consists of several receptors with tyrosine kinase activity which has an impact on cell proliferation and survival. The dimerization of HER family members results in the autophosphorylation of tyrosine residues in the cytoplasmic domain and induces cell proliferation and tumorigenesis.[13] While HER family members do not have a natural ligand for signalling, several synthetic ligands have been developed and they are shown to be effective in drug delivery. Of all-family members, HER2 is a crucial molecule and expression of HER2 is increased in several cancer types. HER2 amplification is among the most common genetic alterations in BC.[14] HER2 amplification is an adverse prognostic factor and a predictive biomarker of response to HER2-targeted treatment.[14] Furthermore, HER2 amplification is functionally proposed as a driver of genomic instability and thus may simultaneously cause amplification and activation of other genes.[15] Coamplified genes found in the smallest region of amplification of HER2 amplicon include *MED1, STARD3, GRB7, THRA*, and *RARA*.[16] TOP2A, located in a separate amplicon downstream to HER2 amplicon, is often modified in HER2-amplified tumors.[16] Targeted inhibition of Topoisomerase II alpha enzyme at a molecular level ac-

Table 2 Characteristics of tumor samples according to HER2 gene status

	HER2 gene			
	Normal n (%)	**Amplification n (%)**	**χ^2**	**p**
Histopathological Type				
Invasive ductal	34 (75.6)	15 (100.0)	4.490	0.095
Invasive lobular	5 (11.1)	0 (0.0)		
Other	6 (13.3)	0 (0.0)		
Grade				
Unknown	16 (35.6)	2 (13.3)	18.126	**0.001**
I	6 (13.3)	0 (0.0)		
II	19 (42.2)	4 (26.7)		
III	4 (8.9)	9 (60 .0)		
ER				
ER (–)	16 (35.6)	9 (60)	2.766	0.096
ER (+)	29 (64.4)	6 (60)		
PR				
PR (–)	18 (40)	11 (73.3)	5.006	**0.025**
PR (+)	27 (60)	4 (26.7)		
TOP2A				
Normal	37 (82.3)	5 (33.3)	12.889	**0.004**
Amplification	6 (13.3)	8 (53.4)		
Deletion	2 (4.4)	2 (13.3)		
RASSF1A				
Unmethylated	2 (4)	0 (0)	2.415	0.811
Methylated	43 (96)	15 (100)		
APC				
Unmethylated	19 (42.1)	8 (53.4)	6.373	0.172
Methylated	26 (57.9)	7 (46.6)		

Table 3 TOP2A gene aberrations according to APC gene methylation

	APC gene			
TOP2A	**Methylated n (%)**	**Unmethylated n (%)**	**χ^2**	**p**
Normal	15 (36)	27 (64.0)	21.335	0.026
Amplification	9 (64)	5 (36.0)		
Deletion	2 (50.0)	2 (50.0)		

counts for the cytotoxic effect of the TOP2A inhibitors, such as the anthracycline class.

In the present study, the findings showed that HER2 gene amplification was 25% and TOP2A gene aberrations were 30% (6.6% deletion and 23.4% amplification). Several studies have also reported that TOP2A aberrations are rare in patients with normal HER2.[17] It was reported that TOP2A aberration was present in 50-80% of the patients with HER2 amplification.[18] In the present study, TOP2A aberrations occurred in 17.7% of HER2 non-amplified cases (13.3% deletion and 4.4% amplification), while TOP2A aberration was present in 66.6% (13.3% deletion and 53.3% amplification) of HER2 amplified cases (p=0.004). These results support many previous studies reporting a close relationship between HER2 and TOP2A genes, whereas HER2/TOP2A co-amplification was reported as 35% by Press et al.,[19] as 39% by Bhargava et al.,[20] in the present study, HER2/TOP2A co-amplification was found in 13.3% of the patients. This result may be due to the diversity in methodology and/or established cut-off values. Moreover, although being statistically insignificant, we found that HER2 and TOP2A co-amplification was more common in patients with advanced age.

Epigenetic events are crucial factors in the pathogenesis of human cancers. Aberrant methylation in the promoter regions of tumor suppressor genes is associated with carcinogenesis via transcriptional silencing of gene expression, resulting in the onset and development of cancer.[21] RASSF1A promoter methylation provides significant prognostic information in early-

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stage BC patients.[22] Vu et al. and Spitzwieser et al. reported methylation of RASSF1A in 74.68 %, and 94%of invasive BC.[7,23] In another study, Jezkova et al. found that RASSF1A hypermethylation occurred in 92.2% of the cases.[24] In the present study, we found that RASSF1A methylation was 96.6%. Our result is consistent with the research findings of Spitzwieser et al. and Jezkova et al. The higher ratio of RASSF1A methylation is attributed to that the high-risk patients were included in this study and that MS-HRM is such a sensitive analysis measuring a difference as small as 1/1000.

APC gene inactivation causes dysfunction of β-catenin protein breakdown, and then, induces Tcf/Lef and results in abnormal transcription of oncogenes, including c-myc, c-jun and cyclin D1, eventually leads to carcinogenesis.[25] Methylation in the APC gene has been examined in various types of carcinomas, such as BC, gastric, esophagus, pancreatic, and lung cancer.[26] Although numerous studies have been conducted, the relationship between APC promoter methylation and BC still remains unclear. He et al. reported that the APC promoter methylation was associated with cancer stage, lymph node metastases and ER status in BC.[27]

Jin et al.[28] and Shinozaki et al.[29] reported that APC methylation was associated with BC ($p<0.05$); however, Park et al. and Sturgeon et al. suggested APC methylation had no correlation with BC.[30,31] In a meta-analysis (2483 BC patients and 1218 controls), Zhou et al. demonstrated that the frequency of APC methylation was significantly higher in BC cases than controls under a random effect model.[32] It was found that APC gene promoter methylation was 52.1% in sporadic BC cases, and there was a significant relationship of APC hypermethylation with tumor stage and 3-year survival ($p<0.05$).[33] In the present study, the APC gene methylation was 43.3%. No association was found between RASSF1A/APC methylation status and histopathological type, grade and ER/PR status. However, there was a significant difference between APC gene methylation and TOP2A aberrations. The samples with a normal copy number of TOP2A showed 35.7% APC methylation while samples with TOP2A aberration represented 61.1% APC methylation ($p=0.026$) (Table 3).

Our results did not show a statistically significant relationship between HER2/TOP2A gene aberrations, RASSF1A/APC gene methylation status and survival.

Conclusion

Our results suggested that APC gene promoter hypermethylation was associated with TOP2A gene aberrations. These results suggest that TOP2A aberrations contribute to the epigenetic mechanisms in BC. Our data can provide a new option for individualized treatment. Additionally, in this study, it was confirmed that there was a significant relationship between HER2 amplification and TOP2A gene aberration.

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Conflict of Interest: None declared.

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