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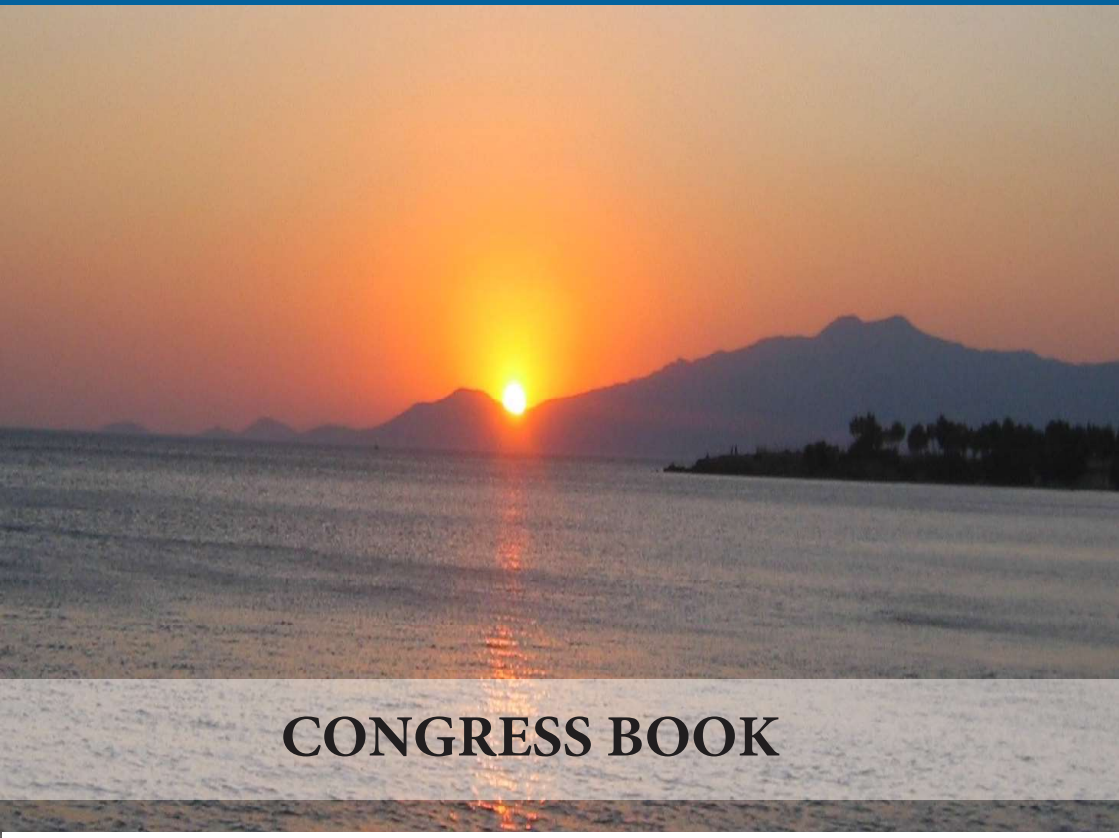

CONGRESS BOOK

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4. Uluslararası Sağlık Bilimleri ve Aile Hekimliği Kongresi
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Relationship Of Her2/Top2a Gene Aberrations With Rassf1a/Apc Gene Methylation Status In Breast Cancer

Abstract

Introduction: Breast cancer (BC) is a heterogeneous and complex disease characterized by the combination of multiple genetic alterations. HER2 gene amplification have prognostic and therapeutic implications in invasive BC. TOP2A is an essential nuclear enzyme that changes DNA topology. RASSF1A /APC genes are putative tumor-suppressor genes that frequently inactivated epigenetically in BC. The purpose 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) sections of tissue from 60 high-risk BC patients were obtained. The inclusion criteria of samples were applied to include the BC patients with (1) tumor size ≥2 cm (2) lymphatic metastases and/or distant metastases (3) patients under 40 years. HER2/TOP2A aberrations were evaluated using Fluorescence In Situ Hybridization (FISH) method. We examined the promoter methylation status of RASSF1A, and APC genes by Methylation-sensitive high resolution melting (MS-HRM) analysis .

Results: HER2 amplification and TOP2A aberration were observed in 15/60 (25%) and 18/60 (30%) cases, respectively. 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 gene methylation and TOP2A aberrations. APC gene methylation was significantly higher 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 relationship of TOP2A gene aberrations with the HER2 gene amplification.

Key words: breast cancer, HER2, TOP2A, methylation.

Introduction

Breast cancer (BC) is the most common cancer type that effects women in world population. Approximately 1.3 million women are diagnosed with breast cancer annually worldwide (1). Similar to other type 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). It is overexpressed and/or amplified approximately in 15-20% of 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).

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 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 breast cancer cells (10).

The oncogenic issues and signaling 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, we aimed to investigate relationship between HER2/TOP2A aberrations which in predictive markers in BC and methylation status of RASSF1A/APC genes in high-risk patients with BC.

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, Eskisehir Osmangazi University, Medical Faculty, Department of Medical Pathology. The inclusion criteria of samples were applied to include the BC patients with (1) tumor size ≥2 cm (2) lymphatic metastases and/or distant metastases (3) patients under 40 years. Use of FFPE samples for this research was approved by the clinical studies local Ethics Committee and the study were done in accord with the Helsinki Declaration.

Flouresence In Situ Hybridization (FISH) Analysis

Fluorescence In Situ Hybridization (FISH) analysis was performed on 4 μm thick sections of FFPE samples. Commercially available FISH assays of CEP17, HER2, TOP2A were done according to the manufacturer's protocols (Zytovision, Germany). 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 deparafinization, 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). Sub-

sequently, 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 manufacturer recommendations. In order 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 µl Light Cycler 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 previous report (12) as follows; Methylated RASSF1A; F- 5'-GTGTTA-ACGCGTTGCGTATC-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 (Resol Light™, 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 the clinicopathological 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 Kaplan-Meier method (Logrank Test).

Results

A total of 60 cases were included in this study. All cases were females. 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. Baseline clinicopathological features of the tumor samples are presented in Table 1.

HER2/TOP2Aaberrations

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). TOP2A aberration was found in 18/60 (30.0%) (6.6% deletion and 23.4% amplification). Although there was no statistically difference, majority of the patients with HER2 and TOP2A aberration were over the age of 45 years. Kaplan Meier Survival analysis and Logrank test were used to assess the survival time in 19 living patients under follow-up. Five-year overall survival (OS) rates were not different between patients according to HER2/ TOP2A gene aberrations.

RASSF1A/APC methylation

RASSF1A and APC promoter methylation were observed in 58/60 samples (96.6%) and 26/60 (43.3%), respectively. It was found 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). Kaplan Meier Survival analysis and Logrank test revealed no statistical difference between five-year OS rates and RASSF1A/APC methylation. The characteristics of tumor samples according to HER2/TOP2A gene status are summarized in Table 2.

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 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 impact on cell proliferation and survival. Dimerization of Her family members results in autophosphorylation of tyrosine residues in the cytoplasmic domain and induces cell proliferation and tumorigenesis (13). 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 can 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 accounts for the cytotoxic effect of the TOP2A inhibitors, such as the anthracycline class.

In present study, it was found 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 a 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 those of many previous studies reporting a close relationship between HER2 and TOP2A genes. Whereas, HER2/TOP2A co-amplification has been 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 an 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 gives significant prognostic information in early stage BC patients (22). Honorio et al. and Pfeifer et al. reported methylation of RASSF1A in 65 % and 60% of invasive BC (23, 24). In another study, it was found that RASSF1A methylation occurred in 95% of the cases (25). In present study, we found that RASSF1A methylation was 96.6%. Our result is consistent with those of Yeo et al. The higher ratio of RASSF1A methylation is attributed to the facts that the high-risk patients were included in the 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 oncogenens, including c-myc, c-jun and cyclin D1, eventually leads to carcinogenesis (26). Methylation in APC gene has been examined in various types of carcinomas, such as colorectal cancer, prostate cancer, hepatocellular carcinoma, and BC (27). Although numerous studies have been conducted, the relationship between APC promoter methylation and BC still remains unclear. Jin et al. (28), and Shinozaki et al. (29) believed that APC methylation was associated with breast cancer ($P < 0.05$), however Park et al. and Sturgeon et al. suggested APC methylation had no correlation with BC (30, 31). In present study, 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 ($p=0.026$).

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. Our data can provide a new option for individualized treatment. Additionally, it was confirmed that there was significant relationship between HER2 amplification and TOP2A gene aberration.

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