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Clinicopathological Significance of the Proliferation Markers Ki67, RacGAP1, and Topoisomerase 2 Alpha in Breast Cancer

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Abstract

Objectives. The aims of this study are to evaluate expressions of Ki67, RacGAP1 (MgcRacGAP) and topoisomerase 2 alpha (TOP2a), the markers related with cell proliferation that have been proposed to affect the prognosis in the literature and correlate the results with clinicopathological parameters of breast cancer patients. *Methods*. Ki67, RacGAP1, and TOP2a antibodies were applied immunohistochemically to the tissue micrarray blocks of 457 female breast cancer patients. The results were correlated with clinical, prognostic, histopathological features, and other immunohistochemical findings (estrogen receptor [ER], progesterone receptor [PR], HER2, cytokeratin [CK]5/6, CK14, epidermal growth factor receptor [EGFR] and vimentin), statistically. *Results*. Ki67 expression demonstrated direct correlation with TOP2a expression, mitotic count, tumor grade, geographic necrosis, basal-like phenotype. RacGAP1 expression was directly correlated with TOP2a expression, nipple invasion, and number of metastatic lymph nodes, and it was inversely correlated with PR expression. TOP2a expression was directly correlated with vimentin and Ki67 expressions, mitotic count, tumor grade, and geographic necrosis, and nipple invasion, and negatively correlated with ER and PR expressions. Higher TOP2a and Ki67 expressions were correlated with shorter overall survival. Higher TOP2a expression and RacGAP1 positivity were directly correlated with shorter disease-free survival. *Conclusion*. This study showed that the overexpressions of Ki67, RacGAP1, and TOP2a affect the prognosis adversely, thus to develop target therapies against RacGAP1 and TOP2a as well as using Ki67 as a part of routine pathology practice might be beneficial in breast cancer therapy and prediction of prognosis.

Keywords

breast carcinoma, immunohistochemistry, Ki67, RacGAP1, topoisomerase 2 alpha

Introduction

Breast cancer is the second most common cause of cancer-related death.[1] Therefore, the studies are being carried out to develop effective treatments targeting some molecules involved in the pathogenesis and the prognosis of breast cancer seriously. In addition, some clinical, genetic, histopathological, and immunohistochemical findings that might be associated with prognosis are being investigated.

One of the well-known major characteristics of malignant tumors is the high mitotic rate.[2,3] Ki67 is a protein expressed by proliferating cells.[2,4] High Ki67 index has been observed to be associated with poor prognosis in breast cancer and it has been suggested to have independent prognostic significance in some studies.[2,5,6]

RacGAP1, is a Rac guanosin triphosphatase (GTPase) activating protein, located at the metaphase of mitotic spindle of the normal cell cycle and is necessary for cytokinesis.[2,7] RacGAP1 upregulation has been demonstrated in some tumors such as hepatocellular carcinoma, ovarian cancer, and bladder cancer.[2,8-10] Also, some studies have suggested that overexpression of RacGAP1 is associated with poor prognosis in breast cancer patients.[2,7,11]

Topoisomerase 2 alpha (TOP2a) is an important nuclear DNA-binding protein and its expression level is highest in growing cells in the G2/M phase.[2,12,13] TOP2a expression

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is used as an indicator of susceptibility to the anthracycline neoadjuvant therapy of breast cancer in some studies.[2,14,15] TOP2a overexpression has been reported to be associated with shorter disease-free survival and overall survival in some studies, however there are controversial results in the literature on this issue.[2,12,14,16]

The goals of this study are to find out the expressions of Ki67, RacGAP1, and TOP2a that are thought to play a role in the breast cancer prognosis and treatment with some conflicting data in the literature by immunohistochemistry, and investigate the association with the prognostic and histopathological features, and other immunohistochemical antibodies (estrogen receptor [ER], progesterone receptor [PR], HER2, cytokeratin [CK]5/6, CK14, epidermal growth factor receptor [EGFR], and vimentin) performed previously for a thesis.

Materials and Methods

After obtaining informed consents and ethic committee approval, 457 cases of breast cancer diagnosed at Department of Pathology, Gazi University School of Medicine between 2006 and 2010 were included. The tissue microarray paraffin blocks containing 4 samples about 0.1 cm in diameter (about filling the objective of 20× of the light microscope, Olympus BX53F, Tokyo, Japan) from each case previously prepared for a thesis were used in the study. The antibodies of Ki67 (7 mL RTU, mouse anti-human monoclonal antibody, clone K2, Leica Biosystems, Danvers, MA, USA), RacGAP1 (1: 500, rabbit polyclonal antibody, Abcam, Cambridge, MA, USA) and TOP2a (1:150, mouse monoclonal antibody, Abcam, USA) were applied to the 4-μm thick sections prepared from tissue microaaray blocks by immunohistochemistry at the Department of Pathology, Bozok University School of Medicine. The immunohistochemical staining was evaluated by a pathologist under a light microscope (Olympus BX53F, Tokyo, Japan). Nuclear staining for TOP2a and Ki67, and cytoplasmic staining for RacGAP1 were considered as positive. The extent of TOP2a and RacGAP1 staining (score 0 [negative], ≤10%; score 1, 11% to 80%; score 2, 81% to 100%) were evaluated. Ki67 expression was evaluated by counting the number of the nuclei showing positivity in the 4 tissue microarray samples each filling the objective of 20× of the light microscope. The number of Ki67-positive nuclei were scored as follows: score 0, negative; score 1, ≤10 nucleus/nuclei; score 2, 11 to 50 nuclei; score 3, 51 to 100 nuclei; score 4, 101 to 200 nuclei; score 5, 201 to 400 nuclei; score 6, 401 to 600 nuclei; score 7, 601 to 1000 nuclei; score 8, >1001 nuclei. The immunostaining results were correlated statistically with the clinicopathological features and the expressions of other immunohistochemical antibodies (ER, PR, HER2, CK5/6, CK14, EGFR, and vimentin) performed previously for a thesis. Membranous staining for EGFR; cytoplasmic staining for vimentin, CK5/6 and CK14 had been considered as positive. The cases that showed no staining had been considered as negative. Nuclear staining more than 1% for ER and PR had been considered as positive. HER2 status had been scored using the system as scores 0 to 3.[1,17] The HER2 status of the cases that had showed score 2 by immunohistochemistry had been evaluated by florescent in situ hibridization.

Statistical Analysis

All data were analyzed using PASW Statistics version 18. The demographic variables were detected using descriptive statistics. The chi-square test, Fisher's exact test, Pearson and Spearman's rho correlation analysis were used for investigating the association between immunoexpressions of antibodies and the clinicopathological parameters. Kaplan-Meier method was used for survival analysis. The effects of associated variables were studied by multiple linear regression analysis using the backward method. $P < .05$ was considered as significant.

Results

All 457 patients included in the study were female. The mean (±SD) age of the patients was 53.3 ± 12.8 years (range 19-86 years). The operation material was mastectomy in 420 patients, and lumpectomy in 37 patients. The tumor size ranged from 0.4 to 20 cm (mean 2.7 ± 1.6 cm). Lymph node metastases were found in 250 of 447 cases performed lymph node dissection. The mean number of metastatic lymph nodes was 2.71 ± 5.09. Nipple involvement was detected in 32 tumors, skin involvement was present in 14, and fascia involvement was found in 38 tumors. There were 371 invasive ductal carcinomas, 22 invasive lobular carcinomas, 11 mixed carcinomas, 11 mucinous carcinomas, 8 metaplastic carcinomas, 5 papillary carcinomas, 5 medullary carcinomas, 1 atypical medullary carcinoma, 5 tubular carcinomas, 4 apocrine carcinomas, 4 micropapillary carcinomas, 4 signet ring cell carcinomas, 3 pleomorphic carcinomas, 2 cribriform carcinomas, and 1 neuroendocrine carcinoma. Geographic necrosis was observed in 66 cases. The mean number of mitosis in 10 high-power fields was calculated as 11.3 ± 9. Disease-free survival and overall survival were evaluated in 254 patients. Disease-free survival ranged from 5 to 84 months (mean 42.58 ± 14.92 months), and overall survival ranged from 13 to 84 months (mean 44.59 ± 14.38 months). Among 254 patients in whom current status was achieved, 239 were dead, and 15 were alive. Pathological tumor stage was pT1 in 186 cases, pT2 in 249 cases, and pT3 in 22 cases. Pathological lymph node stage was pN0 in 197 cases, pN1 in 152 cases, and pN2 in 67 cases. Distant organ metastasis was detected in 31 of 254 patients. According to modified Bloom-Richardson classification,

Table 1. Clinicopathologic Features (n = 457).

Age, years, mean ± SD (range)	53.3 ± 12.8 (19-86)
Gender (female/male)	457/0
Operation materials, n	
Mastectomy	420
Lumpectomy	37
Tumor size, cm, mean ± SD (range)	2.7 ± 1.6 (0.4-20)
Tumor types, n	
Invasive ductal carcinoma	371
Invasive lobular carcinoma	22
Mixed carcinoma	11
Mucinous carcinoma	11
Metaplastic carcinoma	8
Medullary carcinoma	6
Tubular carcinoma	5
Papillary carcinoma	5
Apocrine carcinoma	4
Micropapillary carcinoma	4
Signet ring cell carcinoma	4
Pleomorphic carcinoma	3
Cribriform carcinoma	2
Neuroendocrine carcinoma	1
Nipple involvement, n	
Present	32
Absent	387
Unknown	38
Skin involvement, n	
Present	14
Absent	443
Fascia involvement, n	
Present	38
Absent	392
Unknown	27
Metastatic lymph nodes, n	
Present	250
Absent	197
Unknown	10
Distant metastasis, n	
Present	31
Absent	223
Unknown	203
Geographic necrosis, n	
Present	66
Absent	391
The number of mitosis/10 high-power fields, mean ± SD (range)	11.36 ± 9.04 (1-60)
Tumor grade, n	
Grade 1	130
Grade 2	166
Grade 3	161
Overall survival, months, mean ± SD (range)	44.59 ± 14.38 (13-84)
Disease-free survival, months, mean ± SD (range)	42.58 ± 14.92 (5-84)
The current status of patients, n	
Alive	239
Dead	15
Unknown	203

130 patients had grade 1, 166 had grade 2, 161 had grade 3 tumors.[18] Clinicopathological features of the patients are presented in Table 1. The tumor was accompanied by carcinoma in situ in 347 cases. ER-positivity was present in 355 cases, PR-positivity was present in 339 cases, HER2 positivity was present in 159 cases. There were 248 cases of luminal A (ER+, PR+, HER2−), 125 cases of luminal B (ER+, PR+, HER2+), 34 cases of HER2+ (ER−, PR−, HER2+), 50 cases of basal-like (ER−, PR−, HER2−, CK5/6+, and/or EGFR+) phenotype according to St Gallen International Breast Cancer Conference.[19] Sixty-six patients were positive for EGFR, 235 were positive for CK 5/6, 43 cases were positive for CK14, and 67 case were positive for vimentin.

Eight cases showed score 0, 85 cases showed score 1, and 348 cases showed score 2 for RacGAP1. Seventy-four cases exhibited score 0, 211 cases exhibited score 1, and 148 cases exhibited score 2 for TOP2a. There were 233 cases of score 0, 58 cases of score 1, 47 cases of score 2, 32 cases of score 3, 30 cases of score 4, 19 cases of score 5, 9 cases of score 6, 9 cases of score 7, and 1 case of score 8 for Ki67.

The data about the immunohistochemical, clinicopathological and prognostic parameters that showed statistically significant correlation according to univariate analysis are given in Table 2. TOP2a (Figure 1A and B) expression showed direct correlation with RacGAP1 (Figure 1C and D) (P = .000), vimentin (P = .028) and increased Ki67 (Figure 1E and F) proliferation index (P = .007), number of mitosis (P = .007), high tumor grade (P = .031), geographic necrosis (P = .013), and nipple involvement (P = .042); however, there were inverse correlation with ER (P = .002) and PR (P = .029) positivity. It was detected that when TOP2a expression increased, disease-free survival (P = .018) and overall survival (P = .027) tended to decrease statistically. There were direct correlation between RacGAP1 expression and TOP2a expression (P = .000), nipple involvement (P = .047), and the number of metastatic lymph nodes (P = .013), but there was inverse correlation between PR expression (P = .038). It was detected that RacGAP1 negative (≤10% expression) cases tended to have longer disease-free survival; however, shorter disease-free survival was detected in RacGAP1-positive (>10% expression) cases (P = .000). Ki67 expression showed direct correlation with TOP2a expression (P = .007), number of mitosis (P = .000), tumor grade (P = .000), and geographic necrosis (P = .012). In addition, higher Ki67 expression was observed in the tumors of basal-like phenotype (P = .021). The overall survival of the patients were found to be shorter in the cases that showed higher Ki67 proliferation index and distant metastasis (P = .000 and P = .016, respectively). There were direct correlation between disease-free survival and nipple involvement (P = .026), distant metastases (P = .000), and tumor size (P = .000).

According to multiple linear regression analysis, overall survival was found to be inversely correlated with

Table 2. Statistically Significant Associations Between Immunohistochemical and Clinicopathologic Characteristics According to Univariate Analysis ($P < .05$).

	TOP2a	RacGAP1	Ki67	Overall Survival	Disease-Free Survival
TOP2a	–	+ ($P = .000$, dir)	+ ($P = .007$, dir)	+ ($P = .027$, inv)	+ ($P = .018$, inv)
RacGAP1	+ ($P = .000$, dir)	–	–	–	+ ($P = .000$, inv)
Ki67	+ ($P = .007$, dir)	–	–	+ ($P = .000$, inv)	–
ER	+ ($P = .002$, inv)	–	–	–	–
PR	+ ($P = .029$, inv)	+ ($P = .038$, inv)	–	–	–
Vimentin	+ ($P = .028$, dir)	–	–	–	–
Mitotic count	+ ($P = .007$, dir)	–	+ ($P = .000$, dir)	–	–
Tumor size	–	–	–	–	+ ($P = .000$, dir)
Tumor grade	+ ($P = .031$, dir)	–	+ ($P = .000$, dir)	–	–
Geographic necrosis	+ ($P = .013$, dir)	–	+ ($P = .012$, dir)	–	–
Nipple involvement	+ ($P = .042$, dir)	+ ($P = .047$, dir)	–	–	+ ($P = .026$, dir)
Distant metastasis	–	–	–	+ ($P = .016$, inv)	+ ($P = .000$, dir)
Basal-like phenotype	–	–	+ ($P = .021$, dir)		–
Number of metastatic lymph node	–	+ ($P = .013$, dir)	–	–	–
Disease-free survival	+ ($P = .018$, inv)	+ ($P = .000$, inv)	–	–	–
Overall survival	+ ($P = .027$, inv)	–	+ ($P = .000$, inv)	–	–

Abbreviations: dir, directly correlated; inv, inversely correlated; TOP2a, topoisomerase 2 alpha; ER, estrogen receptor; PR, progesterone receptor.

higher TOP2a expression ($P = .000$, $R^2 = 0.14$, $\beta = 0.19$). Disease-free survival was detected to be inversely correlated with higher TOP2a expression ($P = .000$, $R^2 = 0.29$, $\beta = 0.29$) and presence of distant metastasis ($P = .000$, $R^2 = 0.29$, $\beta = 0.40$). The other associated parameters in univariate analysis did not show any significant correlation by multiple linear regression analysis.

Discussion

High proliferation rate is one of the major characteristics of malignancy, however prognostic gene signatures about proliferation are not the same in different types of cancer.[2,20] The cell proliferation in breast cancer has been suggested to vary according to the molecular subtypes of the tumor.[2] In our study, the expressions of three different proliferation markers as Ki67, RacGAP1, and TOP2a in breast cancer patients were evaluated by immunohistochemical methods and compared with the clinicopathological parameters associated with prognosis.

Ki67 is a nuclear nonhistone protein first defined in 1991.[2,4] It is strongly expressed in all phases of the cell cycle of proliferating cells, but not present in nondividing cells.[2] In the literature, some studies have reported that high Ki67 expression is related with poorer prognosis in breast cancer; however, most of other studies have stated that it is not an independent prognostic factor.[2,5,6] Also, some studies have suggested that high Ki67 expression is associated with chemotherapy response.[2,21] Many studies have proposed that the proliferation markers are important prognostic factors related with disease-free survival and overall survival, particularly for ER+ tumors.[2] Nevertheless, the assess of proliferation markers has not been widely used yet in routine practice.[2] Using time consuming methods, the standardization challenges and problems, the difficulties in reproducibility of the results of those studies are among the reasons for inability to use them routinely.[2,22] The St Gallen guidelines published in 2011 have suggested a cutoff value of 14% for Ki67 proliferation index immunohistochemically for the molecular subtypes of luminal A and luminal B of breast cancer.[2,19] Romero et al[23,24] have proposed a 20% cutoff value for Ki67 expression.[25] However, it is stated that those cutoff values for Ki67 immunohistohemistry are not reliable for routine clinical use due to the intra- and interobserver variability.[2] In our study, Ki67 expression was assessed by counting the number of stained nuclei using tissue microarray method that was thought to be a more objective method rather than the other studies that counted the percentage of staining. In the present study, high Ki67 expression was found in the tumors exhibiting high mitotic count, geographic necrosis, and high histological grade indicating poorer prognosis. Similarly, high Ki67 expression was observed in the tumors with basal-like phenotype implying poorer prognosis. In addition, the tumors with high expression of Ki67 were demonstrated to have statistically significant shorter overall survival and higher TOP2a expression.

TOP2a shows high expression in growing cells in the G2/M phase and overexpression of TOP2a has been reported to be associated with shorter disease-free survival and overall survival in breast cancer in some studies.[2] TOP2a is located on the same chromosome as HER2-neu and often amplified with this receptor.[2] TOP2a is suggested as a target receptor for anthracycline.

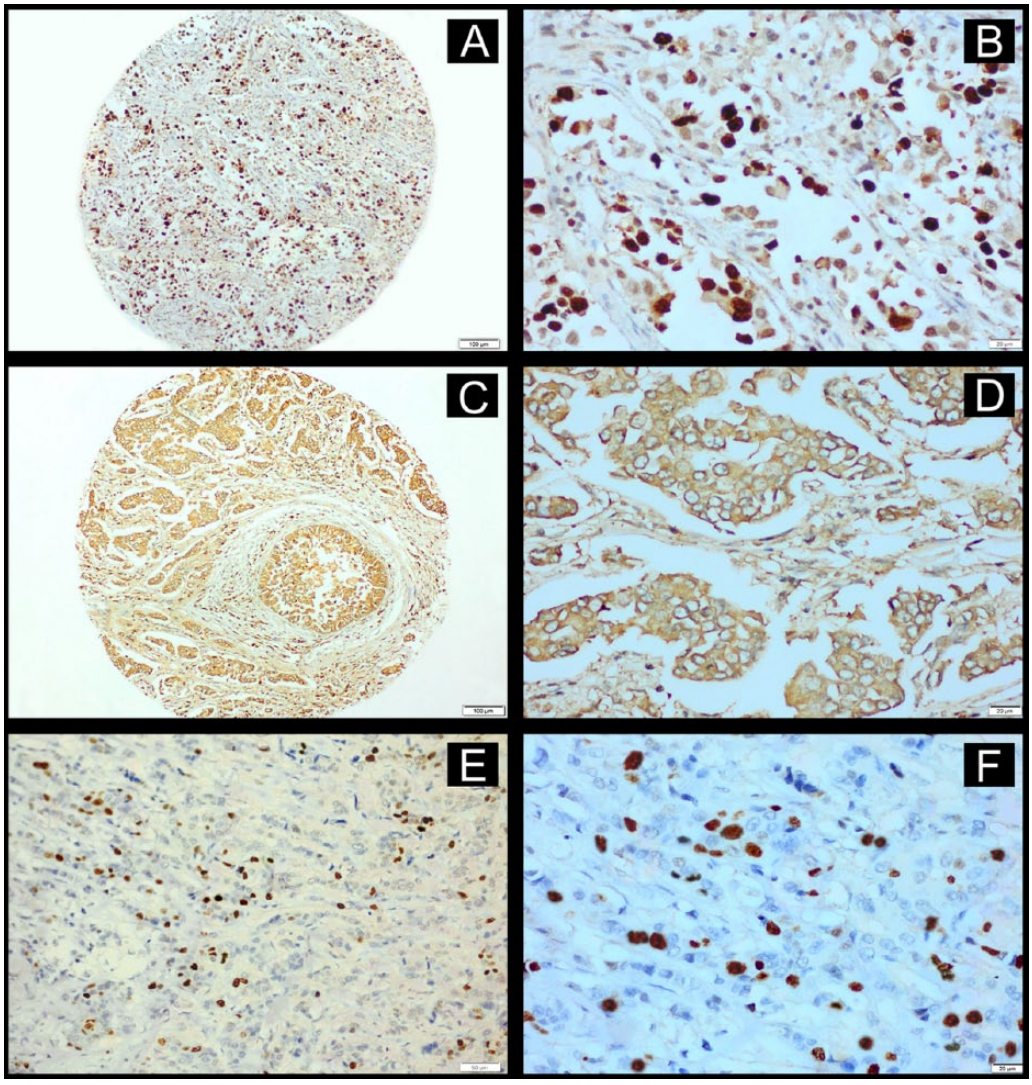

Figure 1. Photomicrographs of immunostaining of breast cancer samples. (A, B) TOP2a positivity (avidin-biotin-peroxidase method, 100×, 400×, respectively). (C, D) RacGAP1 positivity (avidin-biotin-peroxidase method, 100×, 400×, respectively). (E, F) Ki67 positivity (avidin-biotin-peroxidase method, 200×, 400×, respectively).

Therefore, amplification of TOP2a is considered to be an indicator of sensitivity to anthracycline therapy.[2] In the literature, high TOP2a RNA levels have been reported to be related with more short-term metastasis-free survival in breast cancer patients without lymph node metastasis.[2,26] Besides, patients treated with anthracycline have been detected to show high rate of pathological complete response.[2,26,27] Chen et al[16] have investigated the predictive and prognostic value of TOP2a expression in the primary tumors as well as the residual tumors sampled after the treatment of the patients that had locally advanced breast cancer and recieved neoadjuvant chemotherapy with anthracycline. In the literature, most of the studies have advocated that TOP2a expression is a predictive parameter of longer disease-free survival and overall survival of early breast cancer patients received anthracycline therapy; however, there are some other studies with contradictory results that have stated no association on that issue.[2,12-14,16]

The tumors of the patients who did not receive neoadjuvant therapy were evaluated in our study and high expression of TOP2a was detected in the high-grade tumors with high mitotic count, geographic necrosis, nipple involvement, indicating poor prognosis. Similarly, high TOP2a expressions were found in the tumors showing ER negativity, PR negativity, and vimentin positivity[28-32] implying

poorer prognosis in the present study. In addition, high expression of the TOP2a was directly correlated with high RacGAP1 and Ki67 expression, and shorter disease-free survival and overall survival in our study, as previously mentioned.

RacGAP1 is a protein that participates in mitosis.[2,33] It is phosphorylated by Aurora B that is necessary for RhoA GAP.[2,34] Additionally, it plays a role as a nuclear chaperone and participates in nuclear transportation for the STAT transcription factors.[2,35] Milde-Langosch et al[2] have proposed RacGAP1 as a marker indicating poor prognosis in ER+ (luminal) breast tumors in contrast to HER2+ and triple negative tumors. They have demonstrated that the prognostic value of RacGAP1 in luminal tumor is independent from histological grade, clinical stage, and lymph node involvement.[2] RacGAP1 expression was demonstrated to be correlated with both TOP2a and Ki67 expressions; however, the prognostic impact of their expressions were detected to vary between different subtypes of breast cancer.[2] Pliarchopoulou et al[7] investigated the prognostic importance of RacGAP1 mRNA expression on the overall and disease-free survival in high-risk early breast cancer patients and compared with Ki67 expression. The cases with high RacGAP1 expression were found to show higher histological grade and higher Ki67 expression.[7] High RacGAP1 mRNA expression was observed to be related with both shorter disease-free survival and overall survival, also suggested to be an independent prognostic factor.[7] Similar to that study, RacGAP1 positive cases were found to show shorter disease-free survival statistically significantly in our study. Additionally, as favouring poor prognosis, the tumors with nipple and lymph node involvement, PR expression, and high TOP2a expression[16,30-32] were demonstrated to exhibit higher RacGAP1 expression in the present study.

Similar to our study, Milde-Langosch et al[2] studied Ki67, TOP2a, and RacGAP1 expression in different molecular subtypes of breast cancer. Only Ki67 was suggested to be a statistically significant independent prognostic marker for triple negative tumors; however, none of these markers was found to have prognostic significance in HER2+ patients.[2] They advocated that mRNA analysis of RacGAP1 was superior to Ki67 and TOP2a to predict the prognosis of luminal tumors.[2] The effect of proliferation markers was observed to vary in luminal tumors depending on the method of therapy used.[2] Ki67, TOP2a, and RacGAP1 have been demonstrated to be statistically significant independent prognostic markers among untreated patients.[2] In addition, overexpressions of these 3 markers were determined to be predictive for early recurrence for the patients treated with chemotherapy.[2] RacGAP1 was advocated to be the only predictive proliferation marker in the patients treated with endocrine therapy.[2] In our study, significant prognostic significance was detected only for high Ki67 expression in basal-like tumors, there was no relationship between the other molecular subgroups and the expressions of Ki67, RacGAP1, and TOP2a.

In conclusion, our study has demonstrated that overexpressions of Ki67, RacGAP1, and TOP2a have been associated with poorer prognosis in breast cancer, also the effect of TOP2a was supported by multivariate analysis. Thus, performing RacGAP1, TOP2a, and particularly Ki67 by immunohistochemistry in routine practice of pathology while diagnosing breast cancer is strongly advised in order to determine the therapy and predict the prognosis. It is also claimed that comprehensive studies conducted with multivariate analysis are crucial to clarifying the effect of those markers on pathogenesis and prognosis of breast cancer.

Declaration of Conflicting Interests

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