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AMPLIFICATION OF CYCLINE D1, *C-MYC* AND *EGFR* ONCOGENES IN TUMOUR SAMPLES OF BREAST CANCER PATIENTS

AMPLIFIKACIJA CIKLIN D1, C-MYC AND *EGFR* ONKOGENA U TUMORSKIM UZORCIMA PACIJENTKINJA OBOLELIH OD KANCERA DOJKE

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Summary

Background: Breast cancer is the most common form of cancer in women. It arises from multiple genetic changes in oncogenes and tumor suppressor genes. Among so far studied oncogenes relatively few, including epdermal growth factor receptor (*EGFR*), cyclinD1 (*CCND1*)and *c-myc*, have been found to play an important role in progression of this type of human malignancy. The aim of this study was to examine the prognostic potential of *CCND1*, *c-myc* and *EGFR* amplification and their possible cooperation in breast carcinogenesis.
Methods: Copy number analyses of *CCND1* and *c-myc* genes were done by TaqMan based quantitative real time PCR. Amplification status of *EGFR* was determined by differential PCR.

Kratak sadržaj

Uvod: Kancer dojke je najčešći tip maligniteta koji se javljaja kod žena. Tumori dojke nastaju kao rezultat akumulacije genetičkih promena kako u onkogenima tako i u tumor supresorskim genima. Među mnogim onkogenima čija je uloga u genezi tumora dojke ispitivana do danas, samo se neki smatraju značajnim za razviće ovih karcinoma. U tu se grupu svakako ubrajaju receptor za epidermalni factor rasta (*EGFR*), *c-myc* i ciklinD1 (*CCND1*). Cilj rada je bio utvrditi prognostički značaj amplifikacije *CCND1*, *c-myc* i *EGFR* onkogena u razvicu tumora dojke kao i eventualne međusobne koalteracije ovih gena.
Metode: Amplifikacioni status *CCND1* i *c-myc* gena određen je kvantitativnim PCR-om u realnom vremenu, a amplifikacioni status *EGFR* onkogena je definisan diferencijalnim PCR-om.

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List of Abbrevations: EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; QUART, quadrantectomy; SMIR, subcutaneous mastectomy; PCR, polymerase chain reaction; HT, hormone therapy; RT, radiotherapy; CHT, chemiotherapy.

Results: Amplification of *CCND1*, *c-myc* and *EGFR* oncogene has been found in 20.4%, 26.5% and 26.5% of breast cancer cases, respectively. Analysis showed that amplification of *CCND1* oncogene was significantly associated with the stage II of disease while amplification of *EGFR* gene was significantly associated with overexpression of *HER-2/neu*. Tumour stage and expression of *HER-2/neu* appeared to be significant predictors of patient's outcome. Stage I patients lived significantly longer then stage III patients (p=0.04) while patients with *HER-2/neu* overexpression had worse prognoses and lived significantly shorter (p=0.001). Finally, survival of patients who underwent hormone therapy only was significantly longer (p=0.001) then survival of the rest of patients.
Conclusions: Amplification of *CCND1* or *EGFR* oncogene is associated with the progression of breast cancer and bad prognosis. No co-ordination in amplification of *CCND1*, *c-myc* and *EGFR* oncogenes were established in this cohort of breast cancer patients.

Keywords: breast cancer; oncogenes; cycline D1, *c-myc*, *EGFR*

Rezultati: Amplifikacija *CCND1* gena detektovana je kod 20.4%, a *c-myc* i *EGFR* onkogena kod 26.5% ispitanih uzoraka. Analize su pokazale da je amplifikacija *CCND1* onkogena statistički značajno povezana sa stadijumom II tumora dojke kao i da amplifikacija *EGFR*-a značajno korelira sa povećanom ekspresijom *HER2/neu*. Analize kliničkih i histopatoloških parametara su jasno pokazale da stadijum tumora i nivo ekspresije *HER2/neu* gena predstavljaju značajne pokazatelje daljeg toka bolesti, odnosno sudbine pacijenta. Utvrđeno je da pacijentkinje sa tumorima dojke stadijuma I žive značajno duže od onih sa tumorom stadijuma III (p= 0.04) kao i da pacijentkinje sa *HER2/neu* pozitivnim statusom imaju goru prognozu i žive značajno kraće (p=0.001). Na kraju, studija je pokazala da pacijentkinje podvrgnute samo hormonskoj terapiji imaju najbolju prognozu i žive značajno duže od ostalih (p=0.001).
Zaključak: Amplifikacija *CCND1* i *EGFR* onkogena je povezana sa lošom prognozom i progresijom karcinoma dojke. U ispitivanom tumorskom uzorku nisu detektovane nikakve koalteracije *CCND1*, *c-myc* i *EGFR* onkogena.

Ključne reči: tumori dojke, onkogeni, ciklin D1, *c-myc*, *EGFR*

Introduction

Breast cancer is the most common form of cancer in women. It comprises 22% of all cancers (1) and is second only to lung cancer as a cause of cancer related death in women (2). It is a heterogeneous disease arising from multiple genetic changes in oncogenes and tumour suppressor genes with pivotal roles in the control of cell proliferation, differentiation and death. Alterations of these genes lead to clonal expansion with subsequent acquisition of invasive and metastatic phenotypes.

Numerous oncogenes have been characterized in human cancers, but relatively few have been found to play an important role in promotion and progression of breast cancer. Among them are epidermal growth factor receptor (*EGFR*), cyclin D1 and *c-myc*.

EGFR (also known as HER1) is a member of the human epidermal growth factor receptor (HER) family of transmembrane receptor tyrosine kinases that is linked to growth control, cell adhesion, mobility and apoptosis (3). Its role in breast tumors is complicated by the fact that its function may vary according to important clinical features like estrogen receptor (ER) and HER2 status (4). Namely, high expression of *EGFR* has been reported to be associated with low expression of ER (5).

Cyclin D1 is the product of the *CCND1* gene and plays the central role in the regulation of progression from the G1 to the S phase of the cell cycle through the formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6 (6). Consequently, deregulation of cyclin D1 gene expression or function contributes to loss of normal cell cycle control during carcinogenesis. Strong evidence implicates cyclin D1 amplification and overexpression as a driving force in human breast cancer (7).

c-myc protein is a transcription factor which participates in most aspects of cellular function, including replication, growth, metabolism, differentiation, and apoptosis (8). Most, if not all, types of human malignancy have been reported to have amplification and/or overexpression of *c-myc* oncogene.

Thus, amplification and overexpression of these oncogenes and oncogene products are the major mechanisms through which these genes participate in carcinogenesis. A drawback of many studies of oncogenes in human breast cancer is that usually only one oncogene was evaluated. Based on a series of unselected cases, in the present study we aimed to examine the possible prognostic potential of the amplification of *CCND1*, *c-myc* and *EGFR* oncogenes. Moreover, we aimed to determine whether these oncogenes cooperate in breast carcinogenesis. Furthermore we studied whether adjuvant therapies such as chemotherapy and endocrine treatment or no treatment at all had any impact on survival among oncogene amplified breast cancer patients.

Material and Methods

Patients

This prospective study comprised of 49 primary breast cancer tissue samples. 21 patients underwent modified radical mastectomy, 19 underwent quadrantectomy (QUART) and 9 underwent subcutaneous mastectomy (SMIR) at the Institute of Oncology and Radiology of Serbia. All relevant clinical parameters (age, tumor size, lymphonodal status, disease free

survival, overall survival) were retrieved from patients' medical records.

Collected tumor specimens and corresponding normal tissue were formalin-fixed, paraffin-embedded and hematoxylin-eosin (HE) stained. Histological type and grade of each carcinoma sample were determined after hematoxylin-eosin staining. The carcinomas were graded (I – III) according to Scarff-Bloom-Richardson scoring system (9).

For each obtained tumor sample written consent and approval were acquired according to the ethical standards laid down in the1964 Declaration of Helsinki, the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), Geneva 1993, and the Guidelines for Good Clinical Practice CPMP/ICH/135/95), September 1997.

Immunohistochemistry

Labelled streptavidin-biotin-LSAB+ method together with immunoperoxidase was used according to recommended procedure for commercial primary monoclonal mouse antibody: Anti-Human ERα clone (1:50; Clone 1D5; Dako) and Anti-Human PR clone (1:50; Clone PgR 636; Dako), as well as for policlonal rabbit antibody Anti-Human c-erbB2/HER2 Oncoprotein (1:300; Dako) with Dako LSAB^{TM}+/ HRP kit (K0679). Slices were contrasted with Mayer hematoxylin.

The evaluation of steroid receptros (ER, PR) was based on the scoring system which included percentage of stained malignant nuclei (0-5) and their intensity of staining (0–3); positive (high expression) cases were with score ≥4 while negative (low expression) cases were with score <4 (10). HER2 status was determined using DAKO scoring system and HER2 positive status was defined if IHC score was 2+/3+ (11).

Copy number analysis by quantitative real time PCR

Genomic DNA was extracted from 49 fresh frozen tumor and corresponding normal tissue samples according to the standard phenol/chloroform extraction procedure described by Sambrook and colleagues (12). The quality of the extracted DNA was verified by agarose gel electrophoresis and the concentrations were assessed spectrophotometrically. Isolated DNA was stored at +4 °C until further analyses.

Copy number analyses of *CCND1* and *c-myc* genes were done by quantitative real time PCR using TaqMan based assays. Assays included forward and reverse primers for *c-myc* and *CCND1* oncogenes as well as highly specific 6-Fam-TAMRA labeled probes for them. Primers and probe for *CCND1* gene were as follows: F 5'-GGACAACGGGCGGATAGAG-3'; R 5'-CACAGTCATCCCAGGGTTTAACA-3'; Probe 6-FAM-5'-CAGCCTTGTTGTTTACGGCCTCTTTGAG-3'-TAMRA. For the analysis of *c-myc* gene, the following primers and probe were used: F 5'-GGACGACGAGACCTTCATCAA-3'; R 5'-CCAGCTTCTCTGAGACGAGCTT-3'; TaqMan Probe 6-FAM-5'-AGAAGCCGCTCCACATACAGTCCTGG-3'-TAMRA.

RNase-P was used as the internal control, reference gene (accession # 4316831, Applied Biosystems).

Each sample was prepared in duplicate, in total reaction volume of 20 μL, with primers /probe ratio 3:1 (0.1 μmol/L probe : 0.3 μmol/L primers), 1x TaqMan Master Mix and 150ng of tested DNA. Each reaction contained normal DNA controls. Control samples were used as calibrators. PCR reactions were carried out in the ABI Prism 7500 Sequence Detection System at 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycles 3 to 15 plus 10 standard deviations. A mean value of each Ct duplicate was used for further calculation. Each run included a no-template control, as well. The obtained results were analyzed by RQ Study Add ON software for 7500 v 1.3 SDS instrument with a confidence level of 95% ($p<0.05$).

Differential PCR

Amplification status of *EGFR* oncogene was determined by differential PCR (D-PCR) that engaged two pairs of primers, one for the target gene (*EGFR*) and the other for the reference gene (*β-actin*). The primer sequences were as follows: *EGFR*_F 5'-AGCCATGCCCGCATTAGC TC-3' and *EGFR*_R 5'-AACCCTTCAACGTAAGGAAA-3' for *EGFR*, and *ACTB*_F 5'-CTCTTTTCTTTCCCGATAGGT-3' and *ACTB*_R 5'-CTCCAGCTTCTCGTAGGGTC-3' for the *ACTB*. D-PCR was performed in the total reaction volume of 25 μL with 150 ng of DNA, 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris–HCl, pH 8.3, 0.01% gelatin), 1.5 mmol/L MgCl, 0.2 mmol/L each dNTP, 1 μmol/L each of four primers, 1U TaqPolymerase. Thermal cycling included thirty repeats of denaturation at 95 °C/1 minute, annealing at 58 °C/1 minute and extension at 72 °C/1 minute, with initial denaturation (95 °C/10 minutes) before and final extension (72 °C/10 minutes) after the repeating temperature steps. Generated PCR products were applied to 9% polyacrylamide gel for electrophoresis, stained with silver-nitrate, photographed and analyzed by ImageQuant 5.2 by comparing the median pixel intensity in a given/selected area of two bands (*EGFR* and *ACTB*) in the same lane (sample). When median pixel intensity of *EGFR* band was equal or higher then 25% of median pixel intensity of *ACTB*, it was interpreted as gene amplification.

Statistical analysis

Significant differences between the data sets were determined by STATISTICA 6.0 software (StatSoft, Inc., Tulsa, USA). The correlations between clinicopathological parameters and amplification of *c-myc*, *CCND1* and *EGFR* genes were evaluated using Fisher exact test. Survival analyses were performed using Kaplan & Meier product-limit method. The log rank test was used to asses the significance of the difference between pairs of survival probabilities. Overall survival was calculated from the day after surgery to the last follow-up examination or death of the patient. Statistical differences were considered significant when p was < 0.05 (*).

Results

Patient cohort and treatment

We examined breast cancer specimens from 38 postmenopausal and 11 premenopausal women for the amplification status of *c-myc*, *CCND1* and *EGFR* oncogenes. In total of 49 patients, 27 patients had breast carcinomas with histology of invasive ductal carcinoma, while 22 were invasive lobular carcinomas. Patients' characteristics are summarized in *Table I*. Among clinical and histopathological characteristics, stage and HER-2/neu expression were significant predictors of patient's outcome (*Figures 1A* and *1B*). Namely, stage I patients lived significantly longer then stage III patients (p=0.04) while patients with HER-2/neu overexpression had worse prognoses and lived significantly shorter (p=0.001). Most of the samples were steroid receptor (ER and/or PR) positive (96%) but, nevertheless, patients were on different regimens of treatment: 5 of them were on hormone therapy (HT), 8 on combined hormone and radiotherapy (HT+RT), 4 on combined hormone and chemiotherapy (HT+CHT), 17 on combined hormone, chemio- and radiotherapy (HT+CHT+RT) and 15 on other therapeutic protocols (CHT only, RT only, combined CHT and RT). Kaplan-Meier survival curves were generated to evaluate the effects of these treatment regimens on survival. The survival of patients who underwent hormone therapy only was significantly longer (p=0.001) then survival of the rest of patients (*Figure 1C*).

Table I Patients' characteristics.

Age in years (mean)	32 – 82 (61)
Follow-up in months (mean)	36 – 110 (31)
Estrogen receptor status	
positive	47
negative	2
HER-2/neu status	
positive	19
negative	30
Stage*	
I	12
II	20
III	12
Grade	
g 1	0
g 2	44
g 3	5
Lymph node metastasis	
Yes	27
No	22
TOTAL	49

* – unavailable data for 5 patients

Amplification of c-myc, CCND1 and EGFR oncogenes

We determined amplification status of *CCND1* and *c-myc* oncogenes by Quantitative Real Time PCR. Our results revealed that 10 out of 49 samples (20.4%) possessed three to 14-fold amplification of *CCND1* oncogene. *c-myc* gene was amplified three to 10-folds in 13/49 breast cancer samples (26.5%). Further analysis by Fisher exact test showed that amplification of *CCND1* oncogene was significantly associated with the stage II of breast cancer patients (*Table II*). On the contrary, amplification of *c-myc* gene did not show correlation with tumour stage (T*able II*) or any other clinical or pathohistological characteristics of patients (data not shown) including breast cancer subtype, ER or HER2/neu status.

Amplification of *EGFR* oncogene was assessed by differential PCR and we found that it was amplified in 13 patients (26.5%). Interestingly, amplification of *EGFR* gene was significantly associated with overexpression of HER-2/neu (*Table II*). In other words, *EGFR* expressing tumours were more likely to overexpress HER-2/neu. Association with any other clinical or pathohistological characteristic was not obtained.

In order to reveal possible association among three studied oncogenes, we further analyzed whether there were co-alterations between any of them. Our results showed that there was no co-operation among *CCND1*, *c-myc* and *EGFR* gene alterations. Namely, *CCND1* and *c-myc* were co-amplified in four samples (8%), as were *CCND1* and *EGFR* while *c-myc* and *EGFR* were co-altered in only two samples (4%). All three of them were amplified in just one breast cancer sample.

Further, we analyzed possible association between each oncogene alteration and survival and did not find anything of significance (*Figure 2*). Finally, neither of them had any significant influence on response to any applied therapeutic protocol.

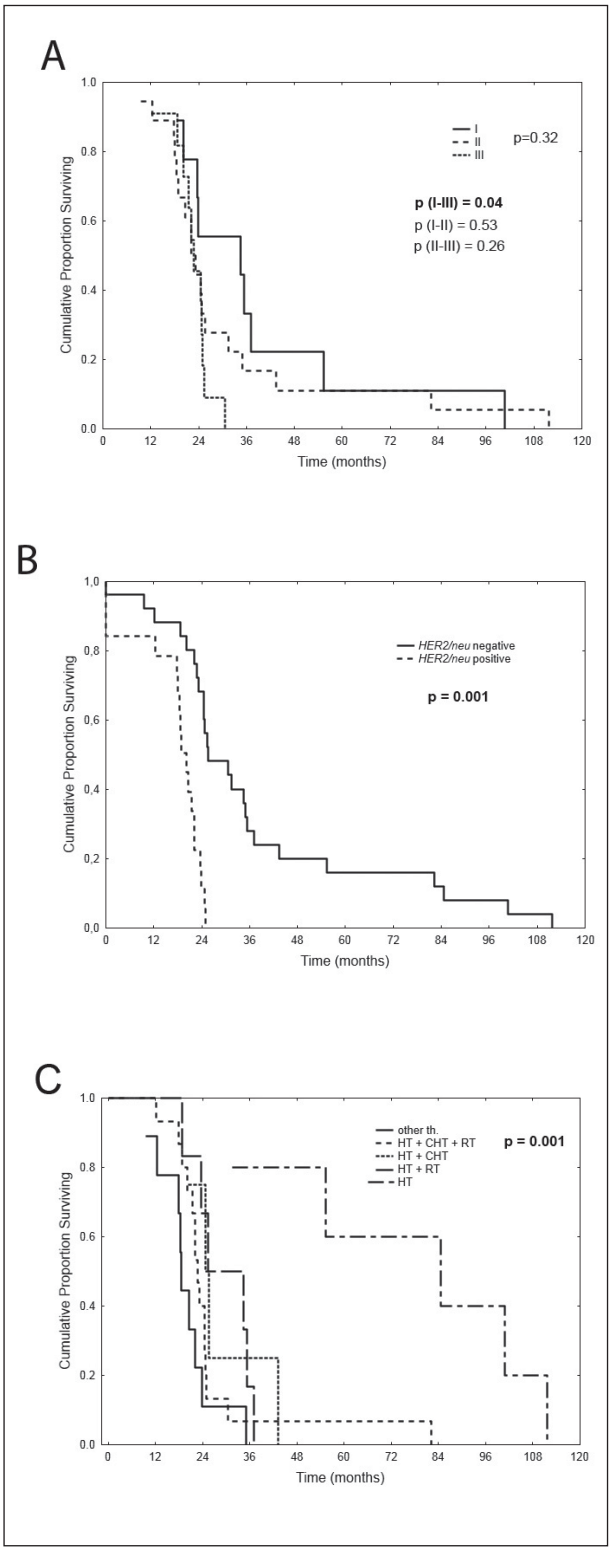

Figure 1 Kaplan–Meier survival curves. (A) Impact of tumor stage on patients' survival. Patients with stage I tumors lived significantly longer compared to stage III (p=0.04); (B) Impact of HER2/neu on patients' survival. Patients with positive status of HER2/neu receptor lived significantly shorter (p=0.001); (C) Impact of therapy on patients' survival. Patients receiving hormone therapy lived significantly longer compared to all other groups (p=0.001).

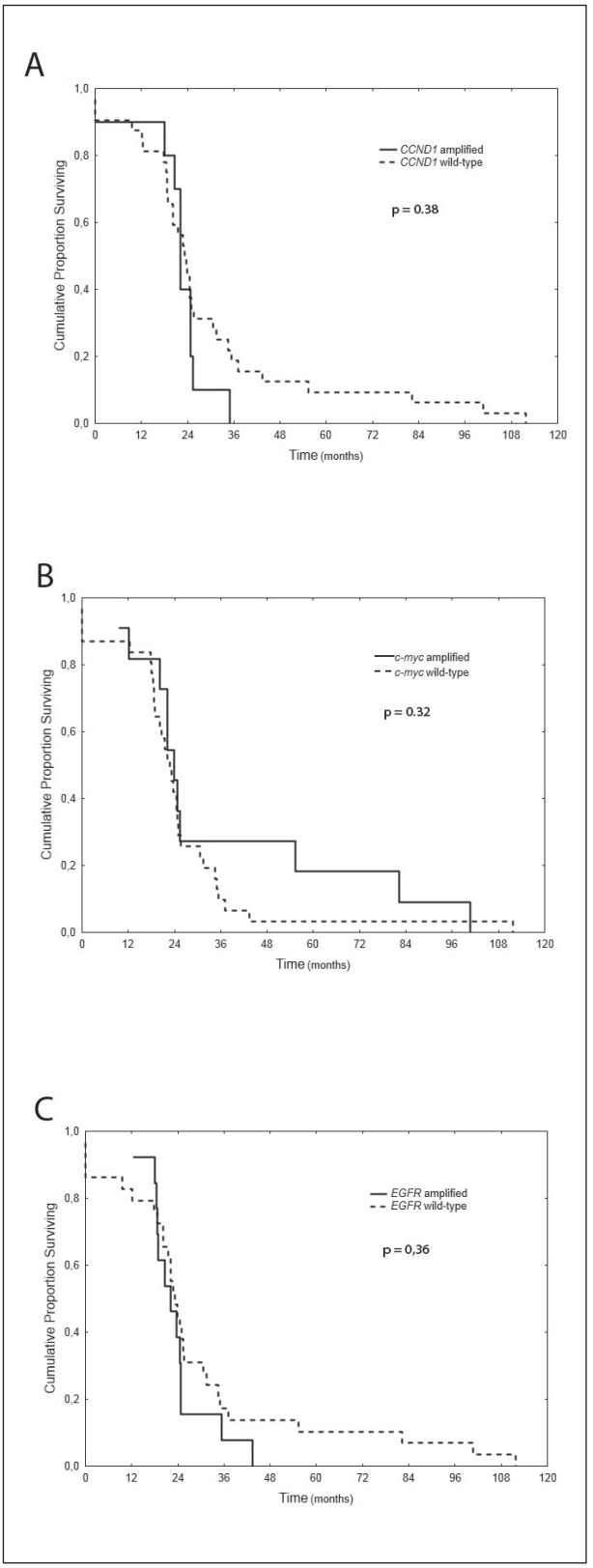

Figure 2 Impact of oncogenes amplification on patients'survival. (A) Patients without amplification of *CCND1* had tendency for better survival; (B) Amplification of *c-myc* seems to have no impact on patients' survival (C) Patients without *EGFR* amplification had tendency for better survival.

Table II Association between amplified oncogenes, stage and *HER2/neu* status.

Parameter	Total aNP	*CCND1*			*c-myc*			*EGFR*		
		NP	%	*p*	NP	%	*p*	NP	%	*p*
Total	49	10	20.4		13	26.5		13	26.5	
Stage [b]										
I	12	0	0	*p1* 0.04[c]	4	33.3	*p1* 0.57	2	16.7	*p1* 0.16
II	20	6	30.0	*p2* 0.55	6	30.0	*p2 0.55*	8	40.0	*p2* 0.32
III	12	3	25.0	*p3* 0.11	3	25.0	*p3* 0.50	3	25.0	*p3* 0.50
HER2/neu status										
positive	19	6	31.6	0.12	4	21.1		8	42.1	0.05[c]
negative	30	4	13.4		9	30.0		5	16.7	

[a] Number of patients; [b] unavailable data for 5 patients [c] Bold indicates statistically significant values; *p1* – statistical significance between stages I and II; *p2* – statistical significancee between stages II and III; *p3* – statistical significance between stages I and III

Discussion

Gene amplification is an important mechanism of oncogene activation and is crucial for the development and progression of cancer. In the present study we used quantitative real time PCR and differential PCR to study gene copy number alterations of *CCND1*, *c-myc* and *EGFR* oncogenes in a cohort of 49 primary breast cancer patients.

CCND1 oncogene was amplified in 20.4% of analyzed breast cancer samples which is consistent with previously reported frequencies of 13%–20%. Quantification results, which revealed that amplification of *CCND1* was three to 14-fold, are consistent with the same reports (7, 13). Interestingly, amplification of *CCND1* gene was significantly associated with the progression of breast cancer, both of ductal and lobular subtypes. Specifically, it was associated with the stage II of the disease. It is important to emphasize that cyclin D1 is always strongly overexpressed when amplified (14–16). Contrary to our findings, immunohistochemical studies of preneoplastic lesions demonstrated that overexpression of cyclin D1 had been already apparent in hyperplasia and increased with increasing malignancy (17). On the other hand, in situ hybridisation studies suggest that cyclin D1 overexpression occurs at the transition from in situ to invasive carcinoma (18). Our results imply that amplification of *CCND1* oncogene could be the marker of the progression to stage II of the disease. In addition, studies on primary breast cancers indicate that overexpression of cyclin D1 was confined to specific phenotypes, implying different roles in different subtypes of the disease. Lobular carcinoma appears to universally overexpress cyclin D1 (19), while overexpression in ductal carcinoma is confined almost exclusively to estrogen receptor positive cases (20). We have not observed any difference in *CCND1* amplification between lobular and ductal breast carcinomas but we are limited in discussing this issue since 96% of our samples are ER positive.

The need for cooperativity with other oncogenic hits is entirely expected for an authentic human oncogene (7), which was the reason why we analyzed the amplification of *c-myc* and *EGFR* in the same cohort of samples. *c-myc* gene was amplified in 26.5% of analyzed samples, slightly above reported frequencies (21, 22), but did not show any co-ordination with *CCND1* amplification. Barnes and Gillette (23) have also shown that *CCND1* gene is amplified in cases were *c-myc* is not. Although this reciprocal amplification seems to be consistent with the observation that *c-myc* represses the transcription of cyclin D1 (24), direct evidence is still lacking for a reciprocal relationship between the expression of *c-myc* and that of cyclin D1.

The role that epidermal growth factor receptor plays in breast cancer has been a subject of intensive study and controversy. Some retrospective immunohistochemical studies have indicated that *EGFR* overexpression in primary tumors is an indicator of poor prognosis (25), whereas other similar studies have failed to establish such a link (26). Collectively, these studies suggest that *EGFR* is expressed in 18–35% of breast cancers (27). Our study revealed that *EGFR* oncogene was amplified in 26.5% of examined samples. No co-alteration with *c-myc* and *CCND1* was observed. However, amplification of *EGFR* gene showed significant association with the expression of HER-2/neu. The largest and the most comprehensive study analyzing *EGFR* expression in breast cancer patients showed that *EGFR* expression was positively correlated with HER-2/neu overexpression, which has been associated with bad prognosis (28). Our results confirm this finding since Kaplan & Meier survival analysis showed that patients with HER-2/neu overexpression had worse prognoses and lived significantly shorter.

Finally, the most promising therapy in this patient's cohort was endocrine (hormone) therapy. We looked for an oncogene signature in the background and found that samples of patients who underwent endocrine therapy did not have amplifications of *CCND1* and *EGFR* oncogenes. This was not a surprise since the overexpressions of both, *CCND1* and *EGFR*, have been associated with the resistance to hormone therapy and chemotherapy in a number of studies (29, 30). However, the therapy groups analyzed in the present study are small, and therefore we recommend careful interpretation of the findings.

In conclusion, amplification of *CCND1* or *EGFR* oncogenes is associated with the progression of breast cancer and bad prognosis. No co-ordination in amplification of *CCND1*, *c-myc* and *EGFR* oncogenes was established in this cohort of breast cancer patients.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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