IMPORTANCE OF COPY NUMBER ALTERATIONS OF FGFR1 AND C-MYC GENES IN TRIPLE NEGATIVE BREAST CANCER
ZNAČAJ PROMENA BROJA KOPIJA FGFR1 I C-MYC GENA U TROSTRUKO NEGATIVNIM KARCINOMIMA DOJKE

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Summary
Background: Triple negative breast cancer (TNBC) is characterized by aggressive clinical course and is unresponsive to anti-HER2 and endocrine therapy. TNBC is difficult to treat and is often lethal. Given the need to find new targets for therapy we explored clinicopathological significance of copy number gain of FGFR1 and c-MYC. Our aim was to determine the impact of FGFR1 and c-MYC copy number gain on clinical course and outcome of TNBC.
Methods: FGFR1 and c-MYC gene copy number alterations were evaluated in 78 archive TNBC samples using TaqMan based quantitative real time PCR assays.
Results: 50% of samples had increased c-MYC copy number. c-MYC copy number gain was associated with TNBC in contrast to ER positive cancers. Our results showed significant correlation between c-MYC copy number gain and high grade of TNBCs. This suggests that c-MYC copy number could be an useful prognostic marker for TNBC patients. c-MYC copy number gain was associated with high pTNM stage as well as lobular and medullary tumor subtypes. 43% of samples had increased FGFR1 copy number. No correlations between FGFR1 copy number gain and clinicopathological variables were observed.
Conclusions: We identified c-MYC copy number gain as a prognostic marker for TNBC. Our results indicate that c-

Kratak sadržaj
Uvod: Trostruko negativne karcinome dojke karakteriše agresivan klinički tok i neosetljivost na endokrinu i anti-HER2 terapiju. Ovi tumori se teško leže i često su lethalni. Zbog potrebe za novim tipovima terapije, ispitali smo kliničko-patološki značaj povećanja broja kopija FGFR1 i c-MYC onkogene. Cilj rada je bio da se utvrdi uticaj povećanja broja kopija FGFR1 i c-MYC na klinički tok i ishod trostrukog negativnog karcinoma dojke.
Metode: Promene u broju kopija FGFR1 i c-MYC gena određene su kvantitativnim PCR-om u realnom vremenu kod 78 arhivskih uzoraka trostruko negativnih karcinoma. Rezultati: 50% ispitanih uzoraka je imalo povećano broj kopija c-MYC. Povećanje broja kopija c-MYC gena je asociirano sa trostrukim negativnim karcinomima dojke u poređenju sa ER pozitivnim karcinomima. Amplifikacija c-MYC je asociirana sa visokim gradusom trostrukog negativnog karcinoma. Iz ovog rezultata proizlazi da bi se broj kopija c-MYC mogao smatrati korisnim progostickim markomerom za TNBC pacijente. Povećanje broja kopija c-MYC gena je asocirano i sa visokim stadijumom tumora kao i sa lobularnim i medularnim podtipom. 43% ispitanih uzoraka je imalo povećan broj kopija FGFR1. Nisu utvrđene nikakve korelacije između povećanja broja kopija FGFR1 i kliničkih i histopatoloških parametara tumora.

List of abbreviations: CISH, chromogenic in situ hybridization; c-MYC; CNA, copy number alterations; DFI, disease free interval; ER, estrogen receptor; FFPE, formalin-fixed, paraffin-embedded; FGFR1, fibroblast growth factor receptor 1; IHC, immunohistochemistry; HER2, human epidermal growth factor receptor 2; OS, overall survival; PR, progesterone receptor; qPCR, quantitative real time PCR; TNBC, triple negative breast cancer
**Introduction**

Triple negative breast cancer (TNBC) is defined by the lack of expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2). It accounts for 10 to 20% of all invasive breast cancer cases (1). TNBC is a highly heterogeneous disease and is usually an invasive ductal carcinoma of no special type with a high histological grade and mitotic index (1). It is characterized by poor prognosis and aggressive clinical course (2–4). Currently the only systemic therapy available for TNBC is conventional cytotoxic chemotherapy but it’s effects seem to be insufficient (5, 6). Identifying molecular targets and devising new therapeutics for these targets is an ongoing effort and imperative for the development of a successful therapy for TNBC.

Fibroblast growth factor receptor 1 (FGFR1) belongs to the fibroblast growth factor receptor family of transmembrane receptor tyrosine kinases. It has an important role in many cellular processes such as cell differentiation, proliferation, migration and apoptosis (7). Aberrant FGFR signaling has been associated with cancerogenesis in several human cancers thus making it a potential therapeutic target (7). Gene amplification is one of the most commonly identified aberrations in breast cancer. FGFR1 amplification was shown to be associated with invasive breast cancer suggesting it could influence breast cancer progression by contributing to the invasive transition processes (8). While FGFR1 amplification was associated with poor prognosis in ER positive breast cancer (9), its role in TNBC is far less clear. To date there have been only a few reports of FGFR1 alternations and their impact on TNBC progression and prognosis.

c-MYC protein is a transcription factor that serves as a key regulator of most aspects of cellular function including metabolism, replication, growth, differentiation and cell death (10). c-MYC overexpression and gene amplification have been detected in a majority of human cancers including breast cancer. c-MYC expression and signaling were found to be elevated in TNBCs compared to hormone receptor positive cancers and linked to poor prognosis (11). c-MYC amplification was shown to be preferentially associated with invasive zones of breast cancer (12) and several studies have indicated that c-MYC may play an important role in aggressive breast cancers with poor prognosis (13, 14).

The main goals of this study were to evaluate the rates and prognostic significance of FGFR1 and c-MYC copy number alterations (CNA) in TNBC.

**Materials and Methods**

**Samples**

Seventy eight breast carcinoma samples obtained from the Institute for Oncology and Radiology of Serbia that were confirmed negative for ER, PR and HER2 were included in this study. ER, PR and HER-2 expression were evaluated using commercial semi quantitative immunohistochemistry (IHC) assays, Dako, according to the manufacturer recommended procedure. The scoring system included the percentage of stained cells on a score from 0 to 5, and the intensity of their staining on a score from 0 to 3. Cases with the overall IHC score <4 were considered negative for ER and PR expression (15). An overall score of 0 or 1+ for IHC staining of HER2 expression was regarded as negative. Score of 2+ was considered equivocal and for these cases HER2 negative status was confirmed by chromogenic in situ hybridization (CISH) (16).

All tumor samples and their corresponding normal tissue were formalin-fixed, paraffin-embedded (FFPE). All relevant histopathologic and clinical parameters (age, tumor type, pN stage, pT stage, pTNM stage, Nottingham combined histologic grade, disease free survival, overall survival) were retrieved from patient’s medical records.

This study was approved by the Institute for Oncology and Radiology of Serbia ethics committee number 4321-01 and carried out in accordance with the ethical standards laid down in the 1964 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.
DNA extraction

Genomic DNA was extracted from all samples using Kapa Biosystems Express Extract Kit (KK7151, Kapa Biosystems, Wilmington, MA, USA) according to the manufacturers recommended protocol. The quality of the extracted DNA was verified by agarose gel electrophoresis. Concentrations and purity were assessed spectrophotometrically using A260 / A280 absorbance ratios (NanoDrop Technologies, Wilmington, DE, USA). Extracted and purified DNA was stored at +4 °C until further analyses.

Copy number analysis by quantitative real time PCR

Copy number analyses of FGFR1 and c-MYC genes were done by quantitative real time PCR (qPCR) using TaqMan based assays. c-MYC assay included highly specific forward and reverse primers as well as a 6-Fam-TAMRA labelled probe: 5’-GAGAGGCAGAGCTCTCATCAA-3’, 5’-CCAGCTCTCCTGAGACGAGCTT-3’, TaqMan Probe 6-FAM-5’-AGAAGCCGCTCCACATACAGTCTCGG-3’-TAMRA. FGFR1 gene copy number was evaluated using Hs00237051_cn TaqMan assay (Applied Biosystems, Foster City, CA, USA). RNase-P was used as a reference gene (4403326, Applied Biosystems). Calibration was done using DNA isolated from normal breast tissue.

Each sample was prepared in duplicate while normal DNA controls were prepared in triplicate. Total reaction volume was 15 μL. For c-MYC, reaction contained primers/probe ratio of 3:1 (0.1 mmol/L probe: 0.3 mmol/L primers), 1x TaqMan Master Mix and 40 ng of DNA. For FGFR1 or RNase-P reaction contained 1x TaqMan Master Mix, 1x TaqMan Copy Number Assay for FGFR1 or RNase-P gene and 40 ng of DNA. Each reaction contained two normal DNA controls that were used as calibrators. PCR reactions were carried out in the ABI Prism 7500 Sequence Detection System at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. A mean Ct value of each duplicate was obtained by qPCR was ≥ 2.0.

Statistical analysis

Data analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc. CA). The correlations between clinicopathologic parameters and CNA of c-MYC and FGFR1 genes were analysed using Fisher’s exact test or the Chi-square test, depending on test conditions. Survival analyses were performed using Kaplan & Meier product-limit method and the log rank test was used to determine the significance of the difference between survival curves. Overall survival (OS) was calculated from the day after surgery to the last follow-up examination or death of the patient and disease free survival (DFS) was defined as time from the day after surgery to first locoregional recurrence, distant recurrence or contralateral disease. Statistical differences were considered significant when p value was < 0.05.

Results

Copy number alternations of c-MYC and FGFR1 oncogenes

We analyzed 78 TNBC samples out of which 34 had increased FGFR1 copy number (43%) and 39 (50%) had increased c-MYC copy number. Comparing this findings with our previous results on receptor positive breast cancer samples (17) it is evident that the triple negative cohort has a significantly higher incidence of copy number gain for c-MYC (p<0.005).

We then correlated copy number gain of examined genes with the clinicopathologic parameters of TNBC (Table I). Copy number gain of c-MYC oncogene was significantly associated with high histological grade (p=0.008) and high pTNM stage (Table I). Patients with lobular breast cancer were represented more in the group with increased c-MYC copy number (p=0.014) and c-MYC copy number gain group tended to have a higher representation of medullary breast cancers (p=0.06). Surprisingly, the group with no FGFR1 copy number gain had a higher percentage of pTNM stage 4 tumors (Table I). FGFR1 copy number gain was not associated with any other clinicopathologic variable. Neither of the analyzed genes had any significant influence on patient OS and DFS (Figure 1).

Next, we analysed whether there were co-alterations between FGFR1 and c-MYC oncogenes. 24 (31%) of samples had increased both FGFR1 and c-MYC copy number, 25 (32%) had copy number gain of one gene and 29 (37%) had no copy number gain for either gene. Simultaneous copy number gain of both genes was significantly associated with high histological grade (p=0.039) and pTNM stage III (Table I). Patients with no copy number gain for either gene had a higher proportion of pTNM stage I tumors compared to other groups while patients with copy number gain of one gene had higher proportion of pTNM stage IV tumors (Table I). No correlation with patient survival was obtained (data not shown).
Table I Clinicopathological correlation according to c-MYC and/or FGFR1 copy number alterations in TNBC.

| Parameters | Total (np=78) | c-MYC np (%) | FGFR1 np (%) | c-MYC and FGFR1 np (%) |
|------------|---------------|--------------|--------------|------------------------|
|            | Gain (np=39)  | p value      | Gain (np=34) | p value                | Both gain (np=24) | One gain (np=25) | p value |
| **Tumor type** |               |              |              |                        |                      |                   |         |
| Ductal     | 46 (59)       | 18 (46)      | 18 (53)      | 11 (46)                | 14 (56)             | pA 0.220          |
| Lobular    | 14 (18)       | 11 (28)      | 6 (18)       | 6 (25)                 | 5 (20)              | pB 0.071          |
| Medullary  | 14 (18)       | 10 (26)      | 9 (26)       | 7 (29)                 | 5 (20)              | pC 0.438          |
| Other*     | 4 (5)         | 0 (0)        | 1 (3)        | 0 (0)                  | 1 (4)               | pD 0.871          |
|            | pE 0.011      |              | pE 1.000     |                       |                      | pE 0.105          |
|            | pF 0.023      |              | pF 0.274     |                       |                      | pF 0.044          |
| **Stage**  |               |              |              |                        |                      |                   |         |
| I          | 10 (13)       | 1 (2)        | 3 (9)        | 1 (4)                  | 2 (8)               | p1 0.106          |
| II         | 38 (49)       | 19 (49)      | 20 (59)      | 14 (59)                | 11 (44)             | p2 0.021          |
| III        | 11 (14)       | 9 (23)       | 7 (20)       | 7 (29)                 | 2 (8)               | p3 0.204          |
| IV         | 19 (24)       | 10 (26)      | 4 (12)       | 2 (8)                  | 10 (40)             | p5 0.284          |
|            | p5 1.000      |              | p5 0.027     |                       |                      | p5 0.079          |
|            | p5 0.139      |              | p6 0.046     |                       |                      | p6 0.009          |
| **pT stage** |             |              |              |                        |                      |                   |         |
| T1 and T2  | 68 (87)       | 34 (87)      | 28 (82)      | 21 (87)                | 20 (80)             | 0.356             |
| T3 and T4  | 10 (13)       | 5 (13)       | 6 (18)       | 3 (13)                 | 5 (20)              |                   |
| **Nodal status** |         |              |              |                        |                      |                   |         |
| Negative   | 32 (41)       | 20 (51)      | 21 (64)      | 14 (59)                | 12 (48)             | 0.708             |
| Pozitive   | 46 (59)       | 19 (49)      | 13 (36)      | 10 (41)                | 13 (52)             |                   |
| **Histologic grade** |     |              |              |                        |                      |                   |         |
| I and II   | 52 (67)       | 20 (51)      | 20 (59)      | 12 (50)                | 16 (64)             | 0.059             |
| III        | 26 (33)       | 19 (49)      | 14 (41)      | 12 (50)                | 9 (36)              |                   |

Abbreviations: np, number of patients per group; * – tubular, mucinous and other rare carcinoma types
pA – statistical significance between ductal and lobular tumors; pB – statistical significance between ductal and medullary tumors; pC – statistical significance between ductal and other tumors; pD – statistical significance between lobular and medullary tumors; pE – statistical significance between lobular and other tumors; pF – statistical significance between medullary and other tumors; p1 – statistical significance between stages I and II; p2 – statistical significance between stages I and III; p3 – statistical significance between stages I and IV; p4 – statistical significance between stages II and III; p5 – statistical significance between stages II and IV; p6 – statistical significance between stages III and IV.

Bold indicates statistically significant values, p < 0.05.
Discussion

Gene copy number gain is an important mechanism of oncogene activation in cancer however the prognostic significance of FGFR1 CNA in TNBC remains unclear. Our study showed that FGFR1 gene copy number was increased in 43% of examined samples which is a higher frequency than previously reported by Lee et al. (18). However, Lee et al. (18) examined FGFR1 amplification in TNBC using fluorescence in situ hybridization (FISH). A recent study compared qPCR with FISH for assessing gene copy number (19). qPCR exhibited excellent correlation with FISH at detecting copy number gain at 8 or more copies. In the range from 2 to 6 copy number gain as detected by qPCR, no copy number gains were found using FISH. We would argue that qPCR is a more sensitive method for detecting lower levels of copy number gain. In our cohort the majority of samples with gene amplification had a low-grade copy number increase. Therefore it is very likely that the higher frequency of FGFR1 copy number gain detected in our study is due to the higher sensitivity of qPCR. In the same study Park et al. (19) demonstrated that FFPE tissue showed lower levels of copy number gain compared to frozen tissue therefore qPCR would be ideally suited for assessing gene copy number gain in FFPE tissue.

FGFR1 expression has impact on overall survival in TNBC but its role in this disease is still controversial (20). Literature data is scarce and offers conflicting results. Cheng et al. (20) found that FGFR1 expression in TNBC was independently predictive for OS with cases with high FGFR1 immunostaining having the worst prognosis. Another study by Lee et al. (18) showed no correlation between FGFR1 expression and patient survival. The two studies differed in immunostaining threshold used which highlights the difficulty of comparing data from various studies. Underlying mechanisms of FGFR1 overexpression in TNBC are not well understood. While several studies reported high level of correlation between FGFR1 protein overexpression and gene amplification, (21, 22), a number of studies observed low protein expression level in FGFR1 amplified tumors (23, 24). We found no significant association between FGFR1 copy number gain and poor prognosis in TNBC or any of the clinicopathologic parameters. Our results support the notion that FGFR1 copy number status may not be an informative independent prognostic factor for TNBC.
c-MYC is frequently deregulated in breast cancer and is thought to contribute to breast cancer progression and poor prognosis. However, the detected frequency of c-MYC amplification and its prognostic significance have been inconsistent (25, 26) with the reported frequency ranging from 1 to 94% (10). We have detected c-MYC copy number gain in 50% of TNBC samples, a frequency significantly higher than in receptor positive breast cancers we previously examined (17). Dillon et al. (27) detected that 75% of TNBCs and 89% of basal-like tumors had c-MYC amplification. However, this study analyzed only 20 TNBC samples. Here we have analyzed a much larger cohort and demonstrated that c-MYC copy number gain is a frequent event in TNBC. High frequency of c-MYC overexpression and copy number gain observed in TNBC, indicates that c-MYC deregulation could be important for TNBC progression.

We have found that c-MYC copy number gain was associated with high grade TNBC. Determining whether c-MYC copy number gain is present may help identify patients with a greater risk of developing high grade TNBC. Therefore, c-MYC could be considered as a prognostic marker of tumour progression.

Copy number gain of c-MYC was significantly associated with stage III of TNBC. These results are in compliance with previous studies that found c-MYC amplification to be associated with invasive zones of breast cancer. These results further confirm that c-MYC copy number gain could be a prognostic marker of tumour progression in TNBC.

Our result that medullary tumors were associated with c-MYC copy number gain is consistent with a previous study (28). This suggests that medullary tumors have a distinct biology conducive to c-MYC amplification. Our finding that lobular tumors were associated with c-MYC copy number gain differs from a study by Green et al. (29) which found c-MYC expression to be more frequent in non-lobular tumors. However, in the study by Green et al. (29) only 15% of samples were TNBC. Our results support the notion that triple negative lobular carcinoma is genetically distinctive from non-triple negative lobular carcinoma. Previous studies have shown that these two types of lobular carcinoma differ in clinicopathologic and IHC characteristics (30).

It has been shown that c-MYC overexpression is associated with TNBC. Understanding the underlying mechanisms of c-MYC expression may open new approaches for therapy of TNBC. One study showed that CDK inhibition effectively induced tumor regression in TNBC tumors that exhibit elevated c-MYC expression (11). c-MYC could represent a promising new target for TNBC therapy.

We have found a high level of concordance between FGFR1 and c-MYC copy number gain as 24 (31%) of samples had increased both FGFR1 and c-MYC copy number. Simultaneous copy number gain of both genes was associated with high grade and high pTNM stage. However, this might be due to the association of c-MYC gain with these parameters. Simultaneous absence of copy number gain for both genes was associated with pTNM stage I which further supports the notion that CNA of these genes occurs at a later phase of tumor progression. One previous study identified frequent coamplification of c-MYC and 8p11-p12 chromosomal region, where FGFR1 is located, in invasive breast cancers (31). Additionally, evidence exists that links c-MYC expression with more favorable response to FGFR inhibiting therapy in lung cancers that exhibit FGFR1 amplification (32). Therefore, examining FGFR1 and c-MYC copy number and expression together may help determine which patients would benefit the most from FGFR inhibiting therapy.

In conclusion, copy number gain of c-MYC is associated with high grade and high stage TNBC as well as lobular and medullary tumor subtypes. FGFR1 gene copy number has low prognostic implication for TNBC. High level of concordance in c-MYC/FGFR1 copy number gain was detected in this cohort. Simultaneous copy number gain of both genes was significantly associated with high histological grade and pTNM stage of TNBC. Though we found no significant implications for patient outcome, a subset of TNBC harbor copy number gain of c-MYC and FGFR1 which could be of interest for TNBC therapy. Patients with no copy number gain for either gene had a higher proportion of pTNM stage I tumors confirming the predictive importance of these genes amplification for tumor progression.

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Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.
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