Co-overexpression of EpCAM and c-myc genes in malignant breast tumors

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Abstract

Purpose: The overexpression of epithelial cell adhesion molecule (EpCAM) proto-oncogene affects progression, treatment, and diagnosis of many adenocarcinomas. C-myc has been shown to be a downstream target of EpCAM and is also one of the most important proto-oncogenes routinely overexpressed in breast cancer. However, co-overexpression of EpCAM and c-myc genes has not been investigated in breast cancer tissues, particularly in Iranian population.

Objectives: The aim of this study was to assess the expression of EpCAM and c-myc genes in malignant breast cancer tissues using RT-qPCR, followed by analyses of the association between the outcomes.

Methods: To this purpose, 122 fresh tissues including 104 malignant and 18 benign samples were disrupted by mortar and pestle and RNA was then isolated from the samples and converted to cDNA. The relative expression levels of EpCAM and c-myc genes were measured by $2^{-ΔΔCt}$ method using RT-qPCR method. EpCAM protein level was also assessed in 66 cases using western blot technique.

Results: Our results showed that EpCAM was overexpressed in 48% of malignant and 11.1% of benign samples, using RT-qPCR method. Evaluating EpCAM protein overexpression in a portion of samples depicted the fully concordance rate between Western blot and RT-qPCR techniques. C-myc expression was first evaluated by RT-qPCR method, showing the overexpression rate of 39% and 28% in malignant and benign samples respectively. These data were also quite concordant with the
clinically available IHC reports of the same samples studied in this study. Importantly, overexpression of EpCAM and c-myc was significantly associated and showed the agreement of 57.3%.

**Conclusion:** This study demonstrated the co-overexpression of EpCAM and c-myc in breast tumors collected from breast cancer patients of the Iranian population. EpCAM and c-myc positive cases were significantly associated with reduced and enhanced risk of ER/PR positivity respectively. However, they both were associated with grade III of breast cancer.

**Keywords:** Breast Cancer; c-Myc; EpCAM; Real Time Reverse Transcriptase-PCR; Western blot

**1. Introduction**

Breast cancer is the most prevalent cancer among Iranian women (Mousavi et al., 2009); clearly an efficient diagnostic method is required to improve the treatment of this disease. Among various well-studied breast cancer-related genes, the overexpression of Epithelial Cell Adhesion Molecule (EpCAM) gene is known to be associated with enhanced proliferation and malignancy in different carcinomas (Balzar et al., 1999; de Boer et al., 1999; Maetzel et al., 2009; van der Gun et al., 2010). EpCAM is a membrane glycoprotein mediating cell-cell hemophilic adhesion interactions (Nübel et al., 2009). Although the squamous epithelium and some specific types of epithelial cells such as hepatocytes and keratinocytes show no EpCAM expression, EpCAM expresses in a majority of normal epithelial cells (Winter et al., 2003). EpCAM is a transmembrane protein comprising three main domains: EpCAM extracellular domain (EpECD), intramembrane domain, and EpCAM intracellular domain (EpICD) (Baenere & Gires, 2007). Although EpCAM is a member of cell adhesion molecules (CAMs), its overexpression has been observed in most types of carcinomas such as breast carcinomas (rate 18% to 46.7%) using immunohistochemistry method (IHC) (Ambrogi et al., 2012; Gastl et al., 2000; Spizzo et al., 2011; Spizzo et al., 2004). Various mechanisms are thought to be associated with the double-faced behavior of EpCAM. First, EpCAM is an E-cadherin antagonist, given that it negatively modulates E-cadherin adhesion by interrupting the linkage between α-catenin and f-actin (Litvinov et al., 1997). Second, the particular complex consisting of the intracellular domain of EpCAM interacting with β-catenin, the adaptor protein FHL2 (Four and Half LIM domain protein2), and the transcription factor Lef1 that is cleaved by presenilin-2 binds to the DNA at the Lef1 consensus site of c-myc and cyclin A/E genes, consequently promoting their expression (Götlinger et al., 1986). Third, EpCAM has been reported to be hyper-glycosylated in carcinoma tissues as compared with that in normal autologous epithelial cells (Munz et al., 2007; Pauli et al., 2003). Fourth, the localization of
EpCAM in a specific subdomain of cell membrane might be important for signal transduction (Kuhn et al., 2007). A number of cell signaling events are facilitated by some cell-cell contacts (Fagotto & Gumbiner, 1996) and by other membrane microdomains such as tetraspanin-enriched microdomains (TEMs), which may play a role as signaling platform (Schnell et al., 2013). EpCAM can be engaged in TEMs by forming a primary complex with tetraspanin CD9 (Le Naour et al., 2006). EpCAM, in metastatic rat carcinoma cell lines, forms a complex with tetraspanins CD9 and CO-029 (Tetraspanin8) as well as CD44V4V7 and plays a significant role in tumor progression (Ponta et al., 2003). Cloudin7 is another protein in the EpCAM-CD44V4V7-tetraspanins complex, which binds to EpCAM via a motif within the transmembrane domain, ultimately leading to the recruitment of EpCAM into TEMs (Kuhn et al., 2007; Ladwein et al., 2005; Nübel et al., 2009). The direct association between claudin7 and EpCAM seems to be mandatory for EpCAM-specific functions. EpCAM has also been reported to enhance apoptosis resistance, cell proliferation, and tumorigenicity with claudin7 (Nübel et al., 2009). In 2013, Baccelli and colleagues showed that the overexpressed EpCAM raises metastases rate in the breast cancer patients (Baccelli et al., 2013). Moreover, studying the EpCAM-targeting antibodies revealed the positive role of Catumaxomab, an efficient monoclonal antibody approved in European Market in 2009, to reduce the rate of metastasis in patients with metastatic breast cancer. Importantly, catumaxomab is effective only for the treatment of EpCAM-positive carcinomas (Linke et al., 2010), reflecting the significance of distinguishing EpCAM positive tumors.

C-myc protein, a multifunctional product of c-myc proto-oncogene, plays a critical role in normal cell processes, including cell cycle, cell growth, metabolism, differentiation, angiogenesis, genomic instability, transformation, and apoptosis (Meyer & Penn, 2008). On the other hand, the dysregulated c-myc expression as a result of genetic alterations has been reported in a variety of neoplasm such as breast cancer (Marcu et al., 1992). The levels of c-myc expression play an important role in induction and progression of human breast cancer. Overexpression of c-myc has been reported in 21.6% to 45% of patients with breast cancer in different studies (Bièche et al., 1999; Chrzan et al., 2001; Lê et al., 1999; Naidu et al., 2002; Scorilas et al., 1999). The c-myc gene is an early target of EpCAM; by which, EpICD forms a complex, binds to the DNA at the Lef1 consensus site and finally promotes c-myc expression (Linnenbach et al., 1993). The c-myc protein level is associated with the effectiveness of some chemical agents. Interestingly, c-myc positive tumors show resistance to cisplatin chemotherapy agent. Thus, the sensitivity to cisplatin is enhanced in tumor cells with low c-myc expression (Biroccio et al., 2001; Sklar & Prochownik, 1991; Tanida et al., 2012).
Therefore, there is a pressing need to distinguish tumor cells expressing a high level of EpCAM and/or c-myc in order to exploit the benefits from catumaxomab and cisplatin chemotherapeutic agent, respectively. Along with this need, the purpose of this study was to analyze the expression of EpCAM and c-myc transcripts in the same samples as well as to evaluate the association between the outcomes, using RT-qPCR method. Following this, c-myc and EpCAM expression status was evaluated in terms of the clinicopathological features of the patients. Here, we showed for the first time in Iranian population that EpCAM and c-myc were overexpressed in 48% and 39% of malignant breast cancer tissues. Furthermore, the agreement of co-overexpression of these two oncogenes was 57.3%.

2. Materials and methods

2.1. Patients and Samples

Fresh tumor tissue samples of 104 patients (72 primary and 32 metastatic breast tumors) as well as 18 benign specimens were collected from the Breast Cancer Research Center of Isfahan over the course of 3 years, from 2012 to 2015. Furthermore, 20 normal breast tissues were also provided from normal mammary tissues adjacent to the malignant tumors. The normality, malignancy and benignity status of all tissues were approved by pathological investigations. The present research was initially approved by the institutional ethics committee of the University of Isfahan. All the participants had been apprised clearly and provided with standardized written permission. For mRNA analysis, an incision of the fresh tumor was immediately frozen in liquid nitrogen after mastectomy operation followed by storage at −70 °C, and the rest section of the tumor was used in the pathology laboratory for determination of breast cancer diagnosis and characteristics. Routine pathological variables consisting of age, tumor grading and type, and also immunohistochemically determination of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and estrogen receptor (ER) status, obtained from Breast Cancer Research Center of Isfahan, are listed in Table 1.

2.2. RNA Isolation and cDNA Synthesis

For RNA isolation, 20 mg fresh frozen sample was accurately dissected on ice from each patient specimen. This amount of mammary tissue was first disrupted with mortar and pestle together with liquid nitrogen and then homogenized by a syringe and needle. The extraction of total RNA was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RNA was then stored in RNase-free water at −70 °C. The quantity and quality of extracted RNA were measured and proved by spectrophotometer.
and electrophoresis in a 2% agarose gel with ethidium bromide staining, respectively. 18s rRNA and 28s rRNA bands were observed as an expected schema. The isolated RNA was then transcribed into cDNA taking advantage of Random Hexamer Primers in RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Synthesized total cDNA was finally stored at –70 °C.

2.3. Primers

The set of GAPDH primers which was designed in the previous study was used for GAPDH cDNA propagation (Tabatabaian & Hojati, 2013). EpCAM and c-myc primers were initially designed by AlleleID7.7 and then evaluated by Oligo7 software to avert primer-dimers, false priming sites, and formation of secondary structures. The selected primers were purchased from BIONEER (South Korea). The sequence of primers to amplify EpCAM, c-myc and GAPDH cDNAs are listed as follow: EpCAM Forward: 5´-TGTGGTTGTGGTATAGCAGTT-3’, EpCAM Reverse: 5´-CCCATCTCTTTATCTAGCCTTC-3’, c-myc Forward: 5´-AGCGACTCTGAGGAGGAACAA-3’, c-myc Reverse: 5´-GTGGGCTGTGAGGAGGTTTG-3’ and GAPDH Forward: 5´-ACCATGTTCGAGGAGGAGCACA-3’, GAPDH Reverse: 5´-GCAAATGAGCCAGCCCGCTTC-3’. The product length of amplified EpCAM, c-myc and GAPDH transcripts is 102, 136, and 115 bp, respectively.

2.4. RT-qPCR Analysis

2.4.1. RT-qPCR

Applied Biosystem StepOnePlus™ Real-Time PCR System and Maxima SYBR Green/ROX qPCR Master Mix (2x) kit (Fermentas) were used for performing RT-qPCR reactions. Amplification reactions were performed in a volume of 20 μl with 0.4 pmol/μl of each primer and 40 ng/μl cDNA. Maxima SYBR Green qPCR Master Mix includes Maxima® Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer which contains SYBR® Green I dye. The thermal cycling conditions consisted of an initial denaturation step for 10 minutes at 95 °C and 35 cycles including of a denaturation step at 95 °C for 30 seconds, an annealing step at 68.3 °C for 30 seconds and an extension step for 50 seconds at 70 °C. Fluorescent measurements were taken just after the extension steps. Each sample was assessed in triplicate and average C_{t} values were used for gene expression analyses. The same amount of cDNA was amplified in single measurement fluorescence for all experiments.

2.4.2. Calculation of the amounts of target genes expression
The relative gene expression was measured by the subtraction of the C\textsubscript{t} value of target (EpCAM and c-myc) and control (GAPDH) genes in the tumor samples relative to the normal samples by the 2\textsuperscript{−ΔΔC\textsubscript{t}} method (Livak & Schmittgen, 2001). To determine the cut-off point for the altered expression of target genes in this study, a normalized expression value of target genes was determined for 20 normal breast tissues. For each gene, values of mean+3SD or more were considered to show overexpression in studied samples, which is 1.76 and 1.95 for EpCAM and c-myc, respectively.

2.4.3. Standard curve

The 2\textsuperscript{−ΔΔC\textsubscript{t}} method can be used in a condition that the amplification efficiency of target and control genes is approximately identical and perfect. The \textit{r\textsuperscript{2}} value for the propagation of EpCAM, c-myc and GAPDH cDNAs was approximately similar between 0.95 and 0.99, demonstrating the dependable values. The efficiency of primer sets was calculated from the threshold cycles obtained with 10-fold serial dilutions (1000 ng to 1 ng) of EpCAM, c-myc and GAPDH cDNAs. After calculation of C\textsubscript{t} values for amplification of all three cDNAs, their standard curves were plotted. In a standard curve, C\textsubscript{t} values from each dilution are plotted versus the log cDNA concentration.

2.5. Protein Analysis

2.5.1 Antibodies

Anti-total EpCAM antibody (#2929) obtained from Cell Signaling Technology Inc. (Danvers, MA, USA) in a dilution of 1:1000, anti-GAPDH (sc-365062) purchased from Santa CruzBiotechnology (Santa Cruz, CA, USA) in a dilution of 1:5000 and anti-mouse horseradish peroxidase-conjugated secondary antibody (A4416) obtained from Sigma-Aldrich (St. Louis, MO, USA) in a dilution of 1:5000 were used for western blotting.

2.5.2 Tissue Lysates Preparation

Tissues were washed once with phosphate-buffered saline (PBS) and lysed in detergent buffer (100 mM Tris, 200 mM sodium chloride, 6 mM sodium azide, 10 mM SDS, 2% Nonidet P-40, 25 mM deoxycholic acid) supplemented with 0.1 mg/ml aprotinin, 2 mM sodium orthovanadate, and 0.2 mg/ml PMSF, as described (Skliris et al., 2002).

2.5.3 Western Blot
40μg of whole cell lysates were separated using SDS/PAGE and transferred to nitrocellulose membrane in a condition of 100 V for 90 minutes at 4°C. Blocking of membranes was done in blocking solution (5% non-fat milk powder, in TBST) at room temperature for 1 hour, followed by the incubation of membranes with the anti-EpCAM antibody (5% non-fat milk powder, in TBST), overnight at 4°C. After three washes with TBST, membranes were incubated with the secondary antibody for 1 hour at room temperature in the blocking solution. EpCAM and GAPDH proteins were visualized, after 3 washes of membranes with TBST, using BioRad Chemidoc Imaging system. The intensity of each EpCAM band in all available samples was first normalized by its related GAPDH band, and then each value was divided by the EpCAM/GAPDH average value of normal tissues to generate the normalized quantitative EpCAM expression level of each available benign and malignant sample. Values of mean+3SD or more, as compared to EpCAM intensity in normal cases, were considered as EpCAM overexpression.

2.6. Statistical Analysis

Data analyses were carried out with the SPSS statistical software, version 19.0 (SPSS Inc., Chicago, Ill., USA). Fisher’s exact test, Pearson Chi-square test, McNemar, Kruskal-Wallis, Mann-Whitney U Test and Kappa test were used for statistical analyses. A p-value of less than 0.05 was statistically regarded significant.

3. Results

3.1 EpCAM and c-myc overexpression in malignant and benign breast tumors

Among 72 primary and 32 metastatic breast cancer tissues, 48% (50 out of 104) were EpCAM-positive in overall, showing 2-8 fold increase in the mRNA copy, whereas 52% (54 out of 104) were EpCAM-negative. The high overexpression rate of EpCAM is due to the metastatic group with 72% rate, while primary breast tumors only displayed the 37.5% overexpression. On the other hand, 11.1% of benign tumors (2 out of 18) were EpCAM-positive, reflecting 2-3 fold enhancement. Among 104 malignant tissues, 40 patients (39%) showed c-myc overexpression with 2-12 fold increase in c-myc mRNA level. Separately, 30% and 56% of primary and metastatic breast tissues showed c-myc overexpression respectively. Among benign tissues, 5 out of 18 cases (28%) have c-myc overexpression showing 2-3 fold increase in mRNA level.
Comparing the median values of EpCAM expression levels in four groups including normal, benign, primary and metastatic breast tissues showed a significant difference (Kruskal-Wallis, p-value < 0.001) (Figure 1). To know which groups have significantly different medians, Mann-Whitney test was recruited, by which, normal and metastatic (p-value<0.001), benign and metastatic (p-value=0.006) and primary and metastatic tumors (p-value<0.001), showed significantly different medians.

To validate the accuracy of the EpCAM overexpression detection, lysates were extracted from 66 available samples, including 9 control samples, 7 benign, 26 primary and 24 metastatic breast tumor specimens. Although we did not have enough tissues for extracting the lysates, assessing the EpCAM protein expression level showed the consistency between the EpCAM\(^*\) cases between RT-qPCR and western blot methods (Figure 2). Moreover, c-myc positive and negative cases were also common between our qPCR results and the available IHC results, from the same samples, reported to us from Breast Cancer Research Center of Isfahan. In terms of these validations, our qPCR results were used for the further analysis.

In terms of c-myc expression, the same test revealed the significantly different median values of c-myc in 4 different groups (Kruskal-Wallis, p-value < 0.001), in which, normal and metastatic (p-value<0.001), benign and metastatic (p-value<0.001) and primary and metastatic breast tumors (p-value<0.001) showed significantly different medians (p-value=0.006) (Figure 3).

3.2 The significant association between EpCAM and c-myc-co-overexpression

Totally, 50 malignant samples were EpCAM-positive, among which, 39 samples also showed c-myc mRNA overexpression (Table 2). According to these results, an agreement of 57.3% was proved between EpCAM and c-myc overexpression (p-value<0.01; Kappa test). In addition, Pearson Chi-square test showed a significant association between the positive EpCAM and c-myc status (P-value<0.001), odds ratio=17 (95%CI: 6.033-47.906).

3.3 Comparing mRNA levels in malignant and benign specimens

Among the 18 benign breast tumor samples studied by RT-qPCR, only two samples showed EpCAM mRNA overexpression, while in 104 malignant tissues, 50 cases were EpCAM positive. Statistically, no significant difference was observed comparing the median values of EpCAM mRNA level between these two groups (P value=0.174; Mann-Whitney U test) (Figure 4-A). On the other hand, c-myc mRNA overexpression was observed in 5 out of 18 benign and 40 out of 104 malignant specimens. In terms of the median values of c-myc
transcript expression between these two groups, a significant difference was observed (P value=0.037; Mann-Whitney U test) (Figure 4-B).

3.4 Comparison between EpCAM/c-myc positivity and the clinicopathological features of patients

To find any potential association between the overexpression of EpCAM/c-myc genes and clinicopathological characteristics of studied patients in this paper, EpCAM* and c-myc* cases were statistically analyzed in terms of their distribution in different categories including HER2*, ER/PR*, lymph node*, breast cancer grade and breast cancer subgroups.

Comparing EpCAM* and ER/PR* cases showed a significant association (Pearson Chi-square test, p-value=0.02), in which, EpCAM overexpression reduced the chance of ER/PR positivity, odds ratio=0.391 (95%CI: 0.176-0.871). Likewise, c-myc and ER/PR positivity revealed the significant association (Pearson Chi-square test, p-value<0.005); however, c-myc positivity increased the chance of ER-PR positivity, odds ratio=5.143 (95%CI: 2.052-12.889).

Moreover, the comparison between EpCAM/c-myc positivity and HER2 status showed no significant association (Pearson Chi-square test, p-value=0.993 and p-value=0.088 for EpCAM and c-myc respectively). Likewise, there was no significant association between EpCAM/c-myc and positivity of lymph nodes (Pearson Chi-square test, p-value=0.054 and p-value=0.096 for EpCAM and c-myc respectively).

With regards to the grade of studied breast cancer cases, EpCAM overexpression significantly reduced the chance of grade II of breast cancer (Pearson Chi-square test, p-value=0.016), odds ratio=0.376 (95%CI: 0.376-0.838), while it increased the risk of grade III (Pearson Chi-square test, p-value=0.020), with odds ratio of 2.573 (95%CI: 1.150-5.758). Grade I of breast cancer had no significant association with EpCAM overexpression (p-value=0.867). Furthermore, c-myc positivity only had a significant association with elevated risk of being in grade III of breast cancer (Pearson Chi-square test, p-value=0.047) with an odds ratio of 2.263 (95%CI: 1.006-5.091).

The distribution of EpCAM* cases in metaplastic, carcinoma in situ (CIS), ductal and lobular subtypes of studies breast tumors depicted the significant association of EpCAM overexpression and metaplastic/CIS subgroups. In fact, EpCAM overexpression increased the chance of being in metaplastic group (Fisher’s exact test, p-value=0.023), odds ratio=2.2 (95%CI: 1.773-2.730), whereas EpCAM negativity enhanced the risk of CIS
tumors (Fisher’s exact test, p-value=0.003), odds ratio=2.111 (95%CI: 1.708-2.610). C-myc, however, was significantly overexpressed inductal breast tumors (Pearson Chi-square test, p-value=0.042), odds ratio=2.564 (95%CI: 1.019-6.455).

4. Discussion

Breast cancer is the most prevalent cancer among Iranian women (Mousavi et al., 2009); and our recent study has shown the up-regulation of two important oncogenes, EpCAM and c-myc, in 48% and 39% of malignancies respectively. The rate of EpCAM overexpression in breast cancer in different studies varies between 17.6% and 70% (El-Maqsoud & El-Rehim, 2014; Niemiec et al., 2012; Schmidt et al., 2008). This dramatic fluctuation observed in different studies may be attributed to the different methods used for the analysis, such as IHC and RT-qPCR, and to the various populations of patients especially in terms of breast cancer classification. According to our RT-qPCR outcomes, 50 out of 104 (48%) malignant breast tumor samples showed overexpressed EpCAM gene. EpCAM overexpression was also observed in two of 18 (11.1%) benign cases. Hypothetically, it might show the pre-malignancy phase of breast cancer particularly, as one of these EpCAM-positive benign carcinomas displayed overexpression of c-myc gene as well. Therefore, we hypothesized that EpCAM expression alterations could be a useful potentiating biomarker of malignancy. In 2010, Raffel et al. found strong (IHC 3+) EpCAM expression in 38% of the benign insulinomas, suggesting that it could contribute to distinguishing high-risk patients for malignancy (Raffel et al., 2010).

The c-myc overexpression rate has been reported between 12% and 100% rates in different studies, which might be due to different antibodies and/or techniques, the assigned cut-off point, molecular subtype of the breast tumor, and patient heterogeneity (Liao & Dickson, 2000; Lutz et al., 2002; Xu et al., 2010). According to our RT-qPCR results, 41 out of 104 (39%) malignant samples showed c-myc overexpression. This rate of c-myc overexpression is in agreement with the study by Le et al. showing 35% overexpression of c-myc, using RT-qPCR method (Lê et al., 1999). C-myc overexpression was also displayed in five of 18 (28%) benign samples. In addition, c-myc overexpression had been noted before in studies by Whittaker et al. and Spandidos et al. reporting the overexpression of c-myc gene in fibroadenoma and fibrocystic benign tumors (Spandidos et al., 1986; Whittaker et al., 1986). This strongly indicates the role of c-myc overexpression in breast tumors, thereby could introduce c-myc as a pre-malignancy biomarker (Liao & Dickson, 2000).
Based on the RT-qPCR method, among the 18 benign breast tumor samples investigated in this study, two and five cases were transcriptionally overexpressed in terms of EpCAM and c-myc genes, respectively. The comparison of the median values of mRNA level of EpCAM in 2 distinct groups, i.e. benign and malignant categories showed no significant difference (Figure 1). This insignificant difference is due to the normal level of EpCAM mRNA in 54 out of 104 tumor tissues, approaching the median values of relative mRNA level in malignant and benign groups. However, comparing the median values of c-myc expression levels in benign and malignant groups showed the significant difference, reflecting the much more expression rate of c-myc in malignant tissues (Figure 3).

There were a significant agreement and correlation between co-overexpression of EpCAM and c-myc genes in the same samples as in 50 EpCAM⁺ malignant specimens studied by RT-qPCR; 34 cases had also c-myc mRNA overexpression. These numbers reflect the agreement rate of 57.3% based on Kappa test outcome, as we as showing the significant association (p-value<0.05, Fisher’s exact test). This report strongly supports that c-myc is one of the most important targets of EpCAM and undergoes up-regulation following by EpCAM overexpression in breast cancer. Likewise, studies by Münz et al. and Martowicz et al. reported the correlation between co-overexpression of EpCAM and c-myc genes in HEK-293 and MCF10A cell lines (Martowicz et al., 2013; Münz et al., 2004). In this study, we have shown this association in malignant breast cancer tissues. Noticeably, there were 16 EpCAM⁺ cases, in which, c-myc was not up-regulated. This inconsistency between EpCAM and c-myc overexpression might be explained by the different mechanisms, by which, c-myc gene is being transcribed in cells. In fact, EpCAM is not the only protein regulating c-myc transcription and there are other well-studied mechanisms underlying c-myc expression level in the cells. For example, the epigenetic modifications on c-myc promoter such as histoneH3-K9-methylation and hypo-acetylation of histones H3 and H4 result in heterochromatinization of c-myc promoter and reducing its transcription level(Gombert et al., 2003). C-myc promoter is also being regulated by other transcription factors including E2F, CTCF, c-Fos/c-Jun, FOXM1, c-Abl, ER and NF-κB, etc.(Wierstra & Alves, 2008). Therefore, c-Myc transcription is regulated by many factors and despite the important role of EpCAM in c-myc up-regulation, EpCAM overexpression might not necessarily result in c-myc overexpression in the cells.

To find any potential association between EpCAM/c-myc positivity and clinicopathological characteristics of patients, the distribution of EpCAM⁺/c-myc⁺ cases in different categories were analyzed. Statistically and importantly, a significant association between EpCAM⁺/c-myc⁺ and ER/PR and grade III of breast cancer was
observed. It could reflect the importance of EpCAM/c-myc overexpression in the progression of breast cancer since statistically, EpCAM\(^+\)/c-myc\(^+\) was observed significantly in patients with grade III of breast cancer.

Clinically, co-overexpression of EpCAM and c-myc might be important because of their association with grade III of breast cancer, and also their influence in breast cancer therapy. Moreover, EpCAM and c-myc proto-oncogenes are the most influential factors in stemness of breast cancer cells (Magee et al., 2012; Wong et al., 2008; Yilmaz et al., 2006). Cancer stem cells (CSCs) are the pathological stem cells possessing the proliferative and self-renewal capability, producing a rapidly dividing tumor mass. Although CSCs account for a small percentage of the tumor mass, they play a role in cancer maintenance, development, drug resistance, as well as cancer recurrence; therefore, these are valuable candidates to be targeted for cancer treatment (Sánchez-García et al., 2007). Taking advantage of catumaxomab which is only useful for the treatment of EpCAM\(^+\) carcinomas (Simon et al., 2013) and cisplatin chemotherapeutic agent which has a better impact on c-myc tumors (Sklar & Prochownik, 1991; Tanida et al., 2012) support the importance of distinguishing EpCAM\(^+\)/c-myc\(^+\) tumors. However, a broader study is required to assess this hypothesis.

The limitation of this study was the absence of a validated and standard technique such as western blot for all cases to provide us with an accurate criterion for comparing RT-qPCR results. In this study, only 66 samples were analyzed in terms of EpCAM protein expression level. The other drawback of this study was the extraction of RNA from heterogenic tumor specimens. Contamination of tumor cells by normal cells (such as blood vessels) can result in the dilution of EpCAM/c-myc positive tumor cells and thus may cover the overexpression status of target genes. Using laser microdissection for separating normal cells can help overcome this problem.

Our study indicates that EpCAM overexpression could contribute to identifying patients at risk for malignancy and might be used as a therapeutic target for monoclonal antibody-based therapies in patients with a positive status of EpCAM overexpression. EpCAM and c-myc are the most important genes undergoing overexpression in breast carcinoma and should be assessed as prognostic factors. They are also important in the prescription of the appropriate chemotherapy and/or monoclonal antibody treatment of EpCAM and c-myc breast cancers, respectively. Consequently, it seems logical to evaluate EpCAM and c-myc genes expression status in terms of breast cancer prognosis and treatment.
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Conflict of Interest: The authors declare that they have no conflict of interest.

6. References


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7. Tables

Table 1. Characteristics of benign (A) and malignant cases (B) based on EpCAM/c-myc expression status

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Table 2. Distribution of EpCAM/c-myc positive and negative cases in the same malignant samples

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8. Figure Legends

**Figure 1.** Comparison of EpCAM mRNA between control (normal), benign, primary and metastatic breast cases. Normal and metastatic, benign and metastatic and primary and metastatic tumors showed significantly different medians.
Figure 2. EpCAM protein expression level in breast tissue control sample, benign, primary and malignant breast tumors. EpCAM+ cases as well as each sample identification number are shown below each Immunoblot.
Figure 3. Comparison of c-myc mRNA between normal, benign, primary and metastatic breast cases. Like EpCAM, normal and metastatic, benign and metastatic and primary and metastatic breast tumors depicted the significantly different medians.
Figure 4-A. Comparison of EpCAM mRNA between benign and malignant cases. No significant difference was observed in median values of EpCAM mRNA levels between benign and malignant groups. B. Comparison of c-myc mRNA between benign and malignant cases. A significant difference was seen by comparing the median values of c-myc mRNA levels between benign and malignant groups.