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RESEARCH ARTICLE

Cooverexpression of *EpCAM* and *c-myc* genes in malignant breast tumours

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Abstract

The overexpression of epithelial cell adhesion molecule (EpCAM), a 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, cooverexpression of *EpCAM* and *c-myc* genes has not been investigated in breast cancer tissues, particularly in Iranian population. The aim of this study was to assess the expression of *EpCAM* and *c-myc* genes in malignant breast cancer tissues using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) followed by analyses of the association between the outcomes. In this study, 122 fresh tissues, including 104 malignant and 18 benign samples, were disrupted by mortar and pestle, and then the RNA was isolated from the samples and converted to cDNA. The relative expression levels of *EpCAM* and *c-myc* genes were measured by $2^{-\Delta\Delta Ct}$ method using RT-qPCR. EpCAM protein level was also assessed in 66 cases using Western blot technique. Using RT-qPCR method, our results showed that EpCAM was overexpressed in 48% of malignant and 11.1% of benign samples. 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 immunohistochemistry reports of the same samples studied in this study. Importantly, overexpression of EpCAM and c-myc was significantly associated and showed an agreement of 57.3%. This study demonstrated the cooverexpression of EpCAM and c-myc in breast tumours 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, both were associated with grade III of breast cancer.

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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 haemophilic 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), a transmembrane domain, and EpCAM intracellular domain (EpICD) (Baeuerle and 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) (Gastl *et al.* 2000; Spizzo *et al.* 2004, 2011; Ambrogi *et al.* 2012). Various mechanisms are thought to be associated with the double-faced behaviour 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

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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 (Göitlinger *et al.* 1986). Third, EpCAM has been reported to be hyperglycosylated in carcinoma tissues as compared with that of normal autologous epithelial cells (Pauli *et al.* 2003; Munz *et al.* 2007). 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 signalling events are facilitated by some cell–cell contacts (Fagotto and Gumbiner 1996) and by other membrane microdomains such as tetraspanin-enriched microdomains (TEMs), which may play a role as signalling 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 tumour progression (Ponta *et al.* 2003). Cloudin7 is another protein in the EpCAM-CD44V4V7-tetraspanins complex, which binds to EpCAM through a motif within the transmembrane domain, ultimately leading to the recruitment of EpCAM into TEMs (Ladwein *et al.* 2005; Kuhn *et al.* 2007; 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 tumourigenicity 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 tumours.

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 and 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; Lê *et al.* 1999; Scorilas *et al.* 1999; Chrzan *et al.* 2001; Naidu *et al.* 2002). The *cmyc* 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 tumours show resistance to cisplatin chemotherapy agent. Thus, the sensitivity to cisplatin is enhanced in tumour cells with low c-myc expression (Sklar and Prochownik 1991; Biroccio *et al.* 2001; Tanida *et al.* 2012).

Therefore, there is a pressing need to distinguish tumour cells expressing a high level of EpCAM and/or c-myc to exploit the benefits from catumaxomab and cisplatin chemotherapeutic agent, respectively. Along with this need, the purpose of this study was to analyse 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 show for the first time that EpCAM and c-myc were overexpressed in 48 and 39% of malignant breast cancer tissues in Iranian population. Further, the agreement of cooverexpression of these two oncogenes was 57.3%.

Materials and methods

Patients and samples

Fresh tumour tissue samples of 104 patients (72 primary and 32 metastatic breast tumours) 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. Further, 20 normal breast tissues were also provided from normal mammary tissues adjacent to the malignant tumours. 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 messenger RNA (mRNA) analysis, an incision of the fresh tumour was immediately frozen in liquid nitrogen after mastectomy operation followed by storing at –70°C, and the remaining section of the tumour was used in the pathology laboratory for determination breast cancer diagnosis and characteristics. Routine pathological variables consisting of age, tumour grading and type, and also immunohistochemically determination of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and oestrogen receptor (ER) status, obtained from Breast Cancer Research Center of Isfahan, listed in table 1.

RNA isolation and cDNA synthesis

For RNA isolation, 20 mg of fresh frozen sample was accurately dissected on ice from specimen of each patient. 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

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

		EpCAM			c-myc		
		Positive	Negative	Total	Positive	Negative	Total
Benign							
Type	Fibroadenoma	1	9	10	2	8	10
	Ductal or lobular hyperplasia	0	3	3	1	2	3
	Lobular carcinoma *in situ*	1	4	5	2	3	5
	Total	2	16	18	5	13	18
Age	50<	2	12	14	3	10	13
	50 ≥	0	4	4	2	3	5
	Total	2	16	18	5	13	18
Malignant							
Her2	Positive	12	13	25	6	19	25
	Negative	38	41	79	34	45	79
	Total	50	54	104	40	64	104
ER/PR	Positive	23	37	60	32	28	60
	Negative	27	17	44	8	36	44
	Total	50	54	104	40	64	104
Lymph node-status	Positive	36	29	65	29	36	65
	Negative	14	25	39	11	28	39
	Total	50	54	104	40	64	104
Grade	Grade I	8	8	16	3	13	16
	Grade II	16	30	46	16	30	46
	Grade III	26	16	42	21	21	42
	Total	50	54	104	40	64	104
Type	Metaplastic	5	0	5	2	3	5
	Carcinoma *in situ*	0	9	9	1	8	9
	Ductal	33	38	71	32	39	71
	Lobular	12	7	19	5	14	19
	Total	50	54	104	40	64	104
Age	50<	18	36	54	23	31	54
	50 ≥	32	18	50	17	33	50
	Total	50	54	104	40	64	104

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^{TM} First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). Synthesized total cDNA was finally stored at −70°C.

Primers

The set of GAPDH primers which was designed in the previous study was used for GAPDH cDNA propagation (Tabatabaeian and 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 (Seoul, South Korea). The sequence of primers to amplify EpCAM, c-myc and GAPDH cDNAs are listed as follow: EpCAM forward: 5′-TGTGGTTGTGGTGATAGCAGTT-3′, EpCAM reverse: 5′-CCCATCTCCTTTATCTCAGCCTTC-3′. c-myc forward: 5′-AGCGACTCTGAGGAGGAACAA-3′, c-myc reverse: 5′-GTGGGCTGTGAGGAGGTTTG-3′ and GAPDH forward: 5′-ACCATCTTCCAGGAGCGAGA-3′, GAPDH reverse: 5′-GCAAATGAGCCCCAGCCTTC-3′. The product length of amplified EpCAM, c-myc and GAPDH transcripts were 102, 136 and 115 bp, respectively.

RT-qPCR analysis

RT-qPCR: Applied Biosystem StepOnePlus^{TM} Real-Time PCR System and Maxima SYBR Green/ROX qPCR Master Mix (2×) 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 min at 95°C and 35 cycles including of a denaturation step at 95°C for 30 s, an annealing step at 68.3°C for 30 s and an extension step for 50 s 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.

Calculation of the amounts of target genes expression: The relative gene expression was measured by subtracting the C_t value of target (*EpCAM* and *c-myc*) and control (*GAPDH*) genes in the tumour samples relative to the normal samples by the $2^{-\Delta\Delta Ct}$ method (Livak and 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.

Standard curve

The $2^{-\Delta\Delta Ct}$ method can be used in a condition that the amplification efficiency of target and control genes are approximately identical and perfect. The r^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 calculating C_t values for amplification of all three cDNAs, their standard curves were plotted. In a standard curve, C_t values from each dilution versus the log cDNA concentration were plotted.

Protein analysis

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

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 in Skliris *et al.* (2002).

Western blot: Forty μg of whole cell lysates were separated using SDS/PAGE and transferred to a nitrocellulose membrane in a condition of 100 V for 90 min at 4°C. Blocking of membranes was done in blocking solution (5% nonfat milk powder, in TBST) at room temperature for 1 h followed by the incubation of membranes with the anti-EpCAM antibody (5% nonfat milk powder, in TBST), overnight at 4°C. After three washes with TBST, membranes were incubated with the secondary antibody for 1 h at room temperature in the blocking solution. EpCAM and GAPDH proteins were visualized, after three 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.

Statistical analysis

Data analyses were carried out with the SPSS statistical software, ver. 19.0 (SPSS, Chicago, USA). Fisher's exact test, Pearson's chi-squared test, McNemar, Kruskal–Wallis, Mann–Whitney U test and Kappa test were used for statistical analyses, a P value < 0.05 was regarded as statistically significant.

Results

EpCAM and c-myc overexpression in malignant and benign breast tumours

Among the 72 primary and 32 metastatic breast cancer tissues, 48% (50 of 104) were EpCAM-positive in overall, showing 2–8 fold increase in the mRNA copy, whereas 52% (54 of 104) were EpCAM-negative. The high overexpression rate of EpCAM is due to the metastatic group with 72% rate, while primary breast tumours displayed only 37.5% overexpression. On the other hand, 11.1% of benign tumours (2 of 18) were EpCAM-positive, reflecting 2–3 fold enhancement. Among the 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,

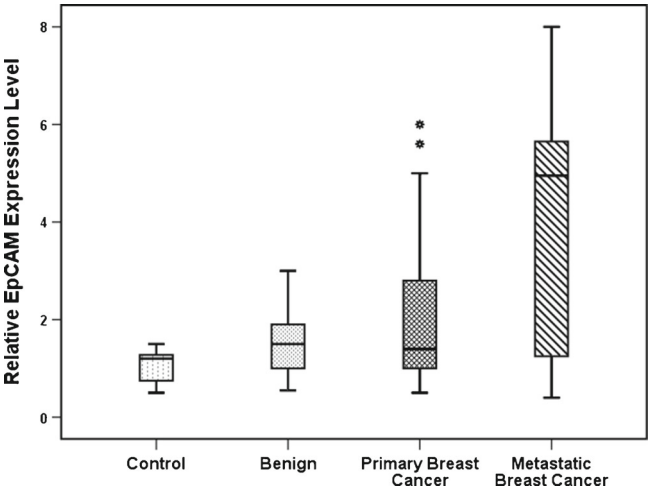

Figure 1. Comparison of EpCAM mRNA between control (normal), benign, primary and metastatic breast cases. Normal and metastatic, benign and metastatic, and primary and metastatic tumours showed significantly different medians.

respectively. Among benign tissues, five 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, $P < 0.001$) (figure 1). To know which groups have significantly different medians, Mann–Whitney test was recruited, by which, normal and metastatic ($P < 0.001$), benign and metastatic ($P = 0.006$) and primary and metastatic tumours ($P < 0.001$), showed significantly different medians.

To validate the accuracy of EpCAM overexpression detection, lysates were extracted from 66 available samples, including nine control samples, seven benign, 26 primary and 24 metastatic breast tumour specimens. Although, we did

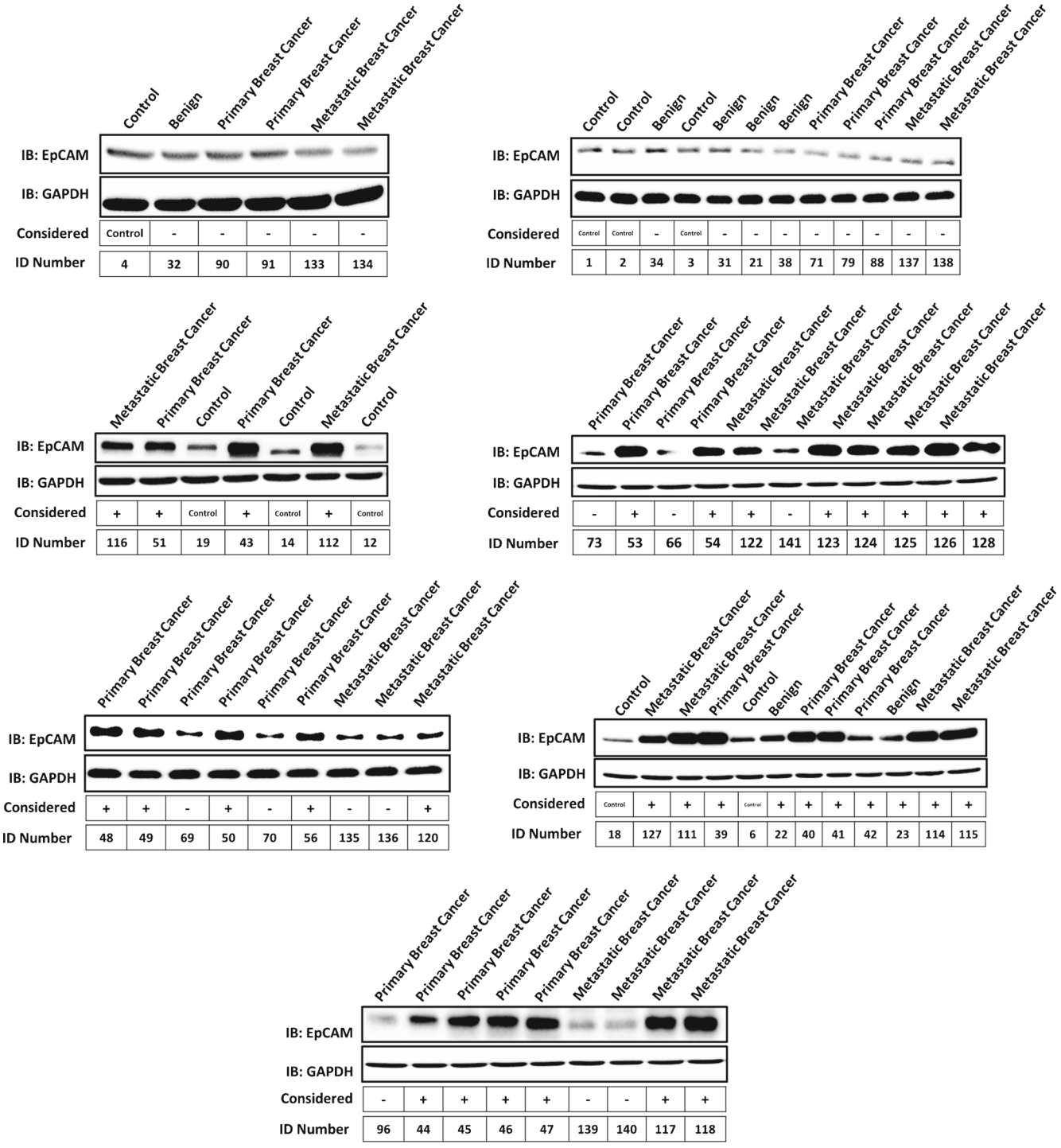

Figure 2. EpCAM protein expression level in breast tissue control sample, benign, primary and malignant breast tumours. EpCAM$^{+/-}$ cases as well as each sample identification number are shown below each immunoblot.

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 further analysis.

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

The significant association between EpCAM and c-myc cooverexpression

In total, 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 ($P < 0.01$; Kappa test). In addition, Pearson's chi-squared test showed a significant association between the positive EpCAM and c-myc status ($P < 0.001$), odds ratio = 17 (95% CI: 6.033–47.906).

Comparing mRNA levels in malignant and benign specimens

Among the 18 benign breast tumour samples studied by RT-qPCR, only two 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 ($P = 0.174$; Mann–Whitney U test) (figure 4a). On the other hand, c-myc mRNA overexpression was observed in five of 18 benign and 40 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 4b).

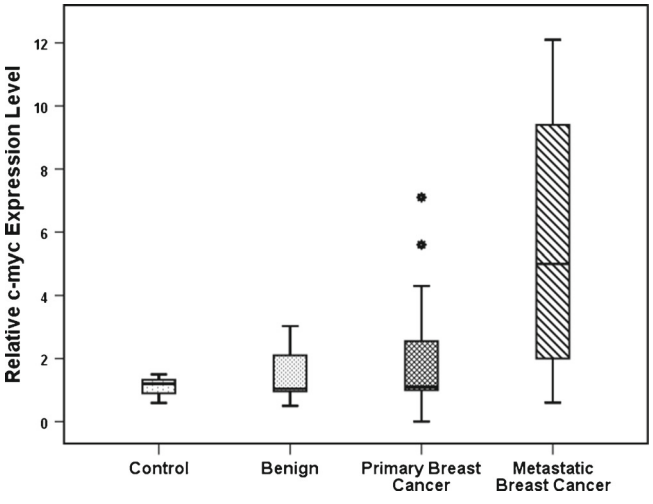

Figure 3. Comparison of c-myc mRNA between normal, benign, primary and metastatic breast cases. Like EpCAM, normal and metastatic, benign and metastatic, and primary and metastatic breast tumors depicted the significantly different medians.

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

		c-myc		
		Negative	Positive	Total
EpCAM	Negative	48	6	54
	Positive	16	34	50
	Total	64	40	104

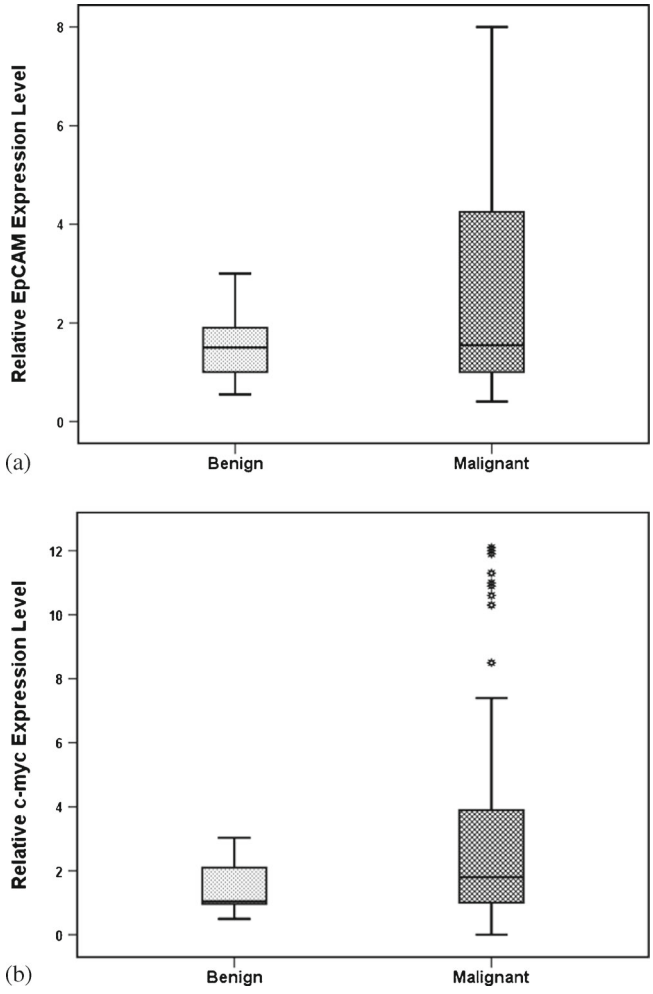

Figure 4. (a) Comparison of EpCAM mRNA between benign and malignant cases. No significant difference was observed in median values of EpCAM mRNA levels between benign and malignant groups. (b) Comparison of c-myc mRNA between benign and malignant cases. A significant difference was seen by comparing the median values of c-myc mRNA levels between benign and malignant groups.

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 $c\text{-}myc^+$ cases were statistically analysed 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's chi-squared test, $P = 0.02$), in which, EpCAM overexpression reduced the chance of ER/PR positivity, OR = 0.391(95% CI: 0.176–0.871). Likewise, c-myc and ER/PR positivity revealed the significant association (Pearson's chi-squared test, $P < 0.005$); however, c-myc positivity increased the chance of ER–PR positivity, OR = 5.143 (95% CI: 2.052–12.889).

Moreover, the comparison between EpCAM/c-myc positivity and HER2 status showed no significant association (Pearson's chi-squared test, $P = 0.993$ and $P = 0.088$ for EpCAM and c-myc, respectively). Likewise, there was no significant association between EpCAM/c-myc and positivity of lymph nodes (Pearson's chi-squared test, $P = 0.054$ and $P = 0.096$ 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's chi-squared test, $P = 0.016$), OR = 0.376 (95% CI: 0.376–0.838), while it increased the risk of grade III (Pearson's chi-squared test, $P = 0.020$), with OR of 2.573 (95% CI: 1.150–5.758). Grade I of breast cancer had no significant association with EpCAM overexpression ($P = 0.867$). Further, c-myc positivity only had a significant association with elevated risk of being in grade III of breast cancer (Pearson's chi-squared test, $P = 0.047$) with an OR 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 tumours depicted the significant association of EpCAM overexpression and metaplastic/CIS subgroups. In fact, EpCAM overexpression increased the chance of being in a metaplastic group (Fisher's exact test, $P = 0.023$), OR = 2.2 (95% CI: 1.773–2.730), whereas EpCAM negativity enhanced the risk of CIS tumours (Fisher's exact test, $P = 0.003$), OR = 2.111 (95% CI: 1.708–2.610). C-myc, however, was significantly overexpressed in ductal breast tumours (Pearson's chi-squared test, $P = 0.042$), OR = 2.564 (95% CI: 1.019–6.455).

Discussion

Breast cancer is the most prevalent cancer among Iranian women (Mousavi *et al.* 2009); and our recent study has shown the upregulation 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% (Schmidt *et al.* 2008; Niemiec *et al.* 2012; El-Maqsoud and El-Rehim 2014). This dramatic fluctuation that are 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 outcome, 50 of 104 (48%) malignant breast tumour samples showed overexpressed *EpCAM* gene. EpCAM overexpression was also observed in two of 18 (11.1%) benign cases. Hypothetically, it might show the premalignancy 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.* (2010) found strong (IHC 3+) EpCAM expression in 38% of the benign insulinomas, suggesting that it could contribute to distinguishing high-risk patients for malignancy.

The c-myc overexpression rate has been reported between 12 and 100% rates in different studies, which may be due to different antibodies and/or techniques, the assigned cut-off point, molecular subtype of the breast tumour, and patient heterogeneity (Liao and Dickson 2000; Lutz *et al.* 2002; Xu *et al.* 2010). According to our RT-qPCR results, 41 of 104 (39%) malignant samples showed c-myc overexpression. This rate of c-myc overexpression is in agreement with the study by Lê *et al.* (1999) showing 35% overexpression of c-myc using RT-qPCR method. 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.* (1986) and Spandidos *et al.* (1986) reporting the overexpression of *c-myc* gene in fibroadenoma and fibrocystic benign tumours. This strongly indicates the role of c-myc overexpression in breast tumours, thereby could introduce c-myc as a premalignancy biomarker (Liao and Dickson 2000).

Based on the RT-qPCR method, among the 18 benign breast tumour 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 two 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 of 104 tumour 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 cooverexpression 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 well as showing the significant association ($P < 0.05$, Fisher's exact test). This report strongly supports that c-myc is one of the most important targets of EpCAM and undergoes upregulation followed by EpCAM overexpression in breast cancer. Likewise, studies by Münz *et al.* (2004) and Martowicz *et al.* (2013) reported the correlation between cooverexpression of *EpCAM* and *c-myc* genes in HEK-293 and MCF10A cell lines. 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 upregulated. 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 histone H3-K9-methylation and hypoacetylation 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 and Alves 2008). Therefore, c-Myc transcription is regulated by many factors and despite the important role of EpCAM in c-myc upregulation, 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 $EpCAM^+$/$c\text{-}myc^+$ cases in different categories were analysed. Statistically and importantly, a significant association between $EpCAM^+$/$c\text{-}myc^+$ 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, $EpCAM^+$/$c\text{-}myc^+$ was observed significantly in patients with grade III of breast cancer.

Clinically, cooverexpression 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 (Yilmaz *et al.* 2006; Wong *et al.* 2008; Magee *et al.* 2012). Cancer stem cells (CSCs) are the pathological stem cells possessing the proliferative and selfrenewal capability, producing a rapidly dividing tumour mass. Although CSCs account for a small percentage of the tumour 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 $c\text{-}myc^-$ tumours (Sklar and Prochownik 1991; Tanida *et al.* 2012) support the importance of distinguishing $EpCAM^+$/$c\text{-}myc^+$ tumours. 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 analysed in terms of EpCAM protein expression level. The other drawback of this study was the extraction of RNA from heterogenic tumour specimens. Contamination of tumour cells by normal cells (such as blood vessels) can result in the dilution of EpCAM/c-myc positive tumour 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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