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Relationship Between PIK3CA Amplification and P110α and CD34 Tissue Expression as Angiogenesis Markers in Iranian Women with Sporadic Breast Cancer

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ABSTRACT

Background and Objective: The PI3K/AKT/mTOR pathway is known to play an important role in regulating angiogenesis both in normal and breast cancer (BC) tissues. *PIK3CA* amplification was reported in various malignancies, including approximately 10% of BC cases. The aim of this study was to identify the frequency of *PIK3CA* amplification in Iranian female patients suffering from BC. Additionally, possible association between *PIK3CA* amplification and P110α expression with microvascular density (MVD) was examined.

Methods: DNA samples were extracted from paraffin embedded tumor tissue blocks and copy number changes were evaluated by MLPA Technique. The results were analyzed by coffalyzer software. The tissue expression of P110α and CD34 was assessed using immunohistochemistry.

Results: Ten out of 40 samples (17.5%) showed amplification in *PIK3CA* gene and 22 out of 40 samples (55%) showed overexpression in P110α. For CD34, from 40 samples, 20 (50%), 15 (37.5%) and 5 (12.5%) had scores 1+, 2+ and 3+, respectively.

Conclusion: No significant association was detected between gain of *PIK3CA* copy number and P110α or CD34 tissue expression.

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Introduction

Breast cancer (BC) is the most common cancer and the leading cause of cancer death in women worldwide (1, 2). In Iran, female BC patients are affected at least 10 years earlier (average age of diagnosis, 47.1-48.8 years old) than those in Western countries (3). The majority of BC-related deaths are the result of metastasis to other organs but not the primary tumor (4). Angiogenesis, the formation of new blood vessels from the existing vasculature, plays an essential role in tumor growth and both local and distant metastasis in breast cancer (5). Different studies have shown that the spread of tumor cells is related to intratumoral microvessel density (MVD). They have also reported that increased MVD is associated with higher incidences of metastasis and poor prognosis in a variety of malignancies, including BC (6-8). The CD34, a transmembrane glycoprotein related to capillary endothelial cells, is a useful angiogenesis marker. Thus the identification of microvessels using CD34 immunohistochemistry can help to identify more aggressive breast tumors (9).

In addition, the PI3K/AKT/mTOR pathway is known to play an important role in regulating angiogenesis both in normal and cancerous tissues (10). Deregulated PI3K/Akt pathway is associated with several human malignancies. Numerous studies have shown that PI3K/AKT/mTOR pathway plays a critical role in cellular functions, including proliferation, adhesion, invasion, growth, and survival (11-13). The PI3K proteins are a family of lipid kinase enzymes that are activated in response to growth factor recep-

tor tyrosine kinases (RTKs) and G-protein-coupled receptor signaling (14). There are three classes of PI3K that catalyze the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3): I, II, and III. The class I PI3Ks consists of two subgroups, IA and IB. Class IA PI3K is consisted of two subunits: a catalytic domain named p110 and p85 regulatory domain (12). Catalytic subunit (P110α) is encoded by *PIK3CA* gene which is located on 3q26. *PIK3CA* is the most frequently implicated gene in human cancer and has been shown to harbor oncogenic mutations or amplifications (15, 16).

The gene amplification of *PIK3CA* was reported in about 10% of BC cases (17). It has also been shown that increased copy number of *PIK3CA* is occurred in different epithelial tumors including ovarian cancer (18), cervical cancer (19), brain tumors (20), gastric carcinoma (21), head and neck squamous cell carcinoma (22) and non-small cell lung carcinoma (23). The vast majority of studies have focused on better understanding of the molecular mechanisms of BC; however, the molecular mechanisms leading to the BC are not fully cleared at present. The study of the genes involved in BC has allowed to improve the existing therapies or to develop effective and new targeted therapies. In this study, we aimed to identify the frequency of *PIK3CA* amplification in Iranian female patients with BC. Additionally, possible association between *PIK3CA* amplification and P110α expression with MVD was examined.

Materials and Methods

Patients and clinical samples

Total of 40 Formalin-fixed, paraffin-embedded (FFPE) breast carcinoma tissue samples was obtained from patients who undergone surgery at Mehrad Hospital (Tehran, Iran) between Jan 2011 and Dec 2012. The samples were selected regardless of age, stage or histological subtype. Normal control tissue samples were obtained from cosmetic and breast reduction surgeries. The consent form was obtained from the participants and the study was approved by Ethics Committee of University of Social Welfare and Rehabilitation Sciences (USWR).

DNA Isolation

DNA was extracted from FFPE tumor and normal breast tissue samples using High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's protocol. The quality and integrity of DNAs was evaluated by agarose gel electrophoresis. The concentration of DNAs was assessed using NanorDop ND2000 spectrophotometer.

Multiplex ligation-dependent probe amplification (MLPA)

DNA samples were amplified by MLPA technique using the P458-B1 Gastric Cancer kit from MRC-Holland according to the manufacturer's protocol. This kit can be used for BC samples as well. This P458-B1 probemix contains 46 probes for the 16 genes including *PIK3CA*. Probes of *PIK3CA* have been designed for the exons 2, 7 and 19. In addition, 15 reference probes have been included in this probemix, detecting autosomal chromosomal locations which are relatively stable by copy number. MLPA was performed by the following steps , **day one:** DNA denaturation at 98°C for 5 min, hybridization reaction at 60ºC for 16-20 hr , **day two:** ligation reaction at 54ºC for 15 min then 5 min at 98ºC for heat inactivation of ligase enzyme and the last step of reaction for 35 cycles. Then PCR products were separated on an ABI3130-XL capillary sequencer. Interpretation of the results was performed according to the manufacturer's guideline. The cut-off values between 0.7 and 1.3 were considered as normal. The values between 1.3 and 2.15 were referred to as low copy amplification (duplication) and the values over 2 were interpreted as high level amplification.

Immunohistochemistry (IHC)

Paraffin-embedded tissue sections with 4-5 μm thickness were obtained using microtome (LEICA RM2135) and mounted on slides. After slide preparation, samples were stained with the specific antibodies. For P110α immunohistostainig, the slides were placed in 37ºC incubator overnight then deparaffinized using xylen and rehydrated in graded alcohols. Antigen retrieval was performed through heating

slides in Tris/EDTA buffer pH 9.0. The slides were then incubated overnight with anti-p110α antibody (Abcam, ab71090) at 4ºC. After incubation, the sections were washed in PBS. Endogenous peroxidase activity was blocked with 2% H_2O_2. Then the slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The immunoreactivity was detected using 3,3' diaminobenzidine (DAB) as a chromogen and the tissue slides were counterstained with hematoxylin. Scoring was performed based on the intensity of the stained cells: no staining (0), weak staining (1+), moderate staining (2+), and high staining (3+).

The CD34 immunohistostaining was also performed to assess tumor angiogenesis using Dako, Clone: QBEnd 10 kit according to the manufacturer's protocol. For scoring CD34, with a light microscope under low magnification, 4 hotspots were determined and microvessels were counted under high magnification and the average MVD of that sample was determined. The results were reported as: score 1+: low (0- 19.9), score 2+: moderate (20-29.9) and score 3+: high MVD (more than 40).

Normal controls and negative controls (staining without primary antibody) were also included in the experiment.

Statistical analysis

Chi-square and Fisher-exact tests were used to study the relationship between *PIK3CA* amplification and P110α and CD34 overexpression. The results at $P < 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS statistical software package (SPSS Inc).

Results

In terms of histological subtype, all patients were included in the category of invasive ductal carcinoma (IDC). The age of patients in this study ranged between 31 and 70 years. Of 40 patients, 24 (60%) were under 50 years old and 32 (80%) were in stage II.

Copy number analysis o*f PIK3CA.*

PIK3CA amplification was identified in 7 of 40 samples (17.5%). All of the samples showed low copy amplification. Seventy percent of the patients with amplification in *PIK3CA* were under 50 years old and all of them were in stage II.

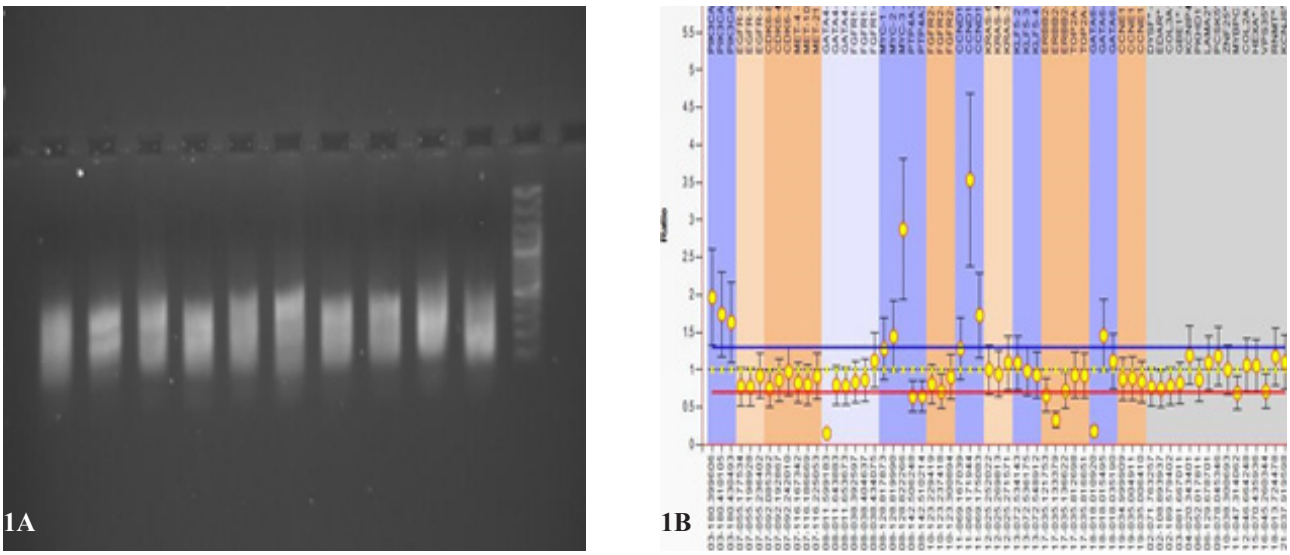

Figure1. A: Electrophoresis of samples on 2% agarose gel B: MLPA result of a sample on coffalyser software which shows amplification of *PIK3CA*

Immunohistochemical analysis of P110α

Since the epithelial layer in some normal breast tissues showed weak cytoplasmic staining, score 0 and 1+ were grouped as negative and tissues with scores 2+ and 3+ were considered to have overexpression. Positive immunoreaction for P110α was detectable in 22 of 40 samples (55%). Only 2 samples with amplification in *PIK3CA* were positive for P110α overexpression (28.6%). We found no significant correlation between PIK3CA amplification and P110α overexpression (P>0.05).

Immunohistochemical analysis of CD34

Twenty of 40 samples (50%) had score 1+, 15 (37.5%) had score 2+ and 5 (12.5%) had score 3+. Of 7 cases with amplification, 4 (57.1%) were low MVD (score 1+), 2 (28.6%) were moderate MVD and 1 (14.3%) was high MVD. This is also worth mentioning that no significant relationship was found between PIK3CA amplification and CD34 expression (P>0.05).

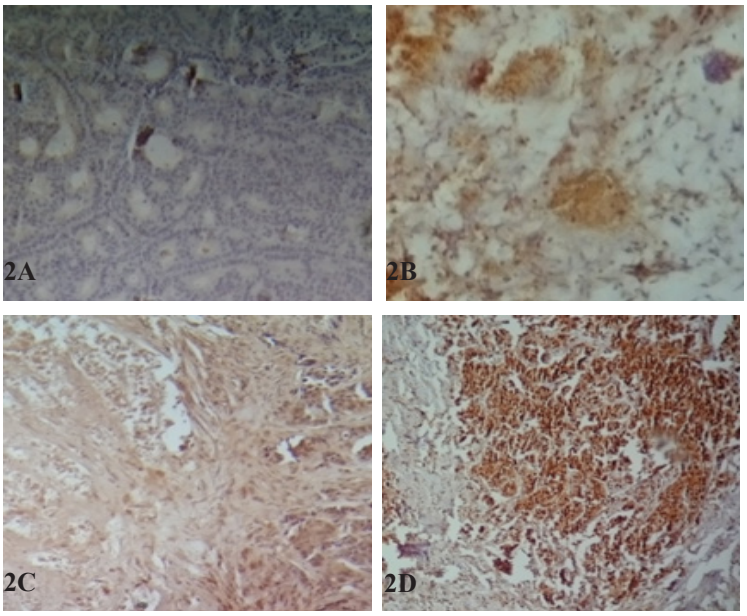

Figure 2. Immunohistochemical staining of P110α on tumor tissues. A: score 0, B: score 1+, C: score 2+, D: score 3+

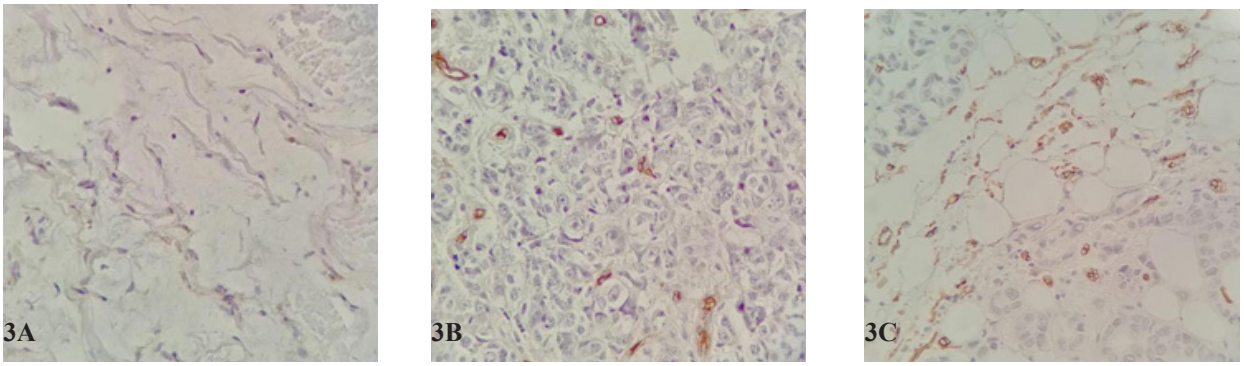

Figure 3. Immunohistochemical staining of P110α on tumor tissues. A: score 1+, B: score 2+, C: score3+

Discussion

To our knowledge, the current study was the first report on the copy number variation of *PIK3CA* in Iranian female patients with breast cancer. We demonstrated the gene amplification of *PIK3CA* in 17.5% of the cases. This result was consistent with data previous published on breast cancer.

Gonzalez-Angelo et al. in 2013 reported that the fre-

quency of *PIK3CA* amplification in BC patients was 13% and gain of *PIK3CA* copy number was related to poor prognosis in BC patients (24). In another study performed by Goujun et al. in 2005, gain of PIK3CA copy number was reported at 8.7% (25).

We then assessed the P110α expression using IHC to explore whether the *PIK3CA* amplification could lead to P110α overexpression. Our data showed overexpression in 55% of cases and 28.6% of cases with amplification in *PIK3CA*. There was no significant association between *PIK3CA* amplification and P110α expression (P>0.05). Published data on association between *PIK3CA* amplification and its protein expression (P110α) varies in different studies and various cancers (Table1). This inconsistency might be due to the modulation of the protein level by transcriptional, post-transcriptional or post-translational mechanisms. For example, it has been shown that the tumor suppressor protein, p53, the oncoprotein Y-box binding protein-1, forkhead box O3a, and nuclear factor-kappaB (NF-κB) regulate transcription of *PIK3CA* (26, 27).

Table 1. Similar studies on different cancers

Study	Cancer	Objective	Results
Yong Lin, et al 2009 (28)	Pituitary carcinoma	CNV detection of *PIK3CA* by real-time PCR and pi3k expression by IHC	*20-40% amplification *Samples with 7 copies showed positive staining
Ichiro Akagi, 2008 (29)	Esophageal cancer	CNV detection of *PIK3CA* by real-time PCR and pi3k expression by IHC	*28% amplification *50% overexpression *No relationship between amp and overexpression
Wui Kien, 2016 (30)	NPC	CNV detection of PIK3CA by Q- PCR and pi3k expression by IHC	20% amplification *44% overexpression *Relationship between amplification and overexpression

In order to investigate the relationship between angiogenesis and PIK3CA and P110α genes, we evaluated the association between PIK3CA amplification and P110α overexpression with MVD. Our data showed no significant relationship between these variables (P>0.05).

However, it is possible that mechanisms rather than amplification in *PIK3CA* gene have caused overexpression in P110α protein in our patients. Further studies with larger sample sizes and different detection methods are required to add more conclusive data which can provide prognostic prediction and ther peutic implications.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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