RESEARCH ARTICLE

Angiogenesis Markers in Breast Cancer - Potentially Useful Tools for Priority Setting of Anti-Angiogenic Agents

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Abstract

Background: Breast cancer is the most common malignancy among women in both developed and developing countries. The burden is increasing in low-income and middle-income countries (LMCs) and threatens the public health of such societies. Introduction of expensive monoclonal antibodies to cancer treatment regimens poses a real challenge in the health systems of LMCs. Despite controversy of cost-effectiveness of bevacizumab in breast cancer, some studies indicate gain of patients from this drug. The present study aimed to propose a priority setting model for administration of anti-angiogenic agents in breast cancer via assessment of tumor angiogenesis by the microvessel density (MVD) method and associations with clinicopathological characteristics (including simultaneous mutations of TP53 and HER-2 genes). Materials and Methods: Age, axillary lymph nodes status, tumor size, stage and grade, estrogen and progesterone receptors status, HER-2/neu status (by immunohistochemistry and FISH test), TP53 mutation, Ki-67 (for proliferation assay) and CD34 (for angiogenesis assay) were assessed in 111 breast cancer patients. The molecular subtype of each tumor was also determined and correlations of simultaneous mutations of HER-2 and p53 genes with angiogenesis and other clinicopathological characteristics were evaluated. Results: There were significant associations between simultaneous mutations of HER-2 and p53 genes and all other parameters except tumor size. The degree of angiogenesis in the ERBB2 subtype was greater than the others. Younger patients showed a higher angiogenesis rate rather those older than 50 years. Conclusions: Our results demonstrated that patients with simultaneous mutations of HER-2 and p53 genes, those with ERBB2 molecular subtype and also younger women (often triple negative) seem more eligible for obtaining anti-angiogenic agents. These results suggest a model for priority setting of patients with breast cancer for treatment with anti-angiogenic drugs in LMCs.

Keywords: Breast cancer - low-income and middle-income countries - anti-angiogenic agents - microvessel density

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Introduction

Breast cancer is the most common malignancy among women in both developed and developing countries. In the United States, the mortality rate of the disease has decreased into 2.2% per year during 1990-2007, which is attributed to screening programs and new therapeutic strategies (Altekruse et al., 2010). However, the global burden of breast cancer is increasing in low-income and middle-income countries (LMCs) and it is considered as a threatening factor for their health systems (El Saghir et al., 2011; Story HL et al., 2012). It's a common misconception that the incidence of breast cancer is high in developed countries. The statistics, however, indicate a bitter truth that the mortality rate of breast cancer is increasing in LMCs because constraints on financial resources in these countries undermine screening managements, and thus the disease is detected in late stages, resulting in increased mortality risk (Pedraza et al., 2012).

In recent years, monoclonal antibodies (mAB) have been included in the treatment protocols of some types of cancer. Although more than a decade has passed since Food and Drug Administration (FDA) approved trastuzumab (Herceptin®, made by Genentech) for breast cancer, there have been some challenges in using this drug due to its high cost (Refaat et al., 2013). It is expected that the number of mABs for the treatment of breast cancer increases. The high expenses of these drugs in one hand, and their effectiveness as well as their side effects on the other hand have made them to be administered based on the cost-effectiveness analysis, even in developed countries. Therefore, its administration to cancer patients in LMCs has always been controversial, for which various global, regional and national guidelines have

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been developed (Yip et al., 2008; Gligorov et al., 2011; Rouhollahi et al., 2013).

According to the World Bank's ranking, Iran, is considered as a upper-middle-income country (UMICs) (World bank., 2010). In Iran, according to the Breast Health Global Initiative Guideline (BHGI-2008) (Yip et al., 2008; El Saghir et al., 2011), which is a resource-sensitive guideline, HER-2/neu assessment and trastuzumab treatment are recommended for breast cancer. Several studies in Iran show that both the incidence of breast cancer and its mortality rate are increasing (Mousavi et al., 2006; Mousavi et al., 2009; Taghavi et al., 2012). On the other hand, the direct medical costs for treatment of breast cancer is very high and therefore, the burden of disease is increasing in our country (Davari et al., 2013). The relationships between tumor angiogenesis and HER-2/neu gene mutation as well as TP53 gene somatic mutation and increased tumor angiogenesis are clearly shown in breast cancer (Kumar et al., 2001; Sopel et al., 2005; Wen et al., 2006; Teodoro et al., 2008; Biesaga et al., 2012). The present study aims to determine how angiogenesis and other clinicopathological characteristics of patients will change if HER-2/neu and TP53 genes mutation simultaneously occurs in breast cancer. On the other hand, if the government policy of UMICs on breast cancer treatment is made to allocate financial resources and support anti-angiogenic drugs such as bevacizumab (Avastin®, made by Genentech), how can it be effective as a scientific criteria for priority-setting?.

Materials and Methods

With relevant ethical considerations, the samples were taken from a hospital in Tehran (Mehrad General Hospital) from April 2011 to August 2012 and then transferred to the Pathology Laboratory of Genetics Research Center (GRC) at the University of Social Welfare and Rehabilitation. The inclusion criteria of this cross-sectional study were determined as follows: (a) Only woman's breast malignant tumors were included; (b) It should be a primary tumor; (c) It should be an invasive tumor; (e) Patients should not have received neoadjuvant chemotherapy; (f) Cold ischemic time and fixation time of the samples should have been carried out according to common protocols; (g) Patients' demographic and clinical characteristics must be specified. Overall, of 121 samples taken, 111 samples were included into the study.

After the samples were coded, tissue processing was performed and paraffin blocks were prepared. Some 4-microns thick sections were prepared from the samples and then stained with H&E and ER (Dako, colone: 1D5), PR (Dako, clone: PgR 636), HER-2 (Dako, clone: mAB), P53(Dako, clone: DO-7), Ki-67 (Dako, Clone: MIB-1) and CD34 (Dako, Clone: QBEnd 10) immunohistochemistry (IHC) markers according to the manufacturer's protocol, respectively. The prepared slides were studied by pathologist and the equivalent results of HER-2 IHC were sent to the cytogenetics ward of Genetics Research Center for FISH studies using HER-2 IHC pharDx kits(Dako, pharmDx™) and all sample preparation steps were carried out according to the manufacturer's brochure prep. considering the following protocols :

Histological evaluation:

1-1 Determining tumor histotype and grade: After determining tumor histotype, tumor grading was carried out using Nottingham system method.

1-2 HER-2/neu status: The results of IHC and FISH were evaluated according to the ASCO/CAP 2007 guideline (Antonio C et al., 2007).

1-3 ER & PR status: Hormone receptors were assessed according to the ASCO/CAP 2010 guideline (Hammond et al., 2010).

1-4 TP53 gene status: Positive and negative criteria were based on counting 1000 epithelial cells in 10 random fields and ratio of immunoreactivity. The cut-off for positivity was determined as 10% (Kobayashi et al., 2013).

1-5 Assessment of tumor angiogenesis using microvessel density (MVD-CD34): With a light microscope under low magnification, 4 hotspots were determined and microvesseles were counted under high magnification and the average MVD of that sample was determined. The results were reported as: Low MVD (0-19.9), Moderated MVD (20-29.9) and High MVD (more than 40) (Dhakal et al., 2009).

1-6 Assessment of tumor proliferation using Ki-67: Under a light microscope, 1000 epithelial cells in 10 random fields were counted and percentage of immunoreactivity was obtained. The results were reported as: low Ki-67 (0-4.9%), moderated Ki-67 (5-14.9%) and high Ki-67 (more than 15%) (Goldhirsch et al., 2011).

1-7 Molecular subtypes in breast cancer were assessed according to St. Gallen 2011 guideline: from the results of HER-2 gene status, ER & PR status and Ki-67 status. Next, they were classified into 4 groups, namely Luminal A, Luminal B, ERBB2 and Triple negative (Goldhirsch et al., 2011).

Clinical assessments

Patients' age, affected side of breast, type of surgery, tumor size, lymph node involvement by pN and absence or presence of metastasis were extracted from their records, and then tumor staging was carried out by TNM (AJCC 2009) system.

Statistical analysis

One-sample kolmogorov-smirnov test was used to find the relationship between simultaneous HER-2 and TP53 mutations and other factors. Moreover, the relationships between simultaneous HER-2 and TP53 mutations and other factors were assessed using Spearman's correlation coefficient. The relationship between age (below and above 50 years) and angiogenesis was determined using Chi-square test. Moreover, one-way ANOVA followed by Tukey's test was performed to compare the angiogenesis between molecular subtypes of breast cancer. Data were represented as mean ± standard error of the mean (SEM). In the present study, the significance level was set at 0.05. All statistical analyses were performed using SPSS version 18.

Results

The age of patients in this study ranged between 31 and 70 years (average age=49.2±1.9). Left breast was involved 55% more than right breast. In terms of surgical method, 62.1% of the patients underwent breast-conserving surgery (BSE) and the rest underwent modified radical mastectomy (MRM). In terms of tumor size, the T2 group with 64.9% involvement had a higher place than the rest. In terms of axillary lymph node status, 46.9% were involved and the staging investigation showed that 70.3% of the patients were in stage II. Histologically, Invasive ductal carcinoma (IDC) was the most frequent type (with 83.8% frequency). From the viewpoint of tumor grading, grade II with 69.1% is located in the prime position (Table 1).

IHC and FISH tests of HER-2 showed that overexpression or amplification of this gene occurred in 22.5% of the patients and they needed to receive Herceptin. TP53 gene mutation was found in 33.3% of the patients and the average percentage of P53-positives was reported to be 7.7±15.1. MVD-CD34 results showed that angiogenesis index in this study was 26.1±12.9 mm-1, the low MVD group was the most frequent (with 44.1% frequency) and the moderated MVD (42.3%) and high MVD (13.6%) groups were the least frequent (Table 1). Ki-67 IHC results showed that the mean tumor proliferation index was 9.7% ± 8.6% and 45.0% of the tumors were in the moderated Ki-67 group (Table 1).

In terms of hormone receptor status, 71.1% were ER positive and 64.7% were PR positive, while 59.4% of the patients were both ER and PR positive. (Table 1).

In terms of molecular subtypes, 47.8%, 26.1%, 9.9% and 16.2% of the patients were in luminal A, luminal B, ERBB2 and Triple negative, respectively (Table 1). Figure -1 shows the average age of each group.

Our study showed that HER-2 and TP53 mutations occurred in 14.1% of the patients simultaneously. Statistical analysis results showed that there was a relationship between HER-2 positivity and tumor staging (p=0.021), Tumor size (p=0.038), lymph node status (p=0.007), MVD-CD34 (p=0.001), and Ki-67 (p=0.001) (Table 3). On the other hand, there is a positive statistical association between P53 positive breast cancer and tumor grading (p=0.036), lymph node status (p=0.011), MVD-CD34 (p=0.002), and Ki-67 (p=0.004) (Table 2).

In HER-2 and TP53 simultaneous mutation, there was a correlation between tumor stage (p=0.041), lymph node status (p=0.005), tumor grade (p=0.044), MVD-CD34 (p=0.001) and Ki-67 (p=0.002), as determined

Table 1. Indicates the Clinicopathological Characteristics of 111 Breast Cancer Patients

		n=111
Age (31-70 years)	> 50	53 (47.7 %)
	≤ 50	58 (52.3%)
Side of involved breast	Left breast	61 (55%)
	Right breast	50 (45%)
Surgical method	MRM	42 (37.9%)
	BCS	69 (62.1%)
Tumor size	T1	34 (30.6%)
	T2	72 (64.9%)
	T3	5 (4.5%)
	T4	0 (0.0%)
Axillary lymph nodes	Free	52 (46.8%)
	Involve	59 (53.2%)
Tumor Staging	I	16 (14.4%)
	II	78 (70.3%)
	III	17 (15.3%)
	IV	0 (0.0%)
Histology Type	IDCa	93 (83.8%)
	ILCa	8 (7.2%)
	Others	10 (9.0%)
Tumor grading	I	21 (18.9%)
	II	76 (68.5%)
	III	14 (12.6%)
HER-2/neu Status	Positive	25 (22.5%)
	Negative	86 (77.5%)
TP53 gene Status	Positive	37 (33.3%)
	Negative	74 (66.7%)
Ki-67 Status	Low	42 (37.8%)
	Moderate	52 (46.9%)
	High	17 (15.3%)
MVD-CD34 status	Low	49 (44.1%)
	Moderate	47 (42.3%)
	High	15 (13.6%)
ER status	Positive	79 (71.2%)
	Negative	32 (28.8%)
PR status	Positive	71 (64.0%)
	Negative	40 (36.0%)
Molecular subtypes	Luminal A	53 (47.8%)
	Luminal B	29 (26.1%)
	ERBB2	11 (9.9%)
	Triple negative	18 (16.2%)

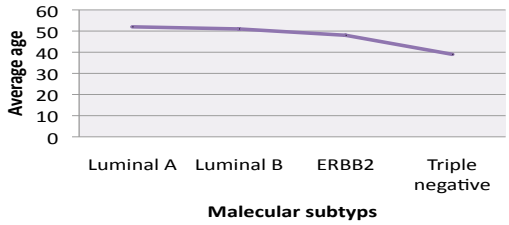

Figure 1. Illustrates the Average Age of Patients in Each Molecular Subtype of Breast Cancer

Table 3. Indicates the Association of Age and Angiogenesis in Women Under and Over 50 years

Tumor Angiogenesis	Above 50-years	(p=0.019)
	Under 50-years	

Table 2. Demonstrates the Associations of HER-2 and p53 with Clinicopathological Characteristics and their sSimultaneous Mutations with Clinicopathological Features

n=111	Tumor size	Axillary lymph nodes	Tumor Staging	Tumor grading	Ki-67 Status	MVD-CD34 status
HER-2 positive (n=25)	(p=0.038)	(p=0. 007)	(p=0.021)	NS*	(p=0.001)	(p=0.001)
p53 positive (n=37)	NS*	(p=0.011)	NS*	(p=0.036)	(p=0.004)	(p=0.002)
HER-2 positive+p53 positive (n=13)	NS*	(p=0.005)	(p=0.041)	(p=0.044)	(p=0.002)	(p=0.001)

*No Significance

by Spearman's correlation test; however, there was no significant correlation between the mentioned simultaneous mutations with tumor size (Table 2).

The comparison of the results of angiogenesis assay between the molecular subtypes of breast cancer showed that ERBB2 subtype (p=0.002) had the highest amount of angiogenesis and there was no significant difference between luminal B (p=0.791) and triple negative (p=0.683) subtypes. On the other hand, luminal A had the lowest amount of angiogenesis between the four molecular subtypes (p=0.003).

As showed in Table-3, there was a statistically significant difference between angiogenesis and the ages under and above 50 years (p=0.019) Moreover, the intensity of angiogenesis was higher in the under 50-year group than in other groups.

Discussion

Statistics indicate that global burden of disease (GBD) of various cancers is increasing in developing countries and this ratio is predicted to increase in the coming decades (Al-Foheidi et al., 2013). It is estimated that by 2008, 690000 out of 1380000 new cases of breast cancer are in developing countries. It is also estimated that more than 269000 out of 480000 deaths caused by breast cancer happen in developing countries. This is due to the fact that survival rate has improved in developed countries (Yip et al., 2008; El Saghir et al., 2011; Story HL et al., 2012).

Health equity and access to health care services is one of the most important challenges for health systems worldwide, especially in LMCs. Given the limited financial resources in these countries, the ultimate goal is to provide patients with the most appropriate treatment. Subscribing and supplying expensive drugs for the treatment of cancers, especially mABs, have caused many problems (Antonio C et al., 2007). In one hand, patients need the access to new treatments and on the other hand, using these drugs in the treatment cycle reduces the financial resources in developing countries, causing severe and irreparable damages to their health systems. To solve this problem, specialists have recommended priority-setting strategies in treatment. Since 2002, BHGI has provided some guidelines on early detection, diagnosis and treatment of breast cancer in developing countries. The main reason to develop a separate guideline by BHGI was that resource constraint in LMCs had not been considered in the developed guidelines (Yip et al., 2008).

In January 2005, a conference was organized by the NCI's Office of International Affairs in Maryland, USA, in which international experts from 33 countries of 5 continents participated. First, the principle called Best Practices with Limited Resources was explained by including the concepts of medical ethics, international health, pharmacoeconomics and sociology. Finally, the guidelines were determined based on the allocation of resources in four levels (basic, limited, enhanced and maximal) and the concepts of these levels were accurately defined (The Breast Health Global Initiative., 2013). Providing these four levels by BHGI was later used as a model for other resource sensitive guidelines.

In the present study, we tried to discuss the priority-setting in breast cancer in terms of taking anti-angiogenesis drugs. One of the objectives of targeted cancer therapies is to decrease or stop tumor angiogenesis. Bevacizumab is one of the most important anti-angiogenic drugs for cancers, which was limited to be used by FDA in 2011 due to lack of strong evidence-based research (Lenzer et al., 2011). However, in two systematic review studies, Xu et al. (2013) and LEE et al. (2011) showed that Avastin treatment is good for the patients with metastatic breast cancer (MBC) who are HER-2 negative (Lee et al., 2011; Xu et al., 2012). In a systematic review study on the clinical trials of anti-angiogenic drugs for breast cancer, Mackey et al., concluded that these drugs improve response rate and progression-free survival, but no increase in overall survival compared to chemotherapy alone is observed (Mackey et al., 2012).

Iran is a developing country whose economy status has been improved in 2010 [according to the World Bank] (World bank., 2010). It's been more than a decade since hormone receptor and HER-2 status in breast cancer is being assessed in the pathology laboratories of Iran. Besides the triple receptors, laboratories assess Ki-67 and P53 status by clinicians' request. Several studies in Iran show that the incidence and mortality rate of the disease as well as the direct treatment costs have been increased. Taghavi et al., showed that the mortality rate of the disease is increasing (Taghavi et al., 2012). Mokarian et al., stated that in Iran, the burden of breast cancer has increased among the patients under 40. Moreover, in a study during 2005-2010 (Mokarian et al., 2011). Davari et al., concluded that the direct treatment cost of breast cancer in Iran (from stage I to stage IV) is estimated as 222.17 $ and 828.52 $, respectively, among which medication therapy is the costliest. The authors concluded that direct economic costs of breast cancer is very high in Iran (Davari et al., 2013).

In this study we found that there is a significant correlation between tumor staging, lymph node status, tumor grading, MVD-CD34 and Ki-67 among the patients HER-2/neu gene and TP53 gene mutations occurred simultaneously. According to Kerebel, the relationship between each HER-2 and TP53 gene mutations and angiogenesis was predictable (Kerebel., 2008). On the other hand, according to Folkman, it was expected that increase in angiogenesis could elevate invasion potential and risk of metastasis, which is true about axillary lymph nodes status (Folkman., 2002). In the present study, there were no relation between tumor stage and HER-2 positivity as well as tumor grade and P53 positivity. However, there was a correlation between both of them in simultaneous mutation.

In this study, luminal A and ERBB2 subtypes accounted for the maximum and minimum frequency, respectively. Our results were rather different from that of Kadivar et al. (Kadivar., 2012). Comparing angiogenesis between the molecular subtypes of breast cancer showed that ERBB2 subtype had the highest angiogenesis. Various studies have shown that often in ERBB2 subtype, patients are also TP53-positive (Calza., 2006), which might be the reason for high MVD in this subtype.

Our results demonstrate a significant relationship between the patients' age and angiogenesis level so that MVD is higher in the patients under 50 than in those above 50. Recently, it has been found that bone marrow has a role in producing endothelial progenitor cells (EPCs) when tumor angiogenesis occurs. Moreover, the origin of tumor endothelial cells (ECs) is attributed to EPCs (Le Bourhis et al., 2010). There is strong evidence that young women have a poorer prognosis of breast cancer than old women (Silva et al., 2008; Arnes et al., 2012). There is also evidence in experimental models of cancer that bone marrow's ability to produce EPCs reduces with increasing age (Singh et al., 2012). Therefore, increased angiogenesis in young women and consequently poor prognosis are partly explained by this hypothesis.

To sum up, it can be said that angiogenesis level in HER-2 positive and TP53 positive breast cancer patients is higher compared to those in HER-2 positive and TP53 negative, HER-2 negative and TP53 positive and HER-2 negative and TP53 negative groups. Moreover, if we are going to discuss the results of this study in terms of angiogenesis level in molecular subtypes of breast cancer and the relationship between age and tumor angiogenesis, and if we want to prioritize patients in terms of taking anti-angiogenic drugs, ERBB2 subtype (given that they are mostly TP53 positive) will be probably the top priority. Our results indicate that there is no significant difference between luminal B and triple negative cancers in younger women, but with regard to the results of the present study indicating that angiogenesis level is higher in young women, it can be concluded that triple negative group has a higher priority over luminal B subtype.

One of the known trastuzumab's mechanisms of action is anti-angiogenesis effects (Albanell et al., 2003). Therefore, tumor angiogenesis is partly inhibited in HER-2 positive patients using this mAB. Hence if health-system policy makers want to support only one expensive drug and prioritize trastuzumab for the ERBB2 subtype, perhaps the triple negative subtype deserves anti-angiogenesis drugs.

In conclusion: the results of the present study can be generalized to other UMICs like Iran, mainly because resource constraints in these countries make health policy-makers apply priority-setting strategies in order to prevent the destruction of resources as well as providing equity in health. Although anti-angiogenic drugs have not played a very successful role in treating cancers for several reasons, a number of anti-angiogenic agents are recently in phase III of their ongoing clinical trial and they will probably obtain marketing authorization within the next few years. Therefore, if the government policies of UMICs for the treatment of breast cancer are made to support anti-angiogenic drugs and to consider the evidence-based side of the 6-facets charter of priority-setting, the results of the present study can be one of the scientific criteria for priority-setting, based on which some clinical trials are recommended to be designed and implemented in future.

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