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Translation of triple-negative breast cancer behavior from the xenograft model to human model

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ABSTRACT

Background: For xenograft models of triple-negative breast cancer (TNBC) to be valuable in the development of molecularly-targeted drugs, careful characterization is essential to their validation. The present study aimed to validate the TNBC xenograft model with a specific focus on angiogenesis.

Methods: Twelve TNBC xenograft tumors and 12 human breast cancer tumors (HTNBCs) were included in this study. Both groups were grade III and p53- positive. Nuclear pleomorphism and mitotic count were analyzed by hematoxylin and eosin (H&E) stains respectively. Basal cytokeratin (CK5/6), vimentin, cathepsin-D, Ki-67 (for proliferation), and MVD-CD34 (for angiogenesis) markers were examined by immunohistochemistry (IHC). The association of mMicrovesseles density (MVD) with Ki-67, nuclear pleomorphism, and mitotic count was assessed in each group separately, and HTNBCs were compared with the xenograft group.

Results: The xenograft models showed a significant correlation between angiogenesis (MVD) and cell proliferation (Ki-67), nuclear pleomorphism, and mitotic count (p= 0.0398; p= 0.020; p=0.001, respectively). The HTNBC group also showed a similar trend, except for nuclear pleomorphism (p=0.193), which did not correlate with angiogenesis. Comparison between the two groups showed significant changes in cell proliferation (Ki-67 and vimentin). The difference in proliferation rate and vimentin expression between the two groups can be due to the biological diversity between human and mice and epithelial-mesenchymal transition (EMT), respectively.

Conclusion: Our results, re-emphasize the significance of angiogenic treatment therapy in patients with TNBC, and further validate the TNBC xenograft model as a valid model for drug discovery and development.

Keywords: Triple-negative breast cancer, Xenograft models, Angiogenesis, Translational research, Validity .

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Introduction

Breast cancer is classified and characterized into four subtypes, each different from the others, based on genetic profile, treatment response, and disease prognosis[1]. One of these subtypes, the triple-negative breast cancer (TNBC), is characterized by lack of expression of the three receptors, estrogen (ER), progesterone (PR), and HER2/neu[2]. Current statistics show that 10%–25% of reported breast cancer cases are TNBC[3-4]. A relatively younger age (<40), family history (in more than 60% of the patients), aggressiveness of the tumor, lack of proper response to hormone receptor blockers, and epithelial growth are the common features of TNBC[5]. Neoadjuvant chemotherapy has been shown to be an effective treatment option for TNBC[6]. Unfortunately, TNBC tumors are of high grades at the time of diagnosis, and due to the lack of triple-receptors, the risk of their recurrence is high. Therefore, the rate of disease- free survival (DFS) and the overall survival rate (OSR) in patients with TNBC are lower than those in patients with other breast cancer subtypes[2-4].

Treatment management is one of the major challenging aspects in treating TNBC. Although tumor cells are chemo-sensitive and respond well to cytotoxic drugs, the recurrence rate is high. Moreover, TNBC tumors are heterogeneous, resulting in varied prognosis among patients[6]. Current research suggests that due to their rapid growth rates, TNBC tumors have an enhanced angiogenesis, and the inhibition of angiogenesis can be beneficial toward for tumor inhibition[7-8].

Xenograft models are valuable cancer models, and have been used extensively to study a variety of tumors. The National Cancer Institute (NCI) in the United States has recommended these models as valid models for pre-clinical studies[9]. Xenograft models are currently available for all 4 subtypes of breast cancer are currently available, and have been widely used in both basic research and clinical trials. Unfortunately, results from the pre-clinical phase are often different than from those of clinical trials, which isthus are a major concern. The heterogeneous nature of the tumor in TNBC adds an additional level of complexity[10]. The MDA–MB-231 cell line is one of the most important TNBC cell lines, and is isolated from pleural effusion of grade III invasive ductal carcinoma (IDC)[11]. The related xenograft model shows the phenotype of triple-negative feature along with the mutated TP53 genes[1, 10, 12-13]. At present, this cell line is widely used in both in vitro and in vivo pre-clinical studies. In the present study, we compare the xenograft model of TNBC with human TNBC, with the primary focus on angiogenesis, and the ultimate aim of providing targeted treatment using anti-angiogenic drugs.

Methods

Cell cultures

The MDA-MB-231 cell line was obtained from the National Cell Bank of Iran (NCBI) and then multiplied in RPMI-1640 culture medium containing 10% FBS.

Xenograft models of TNBC

Ten 6–8-week-old female nude mice with a BALB/c genetic background were purchased from Omid Institute for Advanced Biomodels (Tehran, Iran) and kept in individually ventilated cages. The mice were fed ad libitum with sterile food and water. The experiment received ethical approval from the Institutional Animal Ethics Committee, and the animals were treated in compliance with this Committee's standard procedures. Ten mice were heterotopically inoculated with MDA–MB-231 cells (5 × 106) on their left and right flanks. Thirty-four days after inoculation, the mice were humanely sacrificed and the developed tumors, each with a minimum volume of 1 cm3, were isolated and fixed with 10%

buffered formalin (n=12). Hematoxylin and eosin (H&E) slides were prepared, and the sections were examined under light microscope to confirm tumor malignancy. The paraffin-embedded blocks from malignant tumors were used for immunohistochemistry (IHC).

Human TNBC sections

Files of breast cancer patients at the archives of the Pathology Laboratory at the Genetic Research Center (GRC) of the University of Social Welfare and Rehabilitation were extracted and studied. Twelve paraffin-embedded blocks of breast cancer tissue were selected randomly from patients with grade III invasive ductal carcinoma (IDC), lacking all the three receptors (ER, PR, and HER-2) and positive for TP53. P53-positive patients were chosen because mutations in P53 have been known to promote angiogenesis. Since our xenograft model was TP53-positive, selection of P53-positive patients provided identical conditions. Following H&E staining, slides were examined by a light microscope and then by IHC.

Histopathology

H&E slides were prepared from all the blocks. Four- µm-thick sections were cut from the blocks and stained with HER-2 (Dako, clone: mAB), ER (Dako, colone: 1D5), PR (Dako, clone: PgR 636), basal cytokeratin (CK5/6) (Dako, clone: D5/16 B4), vimentin (Dako, clone: Vim 3B4), P53 (Dako, clone: DO-7), CD34 (Dako, Clone: QBEnd 10), Ki-67 (Dako, Clone: MIB-1), and cathepsin-D (Abcam, clone: EPR3057Y) antibodies, following the manufacturer's IHC protocols. Stained sections were mounted on glass slides and used for IHC. All samples were first analyzed for ER, PR, HER-2, and P53 to confirm the inclusion criteria. More than 5% of immunoreactivity toward P53 was considered as P53-positive.To minimize any experimental errors, each slide was examined by two pathologists, for a maximum of three minutes, using the double-blind technique. The mean of the study conducted on each slide was considered as its score.

Analysis of nuclear malignancy (Pleomorphism)

Ten different fields with aggressive pathological features were selected from each H&E stained slide and examined at a 200× magnification. The number of nuclei that were 2–3 times larger than the neighboring cells was counted, and the mean of the numbers from each slide was calculated.

Mitotic cell count

The 10 abovementioned fields were analyzed for mitotic cells. Hyperchromatic cells were avoided to reduce errors. The mean of the 10 fields was considered as the score of the slide.

Analysis of CK5/6, vimentin, and cathepsin-D

The immunoreactivity of each section toward the respective antibody was determined based on intensity and rate. The scores were assigned as follows: +1 for less than 10%, +2 for 10%–30%, and +3 for more than 30%.

Analysis of tumor proliferation using Ki-67 marker

Ten microscopic fields were randomly selected, 1000 epithelial cells were counted, and the percentage of immunoreactive nuclei toward Ki-67 was determined.

Analysis of angiogenesis or microvessel density (MVD-CD34)

First, using a light microscope at 40× magnification, 10 hot-spot fields were selected from each CD34 stained section. These fields were then examined at 400× magnification. All stained endothelial cells (both single and clustered) and vascular lumens without septa were considered to be vessels. The mean number of these vessels was determined.

Statistical analyses

Data were represented as mean ± SEM. The ordi-

nal data were also presented in medians and modes. Analysis of linear regression was used to evaluate the relationship between MVD and Ki-67, nuclear pleomorphism, and mitotic count. Student's t-test was employed to compare parameters of human TNBC with those of the xenograft model of TNBC. All statistical analyses were carried out using BioStat® 2008.

Results

The growth kinetic curve of the xenograft model of TNBC is shown in **Figure 1** (34 days ± 1.1 SEM). The corresponding statistics of these parameters are presented in **Table 1**.

Results of statistical investigations showed that in the xenograft model, a significant relationship existed between MVD and Ki-67 (r=0.600, p=0.0398), nuclear pleomorphism (r=0.660, p=0.020), and mitotic count (r=0.660, p=0.001) (**Table 1**). The human TNBC also showed significant relationships between MVD and Ki-67 (r=0.811, p=0.015) and mitotic count (r=0.769, p=0.004), However, the relationship between MVD and nuclear pleomorphism was insignificant in human TNBC (p=0.193) (**Table 1**).

As shown in **Table 2**, a comparison of the xenograft model of TNBC and the human TNBC showed that except for Ki-67 (p=0.000) and vimentin (p=0.009), no significant difference existed between these two groups.

Discussion

The diagnosis and treatment of TNBC are among the major challenges in oncology. Gene expression profile analysis in breast cancer patients shows that genetic instability in TNBC group is more than other subtypes of breast cancer, and germ-line mutations in BRCA-1 and BRCA-2 genes account for a major percentage of patients[14]. These genetic features along with other phenotypic features make TNBC patients distinct from other subtypes of breast cancer with respect to clinicopathological features[14]. The treatment options are limited because of the lack of expression of triple-receptors that imposes restrictions on using hormone therapy drugs such as tamoxifen and trastuzumab; hence, the probability of tumor recurrence during the first two years following treatment is 50%–60%[15]. Several studies aimed to elucidate genetic profile of TNBC to improve the DFS and ORS in these patients. Recently, based on tissue array analysis, the TNBC has been classified into two subgroups: basal-like breast cancer (BLBC) and quintuple negative breast cancer (QNBC/5NP), phenotypically represented as BLBCs (ER^-, PR^-, $HER\text{-}2^-$, $CK5/6^+$, and/or $EGFR\text{-}1^+$) and QNBCs/5 NPs (ER^-, PR^-, $HER\text{-}2^-$, $CK5/6^-$, and/or $EGFR\text{-}1^-$)[16]. Anti-angiogenic drugs have been suggested along with other treatment options, and studies show that patients with TNBC benefit from the use of the monoclonal antibody bevacizumab[7]. The role of angiogenesis in tumor cell progression is not clear. Unfortunately, the methods to define and evaluate angiogenesis in tumors are not well established. Among the current methods, the MVD method is the most applicable technique despite its limitations[17].

In our study, we analyzed the relationship between MVD and other histological parameters in human TNBC and the xenograft model of TNBC, and further compared these parameters between the two models. We also aimed to confirm that the biomarkers of human TNBC are also important in the xenograft model of TNBC.

Results of our study showed that both the human TNBC and the xenograft model of TNBC represent a significant correlation between angiogenesis, mitotic count, and proliferation index. Sufficient data

Table 1. Correlation of MVD with proliferation, nuclear pleomorphism and mitotic count in TNBC in both patients and commensurate xenograft models

	Proliferation	Nuclear pleomorphism	Mitotic count
Xenograft models TNBC	+	+	+
Human TNBC	+	-	+

*Plus: significant correlation; asterisk: P<0.05; double asterisk: P<0.01

Table 2. Descriptive statistics of pathological characteristics of human TNBC and commensurate xenograft models

	MVD	Proliferation pleomorphism	Nuclear	Mitotic count	Cathepsin-D	Ck5/6	Vimentin
Xenograft models TNBC	42.5±2.2*	62.5±3.8*	7.25±0.4*	10.5±0.7*	3&3 °°	3&3 °°	3&3 °°
Human TNBC	41.6±2.7*	36.3±3.3*	7±1.8*	10.4±0.7*	3&3 °°	3&3 °°	2&2 °°

*Data are represented as Mean±SEM
°° Data are represented as Medians and Modes

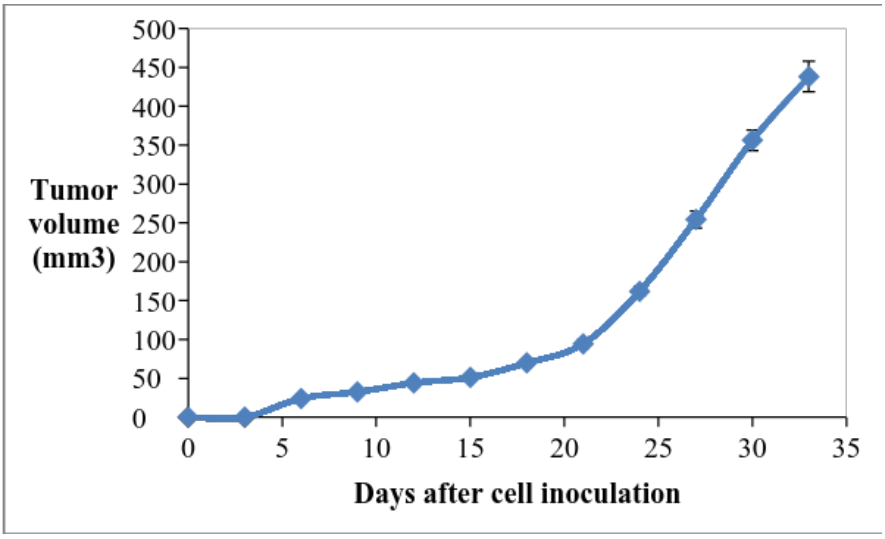

Figure 1. Growth kinetic curve of MDA-MB-231 xenograft tumors in athymic nude mice. Error bars indicate SEM.

exist to support that the TNBC tumors are aggressive; hence, the nuclear pleomorphism and mitotic count scores are high in the Nottingham Grading System (NGS)[3]. Studies also suggest that angiogenesis is high among high-grade tumors. The rise in angiogenesis provides malignant cells with higher amounts of oxygen, resulting in increased proliferation[18]. Mrlkic et al. showed that in TNBC, the tumor proliferation rate significantly correlates with DFS and OSR[19]. Furthermore, Keam et al. showed that TNBC can be categorized into two classes and predicted the extent of pathologic complete response (pCR) in neoadjuvant chemotherapy based on Ki-67 staining[20]. Our findings in human TNBC agree with the abovementioned studies. The xenograft model of TNBC also showed a similar relationship. In a similar study on luminal B breast cancer subtype (in human TNBC and in the xenograft model), Muhammadnejad et al. did not find any correlation between MVD and mitotic count[21], suggesting that different molecular pathways exist between luminal B and triple-negative subtypes of breast cancer. This is potentially interesting and needs more attention.

We also noticed a significant correlation between nuclear malignancy and angiogenesis in the xenograft model; however, human TNBC samples did not show such a correlation. Nuclear pleomorphism, the NGS factor, has been proposed to influence the prognosis of breast cancer at the highest level. Contradictory results are reported by different studies, which might be due to the high ratio of subjectivity to objectivity in pathologists. Even though we expected to see a correlation between the nuclear polymorphism and angiogenesis in both human and xenograft model TNBC, the reasons behind the differences noticed between these two groups are unclear. We hypothesize that the subjectivity of nuclear grade may be a confounding factor and affect the results[22-23]. To avoid this, our future studies will use suitable software.

Another purpose of this study is to compare the xenograft model of TNBC with human TNBC. A suitable model should show minimum differences. The results of our study indicate that there are significant differences in proliferation indices (vimentin and Ki-67) between the two groups. As can be seen in **Table 2**, the proliferation index in the xenograft model of TNBC is higher than that of human TNBC samples. Except for G0, Ki-67 can detect all the phases in cell cycle. Even in aggressive tumors, the doubling time of malignant cells is more than 90 days, and we can expect a large number of cells in the resting phase (G0)[24-25]. In xenograft models, the doubling time of malignant cells is low compared with human TNBC; subsequently, the rate of proliferation of malignant cells is high. This was attributed to the biological differences between the humans and rodents including their size and metabolism[21]. Using the bioinformatics approach, researchers have compared the natural phases of life cycle of rodents and humans, and optimized them to develop suitable clinical models. Therefore, the difference between the proliferation of xenograft models and human TNBC may have little influence on the translation of pre-clinical results to the clinical phase.

In the basal-like type of TNBC, the elevated expression of CK5/6 protein has been reported. Liu et al. reported a significant relationship between CK5/6 and TNBC prognosis, tumor progression, and angiogenesis[26]. In our study, we noticed an elevated CK marker in both groups with no significant difference between them. On the other hand, an increased vimentin expression indicates epithelial mesenchymal transition (EMT), a process in which malignant epithelial cells transform into fibroblast with changes in the expression of a series of genes, to gain motility and aggressiveness[27-28].

Yamashita et al. studied 569 patients with TNBC and showed a relationship between vimentin expression and clinicopathological features of the disease[29]. Based on their data, they suggested vimen-

tin to be a potential biomarker for the prognosis of TNBC. In our study, vimentin expression was high in the xenograft model compared with the human TNBC. This is likely due to the higher rates of proliferation noticed in xenograft models.

Both xenograft models and human TNBC showed similar expression levels of cathepsin D. Sufficient evidence points toward the relationship between cathespin-D expression and tumor metastasis and recurrence[30-31]. Since in our analysis we noticed a strong expression of cathespin-D in both groups, we suggest that it can be a potential biomarker for TNBC, and studies are needed to validate this hypothesis.

Conclusion

The results obtained in this study not only re-emphasize the importance of angiogenic treatment approaches in TNBC patients, but also validate the xenograft model of TNBC with respect to tumor angiogenesis. Despite their limitations, the xenograft models of TNBC are the best models available for pre-clinical studies toward the development of anticancer therapeutics, and their re-validation resolves some of the concerns in oncology, specifically in breast cancer. Future studies on TNBC xenograft models analyzing molecular pathways and mechanisms will be helpful to develop efficient methods for targeted therapy.

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