

## RESEARCH ARTICLE

## Correlation of Microvessel Density with Nuclear Pleomorphism, Mitotic Count and Vascular Invasion in Breast and Prostate Cancers at Preclinical and Clinical Levels

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### Abstract

**Background:** Tumor angiogenesis correlates with recurrence and appears to be a prognostic factor for both breast and prostate cancers. In the present study, we aimed to investigate the correlation of microvessel density (MVD), a measure of angiogenesis, with nuclear pleomorphism, mitotic count, and vascular invasion in breast and prostate cancers at preclinical and clinical levels. **Methods:** Samples from xenograft tumors of luminal B breast cancer and prostate adenocarcinoma, established by BT-474 and PC-3 cell lines, respectively, and commensurate human paraffin-embedded blocks were obtained. To determine MVD, specimens were immunostained for CD-34. Nuclear pleomorphism, mitotic count, and vascular invasion were determined using hematoxylin and eosin (H&E)-stained slides. **Results:** MVD showed significant correlations with nuclear pleomorphism ( $r=0.68$ ,  $P=0.03$ ) and vascular invasion ( $r=0.77$ ,  $P=0.009$ ) in breast cancer. In prostate cancer, MVD was significantly correlated with nuclear pleomorphism ( $r=0.75$ ,  $P=0.013$ ) and mitotic count ( $r=0.75$ ,  $P=0.012$ ). In the breast cancer xenograft model, a significant correlation was observed between MVD and vascular invasion ( $r=0.87$ ,  $P=0.011$ ). In the prostate cancer xenograft model, MVD was significantly correlated with all three parameters (nuclear pleomorphism,  $r=0.95$ ,  $P=0.001$ ; mitotic count,  $r=0.91$ ,  $P=0.001$ ; and vascular invasion,  $r=0.79$ ,  $P=0.017$ ; respectively). **Conclusions:** Our results demonstrate that MVD is correlated with nuclear pleomorphism, mitotic count, and vascular invasion at both preclinical and clinical levels. This study therefore supports the predictive value of MVD in breast and prostate cancers.

**Keywords:** Breast adenocarcinoma - prostate adenocarcinoma - microvessel density - mitotic count - vascular invasion

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### Introduction

Statistics show that the incidence of breast and prostate cancers is growing in women and men, respectively (Siegel et al., 2012). Introduction of new therapeutic approaches has led to an increased survival rate of these patients compared to that in the last decades. However, these two types of cancer have high rates of recurrence, invasion, or metastasis, which ultimately lead to death (Malvezzi et al., 2012). Nowadays, prognostic and predictive factors are used to estimate the survival rate and to determine therapeutic strategies. Some of these factors are clinical, while some others are evaluated by pathological studies. In assessing breast cancer, in addition to the histopathological characteristics of the tumor, other factors such as tumor grade, the expression of

genes related to estrogen receptors (ER) and progesterone receptors (PR), the HER-2/neu gene, the proliferation index, the TP53 gene status, and vascular invasion, are also evaluated (Moise et al., 2011; Song et al., 2011). Since studies have shown that the presence of neoplastic cells inside blood and lymphatic vessels, observed using hematoxylin and eosin (H&E) staining, increases the risk of tumor recurrence, vascular invasion is considered as a potential risk factor in patients with negative lymph node status (Rakha et al., 2011). In prostate cancer, in addition to tumor grade, vascular invasion is also evaluated, because the presence of malignant cells inside the vessels increases the risk of metastasis to the pelvic bones (Yamamoto et al., 2008).

The theory of tumor growth's dependency on angiogenesis directed scientists to focus on inhibition

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of angiogenesis as a way of controlling the growth of neoplastic cells. The amount of angiogenesis of a tumor can be measured quantitatively by microvessel density (MVD) technique. In this technique, endothelial cells are immunoreactivated by immunohistochemistry, and then the vessels are counted by light microscopy. Several studies have shown that there is a correlation between MVD and the risk of tumor invasion in prostate and breast cancers (Rykala et al., 2011). Moreover, correlations of MVD with vascular invasion, nuclear pleomorphism, and proliferation have been observed (Koukourakis et al., 2003). Although the association between MVD and survival rate sometimes seems controversial, most scientists have proposed MVD as a prognostic and predictive factor (Uzzan et al., 2004).

The increased incidence of cancer on one hand and the failure in the treatment of this disease on the other hand have led to developments in basic research on cancer. Established xenograft tumors in athymic nude mice are considered superior models, which can imitate human tumor characteristics in an acceptable way (Jönsson et al., 2007).

In the present study, we aimed to study the correlation of MVD, as a measure of angiogenesis, with nuclear pleomorphism, mitotic count, and vascular invasion in breast and prostate cancers and also in relevant xenograft models. Then, in order to evaluate the validity of xenograft models of cancer, we aimed to compare the mentioned histopathological characteristics of human tumor tissue with those of the relevant xenograft models. Evaluating the validity of xenograft tumor models can assist researchers in judging the results of preclinical studies performed on antiangiogenic and anticancer agents with high confidence.

## Materials and Methods

### Cell cultures

Human breast BT-474 and prostate PC-3 cell lines were purchased from the National Cell Bank of Iran. Cell lines were maintained in a humidified atmosphere of 5% CO<sub>2</sub> and RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA). It merits emphasis that the BT-474 cell line has been established from plural effusion of a grade III invasive ductal carcinoma with luminal B (ER<sup>+</sup>, HER-2<sup>+</sup>) subtype (Jönsson et al., 2007). Also, the PC-3 cell line has been established from the bone metastasis of a Gleason grade IV prostate cancer (Kim et al., 2011).

### Xenograft tumor study

Male and female athymic nude mice (10 each; nu/nu; BALB/c; 4-6 weeks of age) were obtained from Omid Institute for Advanced Biomodels. The animals were treated according to the guidelines outlined by the Institutional Ethical Committee. All animals were kept under optimized hygienic conditions in an individually ventilated cage system. The animals were fed with autoclaved commercial diet and water *ad libitum*. Three days prior to BT-474 cell inoculation, each female mouse was subcutaneously implanted with a 17 $\beta$ -estradiol pellet

(25  $\mu$ g/day, 60-day release; Innovative Research of America, USA) on the upper dorsal side. Also, the BT-474 cell line (5 $\times$ 10<sup>6</sup>) in 200  $\mu$ L of Matrigel/RPMI 1640 (1:1 v/v) (BD Matrigel™ Basement Membrane Matrix, BD Biosciences, USA) was inoculated subcutaneously in both the right and left flank regions. The PC-3 cell line was inoculated in the same manner into the male mice without hormonal supplementation. The tumor volume was measured every 3 days by caliper measurement along two perpendicular diameters of the tumor, and calculated using the formula  $a \times b^2 \times 0.52$ , where  $a$  stands for the long diameter and  $b$  is the short diameter (Tomayko and Reynolds, 1989).

When the volume of xenograft tumors reached about 1000 mm<sup>3</sup>, the animals were sacrificed in a humane manner using CO<sub>2</sub> gas. Tumor tissue biopsies were immediately fixed using 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS). Then, the samples were dehydrated through graded concentrations of ethanol and embedded in paraffin wax. Thereafter, they were stained using H&E. The slides were reviewed by a pathologist in a semiblinded manner. Ten prostate xenograft tumors that sufficiently represented the characteristics of a primary neoplastic tumor were included in the study.

For including breast xenograft tumors, H&E slides were reviewed followed by immunohistochemical staining with HER-2 and ER markers. Then, 10 tumors that were HER-2 positive and showed scattered to strong ER were selected. Since an association between MVD and expression of HER-2 and ER in breast cancer has been proven previously, the present study emphasized upon HER-2 and ER characteristics (Shpitz et al., 2000).

### Study of paraffin-embedded blocks of human tumor

Documents of patients with pathologically confirmed prostate or breast cancer archived in Pathology Division of Cancer Institute of Imam-Khomeini Medical Center from January to June 2011 were reviewed. Paraffin-embedded blocks were included in the study after obtaining written informed consent from patients. It merits emphasis that no coercion was used to solicit subjects. Ten paraffin-embedded blocks from breast cancer subjects, showing grade III non-specific invasive ductal carcinoma (IDC) with luminal B subtype (HER-2<sup>+</sup>, ER<sup>+</sup>), were included.

Ten paraffin-embedded blocks of prostate cancer representing adenocarcinoma (Gleason grade IV) characteristics were also included. Since the association of Gleason grade IV with bone metastasis has been proven, clinical data related to this have not been evaluated here (Yao et al., 2010).

Pathological slides were evaluated again and included in the present study after confirmation.

### Histopathological study

H&E slides were prepared for all blocks. For immunohistochemical study, paraffin-embedded blocks were cooled in an ice-water mixture for 30 min before sectioning. Sections measuring 4  $\mu$ m were cut and placed on slides. After a brief drying period of approximately 15 min, the sections were heat-fixed to the slide at 37°C. After deparaffinization and rehydration of slides

through graded ethanol concentrations, they were stained immunohistochemically according to the manufacturer's instructions (DAKO; Glostrup, Denmark), with CD-34 antibody. In most studies, CD-31 has been used to evaluate MVD in xenograft models. Since it has been demonstrated that CD-34 can identify endothelial cells of lymphatic vessels as well as blood vessels (Li et al., 2009), CD-34 antibody was used in the present study for xenograft tumors.

All slides were examined by light microscopy in a semiblinded manner. To reduce subjective errors, each slide was assessed twice at a 3-day interval. In order to harmonize the study, each slide was reviewed within 3 minutes. The average score of duplicates was calculated for each slide. Microscopic examination methods for each slide were as follows:

**Assessment of MVD:** to calculate MVD, slides immunostained with CD-34 antibody were first scanned at 40 $\times$  magnification to determine 10 "hot spots," areas with the highest microvessel count. Then, these fields were examined at 400 $\times$  magnification. Any darkly stained endothelial cell or cell cluster appearing separate from adjacent structures was considered a single vessel. Vessel lumens were not a prerequisite to define a structure as a microvessel. Necrotic cells were precisely discriminated from immunoreactive cells. The average value of vessels was calculated for each case.

**Assessment of nuclear pleomorphism:** to evaluate nuclear pleomorphism, H&E slides were scanned at 100 $\times$  magnification to select 10 fields that showed more invasive pathological properties. Then they were studied at 400 $\times$  magnification. The number of cells with nuclei that were 2-3 times bigger than other surrounding cells were counted carefully and the average value was calculated.

**Assessment of mitotic count:** to estimate mitotic count, H&E slides were scanned at 100 $\times$  magnification to select 10 fields showing the highest invasiveness. In the selected fields, each cell that showed a stage of mitosis was counted. To reduce errors, hyperchromatinated cells were excluded from counting. The average value of 10 selected fields was considered as the mitotic count score.

#### 4.4 assessment of vascular invasion

H&E slides were scanned at 100 $\times$  magnification to identify 10 blood or lymphatic vessels. Then, these vessels were studied at 400 $\times$  magnification. The number of tumor cells inside the vessels was counted. Cell counting was carried out so carefully that artifacts and stained precipitations were excluded from tumor cells. If there was no evidence of neoplastic cells inside the lumen of vessels, vascular invasion was scored as zero. Finally, the average of each slide was considered as the vascular invasion score.

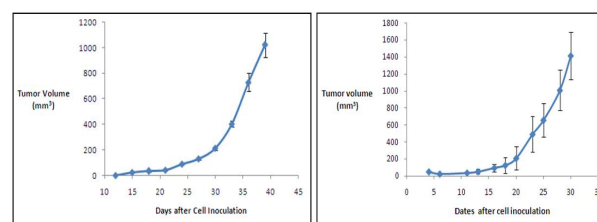
#### Statistical analyses

Data are represented as mean $\pm$ SEM. The level of significance was considered as 0.5. A linear regression analysis was performed to evaluate the correlation of MVD with nuclear pleomorphism, mitotic count, and vascular invasion. The statistical significance of differences was also calculated using Student's t-test.

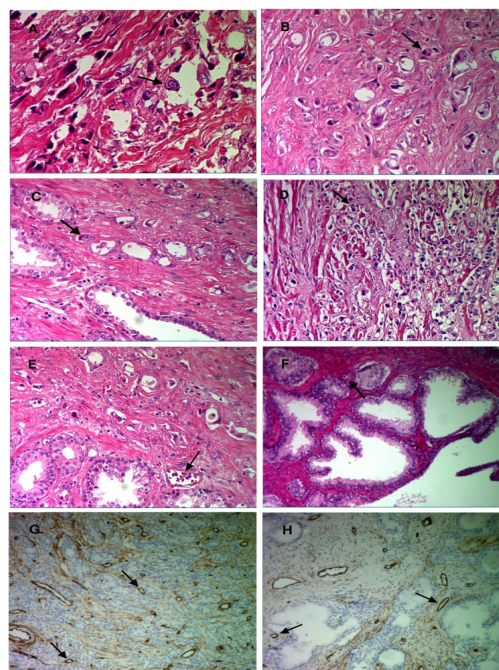
## Results

The growth kinetic curves of breast and prostate xenograft tumors have been illustrated in Figure 1. The photomicrographs obtained from paraffin-embedded blocks or xenograft tumors are depicted in Figure 2. As shown in Table 1, statistically significant correlations were observed between MVD and nuclear pleomorphism ( $r=0.68$ ,  $P=0.03$ ) as well as between MVD and vascular invasion ( $r=0.77$ ,  $P=0.009$ ) in human breast cancer. The correlation between MVD and mitotic count was not statistically significant.

In human prostate cancer, MVD showed significant correlations with nuclear pleomorphism ( $r=0.75$ ,  $P=0.013$ )



**Figure 1. Growth Kinetic Curve of BT-474 (a) and PC-3 (b) Xenograft Tumors in Athymic Nude Mice.** Error Bar SEM



**Figure 2. A and B) Arrows Point Towards the Bizarre Pleomorphic Cells in Human Breast Cancer and Prostate Xenograft Model, Respectively.** These malignant cells have no similarity to the epithelial cells of breast or prostate (original magnification, 400 $\times$ ); **C and D) Arrows Show the Mitotic Cells in Xenograft Breast and Prostate Cancer Models, Respectively.** In all slides, different phases of mitotic cell division were distinguishable (original magnification, 400 $\times$ ); **E and F) Arrows Indicate to Vascular Invasion in Human Prostate and Breast Cancers, Respectively** (original magnification, 100 $\times$ ); **G and H) CD34 Immunostained Photomicrographs of Xenograft Breast Cancer Model and Human Prostate Cancer, Respectively.** Arrows point toward microvessels (original magnification, 100 $\times$ )

**Table 1. Correlation of MVD with Nuclear Pleomorphism, Mitotic Count and Vascular Invasion in Breast and Prostate Cancers in Both Patients and Commensurate Xenograft Models**

	Nuclear pleomorphism	Mitotic count	Vascular invasion
Breast cancer	+	-	+
Prostate cancer	+	+	-
Xenograft Breast cancer	-	-	+
Xenograft prostate cancer	+	+	+

\*Plus significant correlation; Asterisk P<0.05; Double asterisk P<0.01

**Table 2. Descriptive Statistics of Pathological Characteristics of Human Breast Cancer and Commensurate Xenograft Model**

	MVD	Nuclear pleomorphism	Mitotic number	Vascular invasive
Breast cancer	39.6±6.5	5.8±1.3	11.1±2.8	4.3±1.7
Xenograft Model	47.6±7.9	6.8±0.9	12.4±1.5	6.4±1.4

\*Data are represented as Mean±SD

**Table 3. Descriptive Statistics of Pathological Characteristics of Human Prostate Cancer and Commensurate Xenograft Model**

	MVD	Nuclear pleomorphism	Mitotic number	Vascular invasive
Prostate cancer	39.1±9.4	6.1±1.0	10.5±2.6	5.4±1.9
Xenograft Model	48.9±10.3	6.5±1.5	11.5±3.0	5.8±1.3

\*Data are represented as Mean±SD

and mitotic count ( $r=0.75$ ,  $P=0.012$ ). The correlation between MVD and vascular invasion was not statistically significant.

In the breast cancer xenograft model, MVD showed a significant correlation with vascular invasion ( $r=0.87$ ,  $P=0.011$ ), while no such correlation was observed with nuclear pleomorphism or mitotic count.

In the prostate cancer xenograft model, MVD showed significant correlations with all three parameters, nuclear pleomorphism, mitotic count, and vascular invasion ( $r=0.95$ ,  $P=0.001$ ;  $r=0.91$ ,  $P=0.001$ ;  $r=0.79$ ,  $P=0.017$ ; respectively).

Comparison between xenograft tumors and clinical tumors showed no differences in MVD, nuclear pleomorphism, mitotic count, and vascular invasion in both breast and prostate cancers (Table 2 and Table 3).

## Discussion

In 1971, Folkman et al proposed that tumor growth is dependent on angiogenesis and is limited to a size of 1–2 mm in the absence of vascularization. More the angiogenesis in the tumor, more the invasion and metastasis rate will be. If we can control angiogenesis, tumor will be in small size and won't be disturbing anymore (Li et al., 2012). Several studies have shown that subjecting tumor cells to hypoxia leads to regulation of the expression of angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF plays key roles in angiogenesis, including cell migration, proliferation, and survival

(Sakurai and Kudo, 2011). The ability of tumor cells to cope with hypoxia stress through angiogenesis leads to an increase in the proliferation rate of malignant cells. This will cause the appearance of poorly differentiated cells on one hand and will increase metastasis and invasive potential of the tumor cells on the other hand. This results in increased malignancy rate of cancerous cells (Kerbel, 2008; Zerbini et al., 2008; Gordon et al., 2010).

The role of MVD in the survival rate of patients with breast cancer has been proven in several investigations (Uzzan et al., 2004). It has been illustrated that MVD is correlated with tumor grade and the disease stage (Ryakala et al., 2011). Such correlation has been also shown in prostate cancer, suggesting a relationship of MVD with Gleason grade and bone metastasis (Yamamoto et al., 2008). Vascular invasion is defined as tumor cell emboli located within the lumen of lymphatic or other vascular spaces. The presence of such invasion is associated with a high incidence of hematogenous or lymphogenous metastases (Courtneidge, 2012). Some studies have demonstrated an association between MVD and vascular invasion. Although the evidence is not sufficient to propose a theory, the existence of such a relationship can be hypothesized (Tezuka et al., 2007). Due to the lack of a consensus-based protocol to quantify vascular invasion, we used a novel method for quantitative measurement of vascular invasion involving counting the number of tumor cells within vessels.

This study demonstrates that MVD is associated with nuclear pleomorphism and vascular invasion in breast cancer. This finding is consistent with previous studies (Tse et al., 2004; Tezuka et al., 2007). It can be proposed that increased MVD may provide enough oxygen to tumor cells. Thus, wild-type or mutant genes related to cell cycle can overexpress, and as a result, the S phase will be completed in the cell cycle and the cell genetic content will increase. This phenomenon can explain the cause of increased nuclear pleomorphism (Marrinucci et al., 2007; Kerbel, 2008). Another finding of our study indicates the lack of correlation between MVD and the mitotic count in breast cancer. Grading of breast cancer is performed by adding up scores of tubule formation, mitotic count, and nuclear pleomorphism. Several studies have demonstrated the existence of correlation between MVD and proliferation (mitotic activity index) of tumor cells (Lee et al., 2011). Proliferation is often assessed by immunohistochemistry using Ki-67. This protein is present during all active phases of the cell cycle, including G1, S, G2, and mitosis (Luporsi et al., 2011), while in mitotic count, only those cells which are in the mitotic phase are counted. Since breast cancer has a prolonged doubling time (Medina et al., 2011), we can conclude that in the proliferation (mitotic activity index) method, the S phase is identified earlier than other phases, while the mitotic forms are identified later.

In the breast cancer xenograft model, there was no correlation between MVD and the mitotic count. Both these results can be explained by the existence of a prolonged time between S and mitosis phases of the cell cycle in breast cancer. In all groups of the present study, there was a correlation between MVD and nuclear

pleomorphism with the exception of the breast cancer xenograft model. There is no evidence-based reason for the lack of this association in the breast cancer xenograft model. However, it can be proposed that other factors may play roles in nuclear pleomorphism of the breast cancer xenograft model.

In prostate cancer, no significant correlation was found between MVD and vascular invasion, while a significant association between these variables was observed in the xenograft model of prostate cancer. In prostate cancer, vascular invasion depended on extra-capsular extension, seminal vesicle involvement, tumor size, lymph node metastasis, positive surgical margin, and pathological grade (Ng et al., 2012). Although in the present study only the pathological grade was considered as the inclusion criterion for selection of paraffin-embedded blocks; however, in Gleason grade IV lesions, the presence of neoplastic cells in the pelvic bone is anticipated (Yao et al., 2010). It is possible that the study was not large enough to detect a correlation between MVD and vascular invasion in clinical prostate cancer. In the prostate cancer xenograft model, the results were consistent with literature.

The validity of preclinical cancer models for screening and development of anticancer drugs has always been challenging. Even xenograft tumor models that are derived from human cell lines have their limitation. The stroma of xenograft models is murine in origin. So concern for these models has increased because of the probable differences in the biology of xenograft tumors from that of human tumors. Our study has clearly shown that the behavior of breast and prostate tumors in xenograft models with respect to the assessed parameters is the same as that in human tumors. This study highlights that the xenograft tumors can be of excellent value in investigation of angiogenesis. It merits emphasis that xenograft tumors have a shorter tumor doubling time compared to that of human tumors (Fichtner et al., 2004). Our study demonstrates that the shorter doubling time of xenograft tumors cannot confound the predictive value of these models in screening and development of anticancer agents.

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