

RESEARCH ARTICLE

Clinical Study of Tumor Angiogenesis and Perfusion Imaging Using Multi-slice Spiral Computed Tomography for Breast Cancer

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Abstract

Objectives: To explore the correlation between multi-slice spiral CT (MSCT) perfusion parameters and the expression of vascular endothelial growth factor (VEGF) as well as matrix metalloproteinase-2 (MMP-2) in breast cancer. **Methods:** Forty five breast cancer patients and 16 patients with benign breast tumor, both confirmed by pathology examination, were enrolled. All underwent MSCT perfusion imaging to obtain perfusion maps and data for parameters including blood flow (BF), blood volume (BV) and permeability surface (PS). Cancer patients did not receive treatment prior to surgery. The expression of VEGF and MMP-2 were examined with both immunohistochemistry and Western blotting. **Results:** The levels of VEGF and MMP-2 by immunohistochemistry were significantly higher in the breast cancer group ($P < 0.01$) than the benign tumor group. Relative OD values from Western blotting were also higher in cancer cases ($P < 0.05$). Similarly, the mean MSCT perfusion parameters (BF, BV, PS) were significantly higher in the breast cancer group ($P < 0.01$), BF and BV positively correlating with VEGF expression ($r = 0.878$ and 0.809 respectively, $P < 0.01$); PS and VEGF and MMP-2 expression were also positively correlated ($r = 0.860$, 0.786 respectively, $P < 0.01$). **Conclusion:** There is a correlation between breast cancer MSCT perfusion parameters and VEGF and MMP-2 expression, which might be useful for detection of breast lesions, qualitative diagnosis of breast cancer, and evaluation of breast cancer treatment.

Keywords: Breast tumor - multi-slice spiral CT - perfusion - vascular endothelial growth factor - matrix metalloproteinase 2

Asian Pacific J Cancer Prev, 14 (1), 429-433

Introduction

Multi-slice spiral CT (MSCT) perfusion imaging is an imaging technique that was gradually developed from earlier generation that simply provides tumor morphological information and is capable of providing tumor hemodynamic information. The current MSCT technology can quantitatively analyze tumor angiogenesis, provide pathophysiological, and anatomical information associated with the tumor (So et al., 2011). Since the vast majority of malignant tumors are vascular-dependent (Ghasemi et al., 2011), MSCT perfusion imaging plays a significant role in diagnosis and differential diagnosis of malignant tumor, as well as establishing individualized treatment plan for cancer and assessing the treatment effect.

Currently, VEGF is considered the most important cytokine that induces tumor angiogenesis (Jobim et al., 2008), and VEGF has been shown overexpressed in various types of malignant tumors. MMP-2 is major enzyme

in matrix metalloproteinase family that degrades the basement membrane and extracellular matrix (Jezierska et al., 2009). It can degrade the basement membrane collagen and ultimately lead to reduced vascular maturation. Studies have shown that MMP-2 was also overexpressed in breast cancer (Al-Raawi et al., 2011). In addition, for patients with breast cancer VEGF is not only an effective angiogenesis medium, its overexpression is also associated with the change of vascular permeability (Sledge, 2002).

Studies have demonstrated that tumor perfusion parameters were correlated with VEGF overexpression in various types of human cancers, such as lung cancer, kidney cancer, pancreatic cancer, colorectal cancer, etc. The study of relationship between tumor perfusion parameters and the expression of pro-angiogenic factor in breast cancer remains limited to animal model. Research suggested that breast cancer CT perfusion parameters were correlated with high expression of tumor microvessel density (MVD) in rats (Park et al., 2009). Meanwhile the relationship between overexpression of MMP-2 and tumor

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perfusion parameters is still lacking. To our knowledge, the relationship between human breast cancer MSCT perfusion parameters and VEGF, MMP-2 has yet to be reported.

The purpose of this study is to explore whether there is any correlation between MSCT perfusion parameters and VEGF, MMP-2 expression for human breast cancer, and whether breast MSCT perfusion imaging can be an effective approach for the diagnosis, differential diagnosis, and evaluation of treatment for breast cancer.

Materials and Methods

Study Population

A total of 45 breast cancer patients and 16 patients with benign breast tumors, confirmed by surgical pathology exam and underwent MSCT perfusion imaging prior to surgery at Liaoning Medical University affiliated hospital, were consecutively enrolled from June 2010 to June 2011. The patients were all female (mean age 49 years; range 39-71), from middle class family, and divided into either breast cancer or benign breast tumor group. The inclusion criteria are as follows:

I. Clinically diagnosed or highly suspected breast cancer patients who underwent MSCT perfusion scan to acquire perfusion images and perfusion parameters. The subjects in this study were consecutively enrolled, i.e. all patients met the eligibility criteria during the study period (06/2010-06/2011) were enrolled;

II. Patients did not undergo neoadjuvant chemotherapy, radiotherapy and integrated treatment;

III. Patients with complete clinical information, pathology and imaging data.

This study was approved by Liaoning Medical University Institutional Review Board and has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). And informed consent was obtained from each patient.

MSCT perfusion acquisition

Patients took prone position, with elevated support at the neck and breasts sagging naturally. The scan covered the area from the apical of armpit to the lower edge of the breasts. Tumors were initially located with conventional CT axial scan using GE (GE, USA) Light speed 16 spiral CT machine (80 kV, 120mAs, matrix 512 × 512, scan field of vision (FOV) 12cm ~ 16cm, slice thickness 2.5 mm). Then selecting maximum layer of the tumor and 4 adjacent layers, including center layer, for perfusion imaging, the multi-layer dynamic CT perfusion scan (toggling-table) was performed with the following parameters: slice thickness 5.0 mm × 4 i, tube voltage 120 kV, tube current 60 mA, matrix 512 × 512, contrast agent (Europe Omnipaque, 300 mg/ml) injected at the rate of 3.0 ml/s with high-pressure syringe through the median cubital vein, total dose 50 ml, delay time 5.0 s, and the total scan time 50 s.

MSCT Perfusion Imaging and Analysis

Data were transferred to the ADW 4.2 workstation (GE, USA), analyzed with Perfusion 3 tumor perfusion

software. Perfusion images were then selected, calibrated to determine the threshold as -10 Hu ~ +300 Hu. The afferent artery was manually selected to be the thoracic aorta at the level where tumor was identified. The thoracic aorta, cancerous tissue and benign breast tumor are the regions of interest (ROI). Two to five ROIs were chosen to determine perfusion measures and construct perfusion maps for both groups. CT perfusion measures include blood flow (BF), blood volume (BV), permeability surface (PS).

VEGF and MMP-2 Expression Test

VEGF immunohistochemistry Assay: For each slice, the staining intensity and percentage of positive cells were scored separately (Lei et al., 2011). (1) staining intensity scores: 0 being colorless, 1 light yellow, 2 yellow, and 3 being brown or darker; (2) positive cells percentage rating: 0 for no positive cells, 1 for < 10% positive cells, 2 for 11% to 50%, 3 for 51% to 70%, and 4 for ≥ 71% positive cells. The level of VEGF expression was represented by the product of 2 scores: 0 being negative, <3 being weakly positive, and ≥ 3 being strongly positive.

MMP-2 immunohistochemistry Assay: For each slice, the staining intensity and size of positive staining area were scored separately (Shimizu et al., 1990): (1) staining intensity scoring: 0 being colorless, 1 light yellow, 2 yellow, and 3 being brown or darker; (2) positive staining area scoring: 0 being no staining, 1 being staining area less than 1/3, 2 being staining area between 1/3 and 2/3, and 3 being staining area ≥ 2/3. The level of MMP-2 expression was represented by the total of these 2 scores: 0 being negative, ≥ 3 being positive, and ≥ 5 being strongly positive.

Western Blot test and VEGF and MMP-2 protein expression: After ultrasonic fragmentation, tumor tissue samples were centrifuged and supernatant were extracted for electrophoresis. Proteins were then transferred to nitrocellulose membrane. Primary and secondary antibody (Wuhan Boster bioengineering Company, Wuhan, China), appropriately diluted with the hybridization solution, were added for color reaction. Optical density (OD) was acquired under Gel imaging system.

Statistical analysis

Analysis was performed with SPSS (V16.0). Between group perfusion parameters and immunohistochemical assay results were compared using t tests. The relationships between BF, BV, PS and VEGF, MMP-2 expression were analyzed with correlation analysis, including r (correlation coefficient).

Results

VEGF and MMP-2 expression in breast cancer and benign breast tumor groups

VEGF Expression: In breast cancer patients VEGF expression was shown primarily as brown staining of cytoplasm of the tumor cells (Table 1 and Figure1). The mean VEGF expression score was significantly higher than that in patients with benign breast tumors ($P < 0.01$).

MMP-2 Expression: In breast cancer patients MMP-

Table 1. The Results of Immunohistochemical Assay for Two Study Groups ($\bar{x} \pm s$)

	Breast Cancer n=45	Benign Tumor n=16	t	P value
VEGF	10.83±0.38	1.91±0.57	9.65	0
MMP-2	4.96±0.06	1.73±0.86	38.53	0

VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2

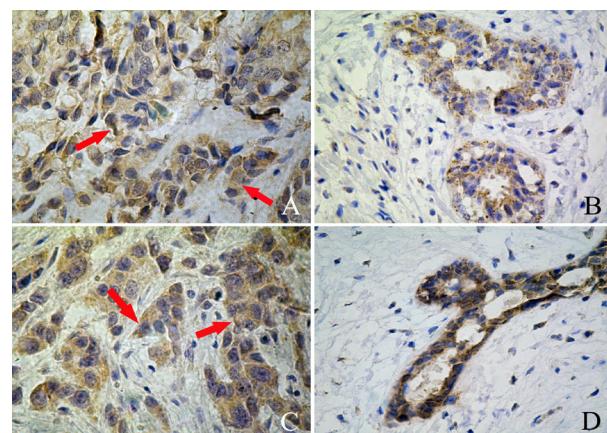


Figure 1. Immunohistochemistry Assay of VEGF and MMP-2 Protein Expression for Breast Tumor(x400).
(A) VEGF Expression in Breast Cancer. Arrow indicates brown particles in the cytoplasm of tumor cells. (B) VEGF Expression in Benign Breast Tumor. There was no brown particle in cytoplasm. (C) MMP-2 Expression in Breast Cancer. Arrow indicates brown particles in the cytoplasm of tumor cells. (D) MMP-2 Expression in Benign Breast Tumor. There was no brown particle in cytoplasm. VEGF: vascular endothelial growth factor. MMP-2: matrix metalloproteinase-2

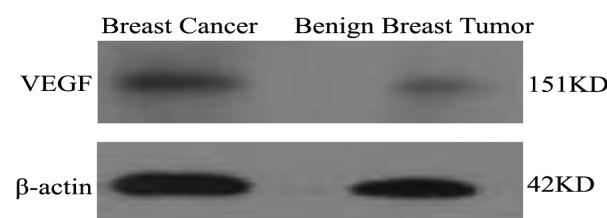


Figure 2. Western Blot Test of VEGF Protein Expression. VEGF: vascular endothelial growth factor

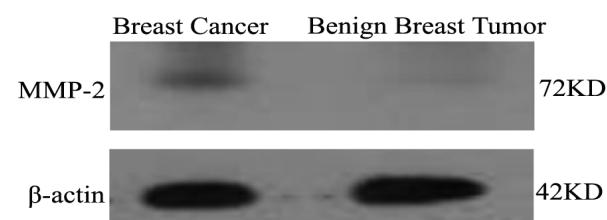


Figure 3. Western Blot Test of MMP-2 Protein Expression. MMP-2: matrix metalloproteinase-2

2 expression was shown primarily as brown staining of cytoplasm of the tumor cells (Table 1 and Figure 1). The mean MMP-2 expression score was also significantly higher than that in patients with benign breast tumors ($P < 0.01$).

The Results of Western Blot Test: The relative OD values of VEGF protein expression (Figure 2), calculated from three western blot tests, were statistically significant higher for the breast cancer group when compared with the

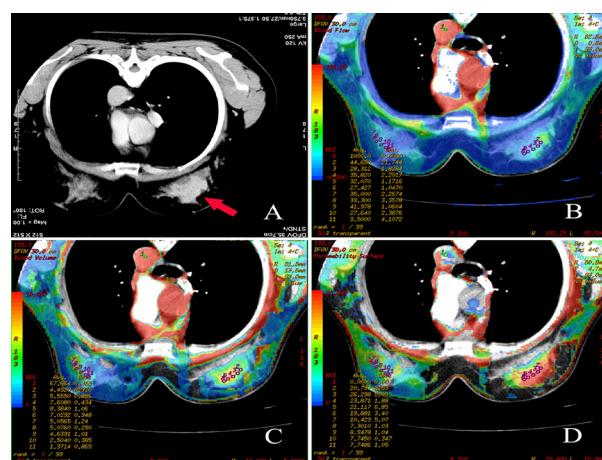


Figure 4. MSCT Perfusion Results of Breast Cancer.
(A) Baseline image for Breast Cancer. Left breast showed irregular mass. (B) MSCT Perfusion (BF) image for Breast Cancer. The region with cancer showed high perfusion state. (C) MSCT Perfusion (BV) Image for Breast Cancer. The region with cancer showed high perfusion state. (D) MSCT Perfusion (PS) Image for Breast Cancer. The region with cancer showed high perfusion state. BF: blood flow. MSCT: multi-slice spiral computed tomograph. BV: blood volume. PS: permeability surface

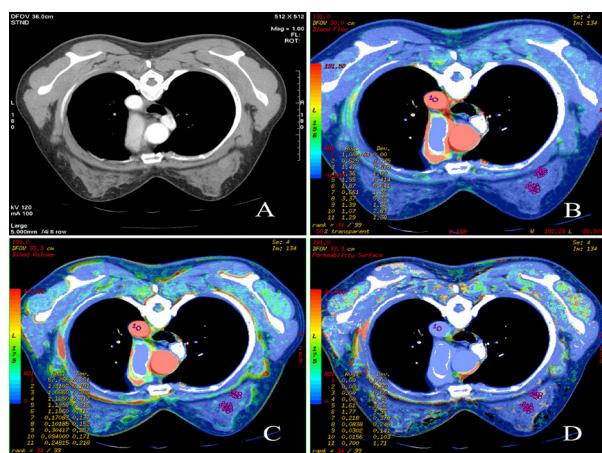


Figure 5. MSCT Perfusion Results of Benign Breast Tumor.
(A) Baseline image for benign breast tumor. Left breast showed round-shaped tumor. (B) MSCT Perfusion (BF) Image for benign breast tumor. The tumor region showed low perfusion state. (C) MSCT Perfusion (BV) Image for benign breast tumor. The tumor region showed low perfusion state. (D) MSCT Perfusion (PS) Image for benign breast tumor. The tumor region showed low perfusion state. MSCT: multi-slice spiral computed tomograph. BF: blood flow. BV: blood volume. PS: permeability surface

benign breast tumor group (0.32 ± 0.08 and 0.17 ± 0.06 respectively, $P < 0.05$). The relative OD values of MMP-2 protein expression (Figure 3), calculated from three western blot tests, were also statistically significant higher for the breast cancer group than those in the benign breast tumor group (0.21 ± 0.05 and 0.06 ± 0.01 respectively, $P < 0.05$).

MSCT perfusion imaging and perfusion parameters in breast cancer and benign breast tumor groups

The perfusion images for both breast cancer and benign tumor groups were satisfactory. The baseline images

Table 2. Perfusion Results for Breast Cancer and Benign Tumor Groups ($\bar{x} \pm s$)

	Breast Cancer (n=45)	Benign Tumor (n=16)	P value
BF [ml/(min×100mg)]	17.81±3.56	7.92±1.62	0
BV (ml/100mg)	5.99±0.81	2.15±0.99	0
PS [ml/(min×100mg)]	17.8±5.21	3.94±1.87	0

BF, blood flow; BV, blood volume; PS, permeability surface

accurately located the tumors. Breast cancer showed high perfusion state, while benign tumor showed low perfusion state (Table 2 and Figure 4-5). The means of perfusion parameters (BF, BV and PS) were all statistically significant higher in breast cancer group than those in benign tumor group.

Correlation Between Perfusion Parameters and Immunohistochemical Results for Breast Cancer

Both BF and BV showed a highly positive correlation with VEGF expression ($r=0.878$ and 0.809 respectively, $P < 0.01$). PS was positively correlated with both VEGF and MMP-2 expression ($r=0.860$ and 0.786 respectively, $P < 0.01$).

Discussion

Our study demonstrated higher VEGF and MMP-2 expression in patients with breast cancer than those with benign breast tumor. This indicated that although there were VEGF and MMP-2 expression in both groups of patients, the extent and density of neovascularization, and permeability of new blood vessels generated in malignant breast tumors were all significantly greater than those in benign breast tumors, suggesting both quantitative and functional changes associated with the angiogenesis of malignant tumor, i.e. a large amount of immature blood vessels formed. The overexpression of VEGF in malignant breast tumor can reflect the potential of angiogenesis in the tumor tissue (Perrot-Applanat et al., 2012). The overexpression of MMP-2 indicates the damage of the integrity of microvascular basement membrane and the change of heterologous tumor vascular function (Vinothini et al., 2011). On the other hand, the low level expressions of VEGF, MMP-2 in benign tumor may have little effect on the amount of angiogenesis and vessel permeability. Thus, the maturity of microvessel is still high, basement membrane remains intact and function is also close to normal blood vessels. Together, these suggest there is essential difference on the expression of pro-angiogenic factors from vascular endothelial cells between benign and malignant breast tumor, which maybe the basis of observed differences on the MSCT perfusion images and perfusion parameters.

In this study, the means of perfusion parameters (BF, BV) were significantly higher in breast cancer group than those in benign tumor group, consistent with the biological phenomena of a large number of tumor angiogenesis seen in malignant breast tumors. At the development of breast cancer, the proliferation of tumor blood vessel increased dramatically (Sachdev et al., 2008), and a variety of pro-

angiogenic factors were overexpressed, which was not seen in benign tumors and may explain the lower BF and BV measures in this group. This study also showed high permeability state and higher PS measure in breast cancer group, suggesting the increased capillary permeability and abnormal, distorted and irregular vascular morphology of the new blood vessels within the cancer tissue. In addition, the degradation of basement membrane and extracellular matrix could also lead to lower tumor vasculature maturity and increased endothelial cell gap, thus causing the extravasation of contrast agent and resulted in a higher PS measure, suggesting the elevated PS measure appropriately reflected the change of vascular permeability in breast cancer tissue. Therefore, MSCT perfusion parameters (BF, BV, PS) reflected the state of hemodynamic changes in both benign and malignant breast tumors from different perspectives, accurately measured the tumor perfusion and angiogenesis, and provided experimental basis for using MSCT in the clinical diagnosis, differential diagnosis and quantitative evaluation of treatment for breast cancer.

In this study, BF, BV were positively correlated with VEGF expression and tumor PS was correlated with both VEGF and MMP-2 expression in breast cancer patients, suggesting tumor angiogenesis in breast cancer patients measured by MSCT perfusion was consistent with the results of pathologic examination, and the changes in perfusion parameters can reflect the changes of tumor tissue VEGF, MMP-2 expression. CT perfusion parameters, BF and BV, reflect the state of dynamic perfusion and blood volume in local tissue respectively (Jain, 2011). BV, in particular, is directly related to the area of local vascular bed (Leiva-Salinas et al., 2011). In the process of tumor angiogenesis, high VEGF expression can promote the generation of new capillary and increase the micro-vascular density of tumor tissue (Ahluwalia et al., 2012), leading to elevated blood flow. BF and BV can also reflect the changes of micro-vascular density and the number of capillary in breast cancer tissue, and their results are consistent with VEGF expression, indicating that MSCT perfusion imaging following intravenous injection of contrast agent can reveal microcirculation distribution and perfusion abnormalities of breast cancer tissue. PS is related to high permeability of tumor blood vessels and can reflect the local tissue vascular permeability, thus can indicate the maturity level of tumor vasculature and the extent of the destruction of the local vascular endothelial cells barrier. PS reflects the changes in breast tumor vascular permeability and the result in this study was consistent with the expression of VEGF and MMP-2, indicating higher degree of malignancy is correlated with lower level of blood vessel maturity; thus higher permeability to macromolecules resulted in higher PS value. The low degree of vascular maturity and the high number of tumor blood vessels are two important characters of breast cancer. Breast perfusion parameters can successfully reflect the level of VEGF, MMP-2 expression through quantitative macroscopic measurement, making it possible to establish reasonable linear relationship between perfusion parameters and tumor angiogenesis. This also serves as the basis for

further clinical application of MSCT in the diagnosis, differential diagnosis and evaluation of treatment for breast cancer.

In conclusion: Breast MSCT perfusion imaging, as a type of functional imaging modality, its perfusion measures have demonstrated a good correlation with the VEGF and MMP-2 expression in breast cancer tissue. While studies have found that among patients with breast fibrous cysts, benign atypical hyperplasia, there were also VEGF and MMP-2 expression (Somiari et al., 2006; Konukoglu et al., 2007), the consecutive enrollment of this study minimized the potential effect of these factors on the VEGF and MMP-2 expression, perfusion measures and their identified association. Therefore, the results support that breast MSCT perfusion imaging can acquire information of tumor morphology and biology and be an effective way to detect breast lesion, diagnose breast cancer and evaluate treatment effect.

MSCT perfusion imaging can accurately reveal the biological characteristics of malignant breast tumor, i.e. high perfusion, high permeability and low maturity when compared with benign breast tumor. Its result is also positively correlated with tumor angiogenesis. However, the proliferation of tumor vascular endothelial cells may not generate the functional blood vessels, and MSCT perfusion imaging may be only sensitive to perfusable capillary, therefore its result may only approximate the state of tumor angiogenesis. In addition, since the breast tissues are exocrine glands, sensitive to X-ray, the scope of using MSCT perfusion imaging is rather limited, especially for the diagnosis and evaluation of treatment for breast disease in younger female. Furthermore, allergy and adverse effect of iodinated contrast agents on patients' renal function when administrated with large dosage also limited the use of MSCT perfusion imaging.

Acknowledgements

The study is funded by 1) Liaoning Medical University Young Investigator Science and Technology Initiation Funds (Project # Y2012Z010), and 2) the First Affiliated Hospital of Liaoning Medical University Young Investigator Science and Technology Initiation Funds (Project # FY2012-16).

References

- Ahluwalia AS, Tarnawski A (2012). Critical Role of Hypoxia Sensor - HIF-1 in VEGF Gene Activation. Implications for Angiogenesis and Tissue Injury Healing. *Curr Med Chem*, **19**, 90-7.
- Al-Raawi D, Abu-El-Zahab H, El-Shinawi M, Mohamed MM (2011). Membrane type-1 matrix metalloproteinase (MT1-MMP) correlates with the expression and activation of matrix metalloproteinase-2 (MMP-2) in inflammatory breast cancer. *Int J Clin Exp Med*, **4**, 265-75.
- Ghasemi M, Emadian O, Naghshvar F, et al (2011). Immunohistochemical expression of vascular endothelial growth factor and its correlation with tumor grade in breast ductal carcinoma. *Acta Med Iran*, **49**, 776-9.
- Jain R (2011). Perfusion CT imaging of brain tumors: an overview. *Am J Neuroradiol*, **32**, 1570-7.
- Jeziarska A, Motyl T (2009). Matrix metalloproteinase-2 involvement in breast cancer progression: a mini-review. *Med Sci Monit*, **15**, RA32-40.
- Jobim FC, Schwartmann G, Xavier NL, et al (2008). [Expression of MMP-9 and VEGF in breast cancer: correlation with other prognostic indicators]. *Rev Bras Ginecol Obstet*, **30**, 287-93.
- Konukoglu D, Turhan MS, Celik V, Turna H (2007). Relation of serum vascular endothelial growth factor as an angiogenesis biomarker with nitric oxide & urokinase-type plasminogen activator in breast cancer patients. *Indian J Med Res*, **125**, 747-51.
- Lei Z, Ma H, Xu N, Xi H (2011). The evaluation of anti-angiogenic treatment effects for implanted rabbit VX2 breast tumors using functional multi-slice spiral computed tomography (f-MSCT). *Eur J Radiol*, **78**, 277-81.
- Leiva-Salinas C, Provenzale JM, Wintermark M (2011). Responses to the 10 most frequently asked questions about perfusion CT. *Am J Roentgenol*, **196**, 53-60.
- Park CM, Goo JM, Lee HJ, et al (2009). FN13762 murine breast cancer: region-by-region correlation of first-pass perfusion CT indexes with histologic vascular parameters. *Radiology*, **251**, 721-30.
- Perrot-Applanat M, Di Benedetto M (2012). Autocrine functions of VEGF in breast tumor cells: Adhesion, survival, migration and invasion. *Cell Adh Migr*, **6**, 547-53.
- Sachdev JC, Jahanzeb M (2008). Evolution of bevacizumab-based therapy in the management of breast cancer. *Clin Breast Cancer*, **8**, 402-10.
- Shimizu M, Saitoh Y, Itoh H (1990). Immunohistochemical staining of Ha-ras oncogene product in normal, benign, and malignant human pancreatic tissues. *Hum Pathol*, **21**, 607-12.
- Sledge GW, Jr. (2002). Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol*, **29**, 104-10.
- So A, Lee TY (2011). Quantitative myocardial CT perfusion: a pictorial review and the current state of technology development. *J Cardiovasc Comput Tomogr*, **5**, 467-81.
- SB, Somiari RI, Heckman CM, et al (2006). Circulating MMP2 and MMP9 in breast cancer -- potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories. *Int J Cancer*, **119**, 1403-11.
- Vinothini G, Aravindraja C, Chitrathara K, Nagini S (2011). Correlation of matrix metalloproteinases and their inhibitors with hypoxia and angiogenesis in premenopausal patients with adenocarcinoma of the breast. *Clin Biochem*, **44**, 969-74.