

Evaluation of clinical significance of endoglin expression during breast cancer and its correlation with ER and PCNA

J.-L. GUO¹, S.-Q. GU², Y. LI³, X.-Y. ZHANG⁴

¹Department of General Surgery, Shouguang People's Hospital of Shandong Province, China

²Digestive System Department, Shouguang People's Hospital of Shandong Province, China

³Department of Pediatrics, Shouguang People's Hospital of Shandong Province, China

⁴Department of Ophthalmology, Shouguang People's Hospital of Shandong Province, China

Abstract. – OBJECTIVE: The present study is aimed to investigate the expression of Endoglin during breast cancer. Moreover, its clinical pathological significance, as well as correlation with estrogen receptor (ER) and proliferating cell nuclear antigen (PCNA), were also studied.

PATIENTS AND METHODS: qRT-PCR and Western blot assays were utilized to study PCNA mRNA, ER, Endoglin and protein expression. Immunohistochemistry analysis was conducted to determine the expression of Endoglin, ER, and PCNA protein in breast cancer tissue and adjacent cancer tissue. The microvascular density (MVD) was indicated by expression of Endoglin protein. The positive cell rate was used to express the protein expression level of ER and PCNA.

RESULTS: The mRNA expression levels of Endoglin, ER, and PCNA were significantly elevated in breast cancer tumor tissues in comparison with the cancer-adjacent tissues. The positive expression rates of Endoglin, ER, and PCNA were 69%, 56%, and 73% respectively in breast cancer tumor tissues. Endoglin MVD values in breast cancer tissues and cancer-adjacent tissues were $(35.18 \pm 9.57)/\text{mm}^2$ and $(7.21 \pm 1.63)/\text{mm}^2$, respectively. The expression of Endoglin protein in breast tumor tissues was positively correlated to lymph node metastasis and TNM stages, but it was not to menopause and tumor size. Endoglin protein was positively correlated to the expression of PCNA protein, but was not correlated to ER expression.

CONCLUSIONS: Endoglin protein expression is positively correlated to PCNA protein expression. So, Endoglin MVD in breast cancer tissues has important clinical significance in the assessment of breast cancer prognosis.

Key Words:

Endoglin, Estrogen receptor, Proliferating cell nuclear antigen, Breast cancer.

Introduction

Breast cancer is one of the most common malignant tumors of women in the world. The epidemiological data of breast cancer show that breast cancer has become the first female malignant tumor in an urban area^{1,2}. Although the diagnosis and treatment technology in recent years has been great progress, the prognosis is still not ideal. Further, the mortality rates have not changed much, so the search for an ideal marker for development and metastasis of breast cancer is of great significance.

Tumor angiogenesis is considered to be a key factor in the development of breast cancer. Many animal and clinical trials showed that tumor growth is dependent on angiogenesis. The angiogenesis is a promoting factor for the development of tumor *in vivo* so that the tumor cells could proliferate rapidly³. Among them, Endoglin (CD105) is the most commonly used and the most reliable tumor microvascular marker and is the prime focus of the present study. The expression of endoglin protein could reflect the ability of tumor angiogenesis and the type of biological behavior.

Endoglin is basically a two-polymer membrane binding glycoprotein with the molecular weight of 180 kDa. Endoglin has high expression in vascular endothelial cells and marginal part of tumor tissue⁴. Researches⁵⁻⁷ showed that Endoglin inhibits the proliferation of vascular endothelial cells by regulating the transforming growth factor TGF β 1. In this way, it is involved in vascular development and remodeling, leading to promotion of tumor angiogenesis. Endoglin could protect tumor cells to escape the inhibition

of TGF β 1, and enhance the ability of invasion and metastasis of tumor⁵. Further, recent studies^{6,7} showed that estrogen receptor α (ER α) protein expression increases and ER β protein expression decreases during breast cancer. ER α promotes the proliferation of human breast cancer MCF-7 cells and up-regulates the expression of the proliferation marker protein PCNA and Ki-67⁷. Inhibition of expression of ER α is the key player in silibinin induced autophagy and apoptosis in breast cancer MCF-7 cells⁸. Bado et al⁹ showed that ER β is able to reduce the invasive ability of three negative breast cancer cells by regulating the carcinogenic effect of mutant p53. However, there are reports that revealed the role of ER β and PEA3 in co-activation of IL-8 expression, leading to the invasion of breast cancer cells¹⁰. These negative breast cancer cells by regulating the carcinogenic eER β in breast cancer are still not clear¹¹. Proliferating cell nuclear antigen (PCNA) lies in proliferating cells and cancer cells. It is closely related to the synthesis of cell DNA and is a key factor as well as a good marker for the initiation of cell proliferation. Previous reports have confirmed that PCNA is directly related to the degree of tumor differentiation, stage of cancer and the prognosis of cancer¹². In this work, we studied the expression levels of Endoglin, Era, and PCNA by qRT-PCR, Western blot, and immunohistochemical staining methods. The present study provided a theoretical basis for the evaluation of tumor invasion and metastasis in clinical practice, and provided an ideal molecular marker for the prognosis of breast cancer.

Patients and Methods

Patients

We collected 62 cases of breast cancer resection from September 2013 to March 2016. All patients were confirmed by pathological biopsy. Referring to the practical surgical pathology carried out the pathological grading of breast cancer patients. Patients with breast cancer had not undergone radiotherapy or chemotherapy before surgery. This study was approved by the hospital Ethics Committee, and obtained the patient's informed consent.

Main Reagents

Trizol kit was bought from Invitrogen Company (Carlsbad, CA, USA). One Step PrimeScript cDNA synthetic reagent kit and SYBR[®]

Premix Ex Taq[™] II were bought from Dalian Treasure Biological Company (Dalian, Liaoning, China). The primers of qRT-PCR experiment were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China). Endoglin antibody, PCNA antibody, and ER α / β antibody were purchased Abcam Company (Cambridge, MA, USA). β -actin antibody and second anti were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase marked second anti and DAB reagent kit were purchased from Wuhan Boster Biological Company (Wuhan, Hubei, China).

qRT-PCR and Western Blot Analyses

Fresh breast cancer tissues and the corresponding adjacent tissues (preserved in liquid nitrogen) were obtained after surgery. The total RNA was extracted by Trizol method. The purity and concentration of RNA were determined by spectrophotometer. Used One Step PrimeScript cDNA reverse transcription kit for the synthesis of cDNA. The amplification reaction was performed at ABI 7500 Real-Time PCR. Used β -actin as a reference gene. beta.1.4 Detection of Ki67, Bax and Bcl-2 protein expression in breast cancer tissues were performed by Western blots.

Immunohistochemical Staining

Dewaxing and dehydration were undertaken, and H₂O₂ was used to block for 30 min. Antigen repair was performed in a microwave oven with a phosphate buffer for 5 min. 10% sheep serum was added and closed at room temperature for 45 min. This was followed by overnight incubation at 4 degrees C. Incubation of horseradish peroxidase (HRP) labeled second anti performed at room temperature for 30 min. DAB was used for color development for 5 min and counterstained with hematoxylin. The results of the experiment were diagnosed and determined through the double-blind method by two doctors.

Positive Result Judgment and MVD Calculation Method

The results of immunohistochemical staining were observed under light microscope. The results showed that yellow or brown cells were positive cells. The nuclei of ER and PCNA positive cells were stained with yellow or brown yellow; CD105 positive cells were stained with yellow or brown yellow. The proportion of positive cells in the total cells expressed the expression levels of Endoglin, ER and PCNA protein. The number

of positive cells >10% was positive (+), > 30% was positive (+ +), > 50% was positive (+ + +), > 75% was positive (+ + + +). The calculation method of MVD value: Carefully observed tumor sections under 100 times light microscope. The region with the highest microvessel density was selected. Then, counted the positive cells of the area under 400 times light microscope. The cells that were not clear or stained blur were excluded regardless of whether there is vascular cavity. It could also be regarded as a microvessel. 5 microvessels in the field of vision were taken, and the average value was recorded as microvessel density (MVD)¹³.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. The protein levels and mRNA levels of Endoglin and PCNA in breast cancer tissues and adjacent normal breast tissues were expressed by $\bar{x} \pm s$, and the *t*-test was used. Correlation analysis used Spearman rank correlation analysis. **p* < 0.05 showed that there was a statistical difference.

Results

The qRT-PCR experiments and statistics showed that Endoglin, ER and PCNA mRNA expression levels in breast cancer tissues were elevated in comparison to adjacent tissues (Figure 1). The Endoglin, ER, and PCNA protein expression were elevated in breast cancer tumor tissues (Figure 2 A-C), but ER protein expression showed decrease. Further, we calculated the ratio of ER α /ER β , and the final results showed that the ratio of ER α /ER β in breast cancer was significantly higher than that in the adjacent tis-

sues (Figure 2 D). The relationship between the expression of Endoglin protein and the clinicopathological parameters of breast cancer. The value of Endoglin MVD in breast cancer was $(35.18 \pm 9.57)/\text{mm}^2$. The value of Endoglin MVD in the tumor adjacent tissues was $(7.21 \pm 1.63)/\text{mm}^2$. Statistical results showed that the Endoglin MVD value in breast cancer tissue was significantly higher than that in the adjacent tissues.

As shown in Table I, Endoglin marked MVD in the breast cancer tissues with lymph node metastasis was significantly higher than that in the breast cancer tissues without lymph node metastasis. Compared with breast cancer tissue in stage I, II, Endoglin marked MVD in breast cancer tissue of TNM stage III, IV elevated. In postmenopausal and postmenopausal breast cancer patients, there was no difference in the MVD values of the Endoglin markers. In breast cancer whose diameter ≤ 3 cm and > 3 cm, Endoglin labeled MVD value had no difference. Therefore, the expression of Endoglin protein in cervical cancer was not related to menopause and tumor size, and the difference was not statistically significant.

Correlation of Expression of Endoglin Protein and ER, PCNA Protein in Breast Cancer Tissues

In 62 cases of breast cancer, the positive expression rate of Endoglin protein in breast cancer was 69%. The positive expression rate of ER in breast cancer tissues was 56%, and there was no significant correlation between Endoglin protein and ER protein expression. The positive expression rate of PCNA in breast cancer tissues was 73%. Compared with PCNA expressed negative breast cancer tissues, the MVD values of Endoglin markers were significantly elevated in

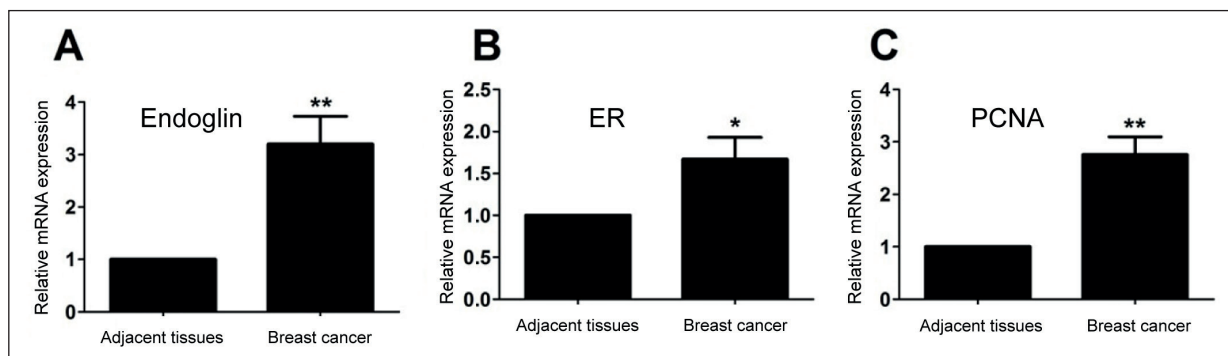


Figure 1. mRNA expression of Endoglin, ER and PCNA were upregulated in breast cancer.

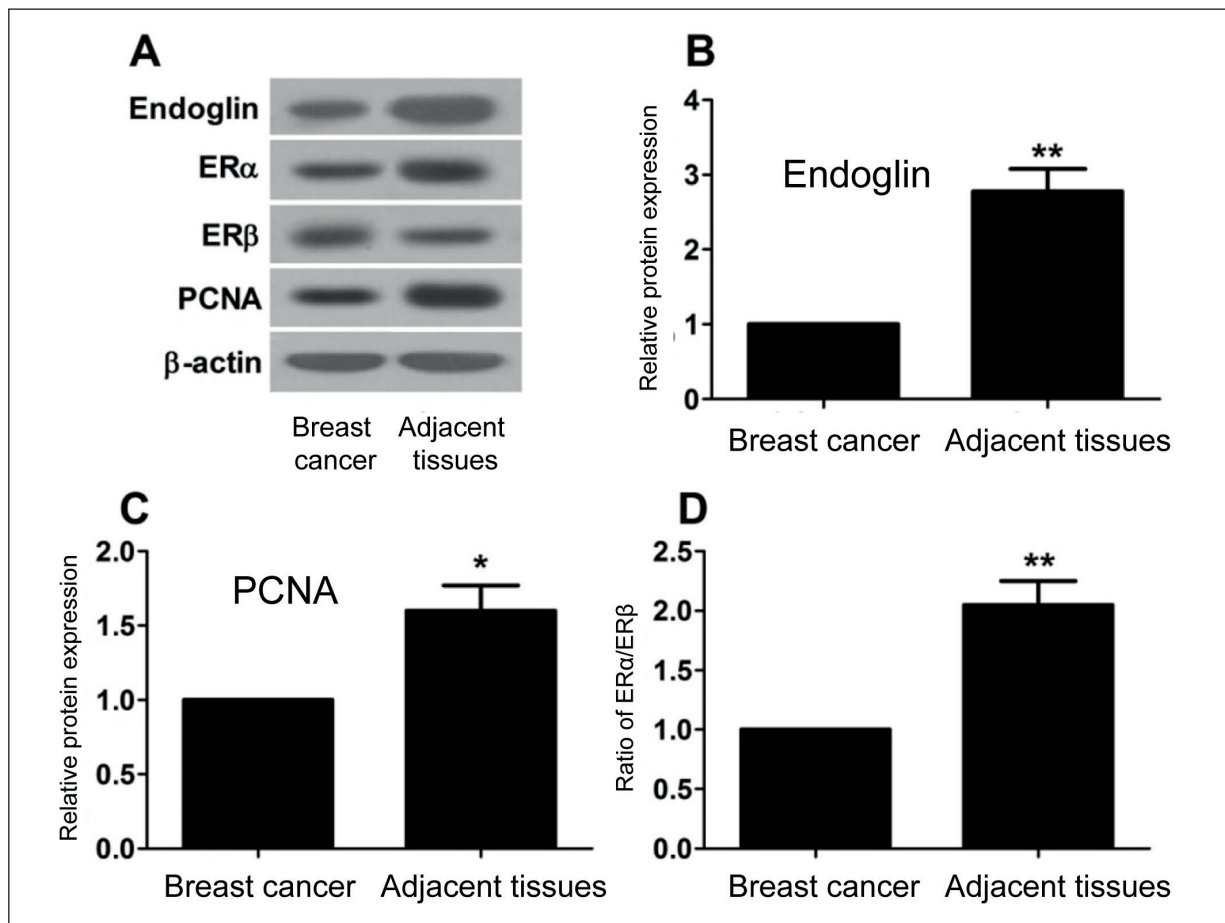


Figure 2. Protein expression of Endoglin, ER and PCNA were upregulated in breast cancer. **A**, Western blot was performed to detect protein expression of Endoglin, ER and PCNA; **B**, Relative protein expression of Endoglin; **C**, Relative protein expression of PCNA; **D**, The ratio of ERα/ERβ.

PCNA positive breast cancer tissues, and were positively correlated (Table II). With the increase of the breast cancer cell proliferation activity,

MVD value showed increase, suggesting that PCNA and Endoglin might be involved in the occurrence and development of breast cancer.

Table I. Correlation between expression of Endoglin protein and clinicopathological feature.

Pathologic feature	Number of cases	Endoglin labeled MVD value	t-value	p-value
Menopause				
Yes	30	37.56 ± 14.83	0.1428	> 0.05
No	32	41.07 ± 16.24		
Tumor diameter (cm)				
≤ 3	24	38.18 ± 12.90	0.2915	> 0.05
> 3	38	40.64 ± 13.66		
Lymph node metastasis				
Yes	37	50.27 ± 23.41	3.8385	< 0.05
No	25	28.56 ± 10.45		
TNM stage				
Stage I, II	34	35.43 ± 13.58	2.7846	< 0.05
Stage III, IV	28	43.35 ± 16.44		

Table II. Correlation between expression of Endoglin and expression of ER and PCNA.

Parameter	Number of cases	Endoglin labeled MVD value	r-value	p-value
ER				
Positive	35	40.86 ± 14.05	0.215	> 0.05
Negative	27	37.79 ± 12.82		
PCNA				
Positive	45	48.31 ± 21.59	0.623	< 0.01
Negative	17	30.62 ± 11.73		

Discussion

The growth of breast cancer tumor depends on the formation of new blood vessels¹⁴. After the tumor in the proliferation of about 10^6 cells, the expansion of tumor cell population needs the function of tumor angiogenesis factor, which could promote the formation of capillary and provide nutrition factors to tumor cell proliferation. The newly formed blood vessels also accelerate the exchange of tumor cells and blood circulation¹⁵. Endoglin is located on the surface of the cell membrane and the cytoplasmic blood vessel for the angiogenesis of promoting factor. Expression of Endoglin enhances the invasion ability of metastatic breast cancer cells¹⁶. Akagi et al¹⁷ confirmed that Endoglin played an important role in the process of tumor angiogenesis and the maintenance of vascular integrity. The expression of Endoglin protein could be determined by immunohistochemical staining. The expression of MVD could also be measured indirectly. After targeting inhibition of Endoglin expression in the animal model of metastatic breast cancer, the tumor formation and metabolism were significantly inhibited¹⁸.

In the present study, we found that Endoglin mRNA and protein expression levels were increased in breast cancer tumor tissues, revealing involvement of Endoglin in the pathogenesis of breast cancer. The value of Endoglin MVD in breast cancer tissue was significantly higher than that in the adjacent tissues. In the breast cancer tissues with lymph node metastasis, the MVD of Endoglin marker was significantly higher than that in the breast cancer tissues without lymph node metastasis. The expression of Endoglin protein in cervical cancer was not related to menopause or tumor size, and the differences were not statistically significant. In the breast cancer tissues, MVD with Endoglin markers was positively correlated to the expression of PCNA protein, lymph node metastasis, and TNM stage.

Therefore, we could speculate that the high levels of MVD in breast cancer tissue promote tumor cells to undergo microvascular metastasis or to be directly infiltrated into surrounding tissue. In addition, the increase in the number of microvessels provides nutrition to tumor cells, so that the proliferation of tumor cells goes more rapidly, which might be a major risk factor for tumor metastasis of breast cancer patients.

In postmenopausal for positive ER α breast cancer patients, ER β overexpression could lead to resistance to endocrine therapy, and the prognosis is poor¹⁹. The IRE1/XBP-1 signaling pathway is inhibited, and the survival rate of breast cancer cells is decreased after the overexpression of wild-type ER β or ER β agonists^{20,21}. Mendoza et al²¹ speculated that the decrease of ER α /ER β ratio resulted in phosphorylation of p38 MAPK and tumor suppressor p53, which inhibited the proliferation of breast cancer MCF-7 cells and promoted the apoptosis of MCF-7 cells in breast cancer. The balance disorder between ER α and ER β might be an important factor affecting the overproliferation of breast cancer cells. In this study, we found that the expression of ER α protein in breast cancer was increased, but the expression of ER β protein was decreased. It was found that the ratio of ER α /ER β was significantly increased in breast cancer tumor tissues. Therefore, the development of new drugs that could selectively regulate the expression of ER α and ER β might become a new strategy to conquer breast cancer.

Conclusions

The expression of Endoglin protein in breast cancer is increased, and is related to lymph node metastasis and TNM stage. Further, there is a positive correlation between Endoglin protein and PCNA protein expression. So, Endoglin and PCNA might have a synergistic relationship in the pathogenesis of breast cancer. Moreover, En-

doglin might be directly related to the genesis and development of breast cancer, but its role in the pathogenesis of breast cancer needs further research. Therefore, Endoglin might be an effective target for targeted therapy of breast cancer.

Conflict of Interest

The authors declare no conflict of interest.

References

- 1) SWAIN SM, KIM SB, CORTÉS J, RO J, SEMIGLAZOV V, CAMPONE M, CIRUELOS E, FERRERO JM, SCHNEEWEISS A, KNOTT A, CLARK E, ROSS G, BENYUNES MC, BASELGA J. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol* 2013; 14: 461-471.
- 2) ZHONG BL, BIAN LJ, WANG GM, ZHOU YF, CHEN YY, PENG F. Identification of key genes involved in HER2-positive breast cancer. *Eur Rev Med Pharmacol Sci* 2016; 20: 664-672.
- 3) GLUZ O, GAUMANN A, WILD P, DIALLODANE BROCK R, LIEDTKE C, TING E, MOHRMANN S, POREMBA C, NITZ U, HARTMANN A. Prognostic and predictive impact of expression of the angiogenic markers CD105 and CD31 expression in patients with high risk breast cancer (HRBC). *Cancer Res* 2010; 69: 2154-2154.
- 4) MARTINEZ LM, LABOVSKY V, DAVIES KM, RIVELLO HG, BIANCHI MS, WERNICKE A, CHASSEING NA. CD105 expression on CD34-negative spindle-shaped stromal cells of primary tumor is an unfavorable prognostic marker in early breast cancer patients. *PLoS One* 2015; 10: e0121421.
- 5) DUMONT N, ARTEAGA CL. Transforming growth factor-beta and breast cancer: tumor promoting effects of transforming growth factor-β. *Breast Cancer Res* 2000; 2: 125-132.
- 6) CHENG L, LI J, HAN Y, LIN J, NIU C, ZHOU Z, YUAN B, HUANG K, LI J, JIANG K, ZHANG H, DING L, XU X, YE Q. PES1 promotes breast cancer by differentially regulating ERα and ERβ. *J Clin Invest* 2012; 122: 2857-2870.
- 7) LIAO XH, LU DL, WANG N, LIU LY, WANG Y, LI YQ, YAN TB, SUN XG, HU P, ZHANG TC. Estrogen receptor α mediates proliferation of breast cancer MCF-7 cells via a p21/PCNA/E2F1-dependent pathway. *FEBS J* 2014; 281: 927-942.
- 8) ZHENG N, ZHANG P, HUANG H, LIU W, HAYASHI T, ZANG L, ZHANG Y, LIU L, XIA M, TASHIRO S, ONODERA S, IKEJIMA T. ERα down-regulation plays a key role in silibinin-induced autophagy and apoptosis in human breast cancer MCF-7 cells. *J Pharmacol Sci* 2015; 128: 97-107.
- 9) BADO I, NIKOLOS F, RAJAPAKSA G, GUSTAFSSON JÅ, THOMAS C. ERβ decreases the invasiveness of triple-negative breast cancer cells by regulating mutant p53 oncogenic function. *Oncotarget* 2016; 7: 13599-13611.
- 10) CHEN Y, CHEN L, LI JY, MUKAIDA N, WANG Q, YANG C, YIN WJ, ZENG XH, JIN W, SHAO ZM. ERβ and PEA3 co-activate IL-8 expression and promote the invasion of breast cancer cells. *Cancer Biol Ther* 2011; 11: 497-511.
- 11) PERONE Y, MAGNANI L. Going off the grid: ERα breast cancer beyond estradiol. *J Mol Endocrinol* 2016; 57: F1-5.
- 12) MALKAS LH, HERBERT BS, ABDEL-AZIZ W, DOBROLECKI LE, LIU Y, AGARWAL B, HOELZ D, BADVE S, SCHNAPER L, ARNOLD RJ, MECHREF Y, NOVOTNY MV, LOEHRER P, GOULET RJ, HICKEY RJ. A cancer-associated PCNA expressed in breast cancer has implications as a potential biomarker. *Proc Natl Acad Sci U S A* 2006; 103: 19472-19477.
- 13) CHEN M, WANG WP, JIA WR, TANG L, WANG Y, ZHAN WW, FEI XC. Three-dimensional contrast-enhanced sonography in the assessment of breast tumor angiogenesis: correlation with microvessel density and vascular endothelial growth factor expression. *J Ultrasound Med* 2014; 33: 835-846.
- 14) XIN X, MAJUMDER M, GIRISH GV, MOHINDRA V, MARUYAMA T, LALA PK. Targeting COX-2 and EP4 to control tumor growth, angiogenesis, lymphangiogenesis and metastasis to the lungs and lymph nodes in a breast cancer model. *Lab Invest* 2012; 92: 1115-1128.
- 15) XIONG J, YANG Q, LI J, ZHOU S. Effects of MDM2 inhibitors on vascular endothelial growth factor-mediated tumor angiogenesis in human breast cancer. *Angiogenesis* 2014; 17: 37-50.
- 16) OXMANN D, HELDFEINDT J, STARK AM, HATTERMANN K, YONEDA T, MENTLEIN R. Endoglin expression in metastatic breast cancer cells enhances their invasive phenotype. *Oncogene* 2008; 27: 3567-3575.
- 17) AKAGI K, IKEDA Y, SUMIYOSHI Y, KIMURA Y, KINOSHITA J, MIYAZAKI M, ABE T. Estimation of angiogenesis with anti-CD105 immunostaining in the process of colorectal cancer development. *Surgery* 2002; 131: S109-113.
- 18) PAAUWE M, HEUKANTS RC, OUDT CH, VAN PELT GW, CUI C, THEUER CP. Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer. *Oncogene* 2016; 35: 4069-4079.
- 19) GUO L, ZHANG YU, YILAMU D, LIU S, GUO C. ERβ overexpression results in endocrine therapy resistance and poor prognosis in postmenopausal ERα-positive breast cancer patients. *Oncol Lett* 2016; 11: 1531-1536.
- 20) RAJAPAKSA G, NIKOLOS F, BADO I, CLARKE R, GUSTAFSSON JA, THOMAS C. ERβ decreases breast cancer cell survival by regulating the IRE1/XBP-1 pathway. *Oncogene* 2015; 34: 4130-4141.
- 21) ZHUGE BZ, DU BR, MENG XL, ZHANG YQ. MASTL is a potential poor prognostic indicator in ER+ breast cancer. *Eur Rev Med Pharmacol Sci* 2017; 21: 2413-2420.
- 22) MENDOZA RA, ENRIQUEZ MI, MEJIA SM, MOODY EE, THORDARSON G. Interactions between IGF-I, estrogen receptor-α (ERα), and ERβ in regulating growth/apoptosis of MCF-7 human breast cancer cells. *J Endocrinol* 2011; 208: 1-9.