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Ki-67 expression in luminal type breast cancer and its association with the clinicopathology of the cancer

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Abstract. The aim of the present study was to examine Ki-67 expression in tissue of luminal type breast cancer and to investigate the association between the Ki-67 expression level and clinicopathology of breast cancer. A total of 62 patients with breast cancer were included in the study group, whereas 30 patients undergoing a health check-up who were diagnosed with breast hyperplasia were included in the control group. Levels of Ki-67 expression in patients of the two groups were assessed using fluorescent quantitative polymerase chain reaction. The association between Ki-67 and the clinicopathology of the cancer was investigated by analyzing cancer cell proliferation and migration by reducing Ki-67 expression in the human MCF-7 cancer cell line. Compared with the controls, Ki-67 expression was significantly increased in the serum and cancer tissue of breast cancer patients ($P<0.05$). Ki-67 mRNA expression was significantly higher in cancer tissue than that in the corresponding paracancerous tissue of breast cancer ($P<0.05$). In addition, a high expression of Ki-67 was positively correlated with the clinical staging of tumor, tumor size and lymphatic metastasis of breast cancer, with statistical significance ($P<0.05$). In MCF-7 cells with a reduced Ki-67 expression, the proliferation activity and migration of breast cancer cells were significantly reduced ($P<0.05$). In conclusion, Ki-67 may be involved in promoting the genesis and development of breast cancer by affecting the proliferation and migration of cancer cells.

Introduction

Breast cancer is the most common malignancy in women, accounting for ~20% of total malignant tumors in females (1). Numerous studies (2) have shown that breast cancer is a highly heterogeneous malignancy with significant differences in histomorphological features, immunophenotype, biological behavior and response to treatment. Currently, breast cancer is classified as luminal tumors [hormone receptor expressing tumors, human epidermal growth factor receptor 2 (HER2)-negative], HER2-negative (hormone receptor negative) and basal-like tumors (absence of hormone receptor and HER2 expression) (3).

The aberrant proliferation and migration of tumor cells are a hallmark of tumor pathology during tumor progression. Studies conducted on proliferative activity and migration of tumor cells facilitate the understanding of biological behavior of tumors and provide clinical evidences for the diagnosis, treatment and prognosis of tumors (4). Ki-67, a nuclear antigen associated with cell proliferation, is expressed in all the phases of the cell cycle except G0. Ki-67 has been identified as a molecular marker for the effective assessment of the cell proliferation index (5). It has been shown that Ki-67 proliferative activity is associated with the extent of tumor differentiation, invasion and metastasis as well as prognosis (6,7). In addition, luminal tumors have been classified into luminal A and luminal B subtypes based on the level of Ki-67 expression. Furthermore, the involvement of Ki-67 expression in luminal breast cancer tissues has been demonstrated through large population-based cohort studies (8).

Between August 2009 and November 2014, 62 patients with luminal type breast cancer and 30 patients with breast hyperplasia, admitted to the Breast Cancer Center, The Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) were included in the present study. The present study aimed to measure the level of Ki-67 expression in luminal type breast cancer tissues and investigate the association between Ki-67 and the clinicopathology of breast cancer. In addition, Ki-67 expression in the human MCF-7 breast cancer cell line was studied to evaluate the proliferation and migration of tumor cells to elucidate the molecular mechanisms underlying the impact of Ki-67 on breast cancer.

Materials and methods

Clinical data. Breast tumor tissues were collected from 62 patients, who were diagnosed with luminal breast cancer

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and underwent surgery at The Affiliated Cancer Hospital of Zhengzhou University, between August 2009 and November 2014. The patients, with an age range of 22-57 years and a mean age of 43.15±9.50 years, had no prior history of radio- and chemotherapy. In addition, 30 patients with breast hyperplasia with an age range of 24-54 years and a mean age of 45.45±11.41 years, served as the controls.

Reagents. RNA was isolated from tissues and serum using the TRIzol RNA isolation kit (Life Technologies, Grand Island, NY, USA). RNA reverse transcription (RT) was performed using the cDNA reverse transcription system (Promega Corp., Madison, WI, USA). An RT-polymerase chain reaction (PCR) kit was purchased from Sunshine Biotechnology Co., Ltd., Nanjing, China.

RT-PCR. Breast cancer tissues were collected from 62 patients during surgery and analyzed. Cancer tissue (200 μg) was homogenized in 1 ml TRIzol solution and RNA was isolated according to the manufacturer's instructions. The isolated RNA was dissolved in 30 μl of diethylpryocarbonate-treated water and quantified using an ultraviolet spectrophotometer (One Drop, Nanjing, China). RNA was reverse transcribed into cDNA and stored in a freezer at -20˚C.

The primer sequences used for Ki-67 amplification were: F: 5'-CGTAGCAGCACAGAAAT-3' and R: 5'-TGA TGGTTGAGGTCGTTCCTTGATG-3' (9). U6 was used as an internal control, with the following primers: F: 5'-CTCGCT TCGGCAGCACA-3' and R: 5'-AACGCTTCACGAATT TGCGT-3'. The PCR reaction conditions used were: denaturation at 95˚C for 20 sec, followed by 50 cycles of 60˚C for 20 sec and 70˚C for 1 sec. RT-PCR data were analyzed on an ABI 7300 RT-PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA) and the relative quantitative analysis of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (10).

Immunohistochemical study and evaluation criteria. Breast cancer specimens were fixed in 10% formalin for 48 h, paraffin-embedded and cut into 4 μm thin sections for SP IHC staining using anti Ki-67 antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Phosphate-buffered saline was used as the negative control. Ki-67 staining was analyzed under optical microscopy (Olympus, Barrington, NJ, USA).

A positive Ki-67 expression was detected in the nuclei of tumor cells identified as brown particles. The IHC staining result was evaluated based on the extent of staining of tumor cells and the intensity of staining reaction. Ki-67-positive tumor cells were counted in 10 randomly selected high-power fields and evaluated according to the following criteria: <5% was defined as negative; 5-25% as +; 25-50% as ++; 50-75% as +++.

Cell transfection. Knockdown of Ki-67 was performed in the human MCF-7 breast cancer cell line through transfection of Ki-67 siRNA using Lipofectamine® 2000 transfection reagent (Invitrogen Life Technologies, Shanghai, China). Ki-67 siRNA sequences used were: 5'-CCACCAGAGCCAAUAGAUACU UCAG-3' and 5'-CUGAAGUAUCUAUUGGCUCUGGUGG UGA-3'. The control sequence was designed as 5'-UCA UAAGUGAUGCUGGAGCTT-3'. Transfection efficiency was evaluated using RT-PCR.

Cell invasion assay. Transfected MCF-7 cells in the logarithmic phase were suspended in solution and cell density was adjusted to 3-5x10^5/ml. Cell suspensions were seeded in the upper chamber of transwell at a concentration of 0.1 ml/well, and 1 ml culture medium containing 10% serum was plated in the bottom chamber. After 24 h culture, transwell chambers were retrieved and the upper layer of the culture media were aspirated to terminate the assay. The cells were air-dried at

Table I. Association between Ki-67 expression and TNM staging of breast cancer.

	Ki-67 level, no.				
Pathological stage	+	++	+++	*r*	P-value
I	2	3	6	0.589	0.004
II	4	6	10		
III	7	10	14		

Table II. Correlation of Ki-67 expression in breast cancer with tumor size and lymphatic metastasis.

	Ki-67 level, no.				
Clinical features of cancer	+	++	+++	*r*	P-value
Tumor size				0.452	0.016
T1 (T≤2 cm)	1	2	6		
T2 (2<T<5 cm)	5	7	10		
T3 (T≥5 cm)	5	12	14		
Lymphatic metastasis				0.331	0.021
N1 (1-3)	2	2	5		
N2 (4-9)	5	8	8		
N3 (>9)	8	8	12		

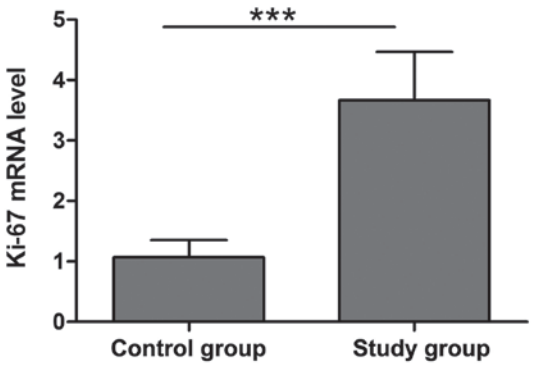

Figure 1. The expression level of Ki-67 mRNA in tissues of breast cancer patients (***vs. control group, t=18.41, P<0.001).

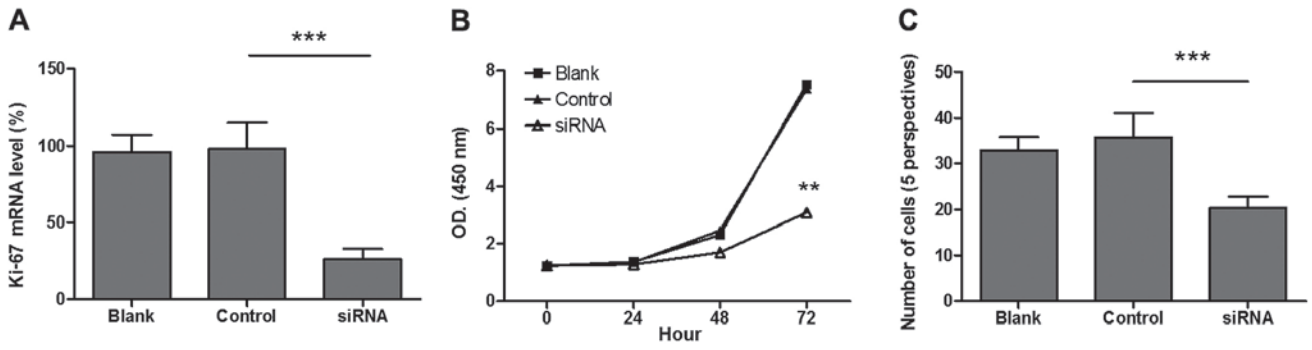

Figure 2. Effect of low expression of Ki-67 on the proliferation and migration of breast cancer (***vs. control group, P<0.001; **vs. control, P<0.05). (A) Knockdown efficiency of Ki-67. (B) Effect of Ki-67 knockdown on cell proliferation. (C) Effect of Ki-67 knockdown on cell migration.

room temperature (20˚C), fixed in ethanol and stained using 0.1% crystal violet for 30 min at room temperature. Cells that migrated to the bottom chamber were analyzed under an inverted microscope (Olympus) and the cells in the bottom chamber were counted in five randomly selected fields.

Cell proliferation assay. Transfected MCF-7 cells at 0, 24, 48 and 72 h post-transfection were plated in a mixture of Dulbecco's modified Eagle's medium (Invitrogen Life Technologies). The cell counting kit-8 assay (Beyotime, Wuhan, China) was used at a volume ratio of 1:10. The optical density values of samples were measured using a spectrophotometer at 450 nm to generate the growth curve of cells.

Statistical analysis. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Quantitative data were presented as mean ± standard deviation. Differences between groups were analyzed using independent samples t-test. The correlation between Ki-67 and tumor pathology was analyzed using the Spearman rank correlation test. P<0.05 was considered to indicate a statistically significant difference.

Results

Increase in Ki-67 levels in the serum and tissues of breast cancer patients. Compared with the control group, Ki-67 mRNA expression was significantly increased in tissues of breast cancer patients, indicating an association of Ki-67 in the progression of breast cancer. The high expression of Ki-67 may be correlated with the invasion and metastasis of breast cancer, suggesting a poor prognosis (Fig. 1).

Ki-67 expression in breast cancer tissues with different histopathological characteristics. According to TNM classification systems in the 7th edition (2010) of the cancer staging manual of the American Joint Committee on Cancer, 11 (17.7%) patients were classified as stage I, 20 (32.3%) as stage II and 31 (50.0%) as stage III. The level of Ki-67 expression was positively associated with TNM stages (r=0.589, P<0.01) (Table I). The Spearman correlation analysis showed that Ki-67 expression levels in breast cancer tissues were positively associated with tumor size and the number of metastatic lymph nodes (Table II).

Correlation of low expression of Ki-67 with breast cancer staging. Ki-67 expression was reduced by the knockdown of Ki-67 in the human MCF-7 breast cancer cell line in an effort to examine the mechanism by which Ki-67 influences breast cancer. The results demonstrated that Ki-67 mRNA expression was significantly reduced in MCF-7 cells 48 h after transfection (Fig. 2A). Compared to the control group, cell proliferation activity was significantly decreased in breast cancer cells with low expression of Ki-67 (P<0.05) (Fig. 2B). In addition, cell migration was significantly reduced in breast cancer cells with low Ki-67 expression compared to that of controls (P<0.05) (Fig. 2C).

Discussion

Breast cancer is one of the most common malignancies in middle-aged and elderly women globally, representing 1.20 million cases per annum in women worldwide (11). In some regions of China, breast cancer has become the leading cancer in women, causing serious damage to their physical and mental health (12). Due to the increase in number of breast cancer patients and younger age of patients, increasing attention has been paid to the treatment and prognosis of breast cancer. In recent years, significant progress has been made in the treatment of breast cancer as further basic and clinical studies were conducted, and the study on breast cancer treatment has become one of the hot topics of cancer research (13).

Invasion and migration is the hallmark of malignant tumors as well as the major cause for breast cancer death (14,15). However, the development details and underlying pathological mechanisms remain to be determined. Ki-67, a gene located on chromosome 10, can detect a nuclear protein in the nuclear matrix of proliferating cells and its function has been shown to be associated with mitosis. In particular, closely associated with tumor cell proliferation (16-18). Currently, however, Ki-67 expression and its function in luminal breast cancer has not been completely elucidated.

Results of the present study have shown that Ki-67 mRNA expression was significantly higher in tissues of breast cancer

than that of breast hyperplasia, indicating that Ki-67 is closely associated with the pathogenesis and development of breast cancer. Warth *et al* (19) have shown that Ki-67 expression is increased in non-small-cell lung cancer and suggested that Ki-67 can serve as a predictor for cancer prognosis (19). Pollack *et al* (20) and Khor *et al* (21) have also shown increased Ki-67 expression in prostate cancer. In addition, Inwald *et al* (22) have shown a significantly increased Ki-67 expression in breast cancer and suggest its role as a prognostic parameter in breast cancer. Taken together, those findings corroborate the results of the present study.

Further analysis was performed to investigate the relationship between Ki-67 expression in breast cancer tissues and clinicopathological characteristics of the cancer. The results revealed that the level of Ki-67 expression in breast cancer tissue was positively correlated with the TNM stages of the cancer. In addition, the Ki-67 expression level was positively correlated with tumor size and the number of metastatic lymph nodes. These findings suggest that a high expression of Ki-67 plays a central role in the promotion of the pathogenesis and development of breast cancer. In addition, Ki-67 plays important roles in promoting the genesis and metastasis of breast cancer.

The mechanism underlying the tumor-promoting function of Ki-67 in breast cancer was further studied by reducing Ki-67 expression in the human MCF-7 breast cancer cell line. The results demonstrate that the proliferation activity and migration of cancer cells were reduced in breast cancer cells with a low expression of Ki-67, indicating that Ki-67 may affect the malignancy of breast cancer cells. Zheng *et al* (23) have revealed that the knockdown of Ki-67 in renal carcinoma cells significantly inhibits cancer cell proliferation and promotes cell apoptosis, thereby indicating that Ki-67 may be involved in the progression of renal carcinoma by influencing the growth and apoptosis of the cancer cells. Furthermore, the knockdown of Ki-67 in bladder cancer cells has been shown to inhibit tumor growth (24). Taken together, the results of the present study were corroborated with those findings, which suggest that interference with Ki-67 expression is involved in the progression of breast cancer probably by affecting the proliferation and migration of cancer cells.

In summary, a high expression of Ki-67 is important in the genesis and development of breast cancer, possibly by influencing the proliferation and migration of cancer cells. Detection and interference of Ki-67 have a certain implication for guiding the treatment and prognosis of breast cancer in clinical practice.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Karuturi M, VanderWalde N and Muss H: Approach and management of breast cancer in the elderly. Clin Geriatr Med 32: 133-153, 2016.
3. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ and Loi S: Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol: Dec 15, 2015 doi: 10.1038.
4. Sun Z, Shi Y, Shen Y, Cao L, Zhang W and Guan X: Analysis of different HER-2 mutations in breast cancer progression and drug resistance. J Cell Mol Med 19: 2691-2701, 2015.
5. Kim DK, Kim DW, Kim SW, Kim DY, Lee CH and Rhee CS: Ki67 antigen as a predictive factor for prognosis of sinonasal mucosal melanoma. Clin Exp Otorhinolaryngol 1: 206-210, 2008.
6. Cuzick J, Dowsett M, Pineda S, Wale C, Salter J, Quinn E, Zabaglo L, Mallon E, Green AR, Ellis IO, *et al*: Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. J Clin Oncol 29: 4273-4278, 2011.
7. Krüger K, Stefansson IM, Collett K, Arnes JB, Aas T and Akslen LA: Microvessel proliferation by co-expression of endothelial nestin and Ki-67 is associated with a basal-like phenotype and aggressive features in breast cancer. Breast 22: 282-288, 2013.
8. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B and Senn HJ; Panel members: Strategies for subtypes - dealing with the diversity of breast cancer: Highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22: 1736-1747, 2011.
9. Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z and Cao X: MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol 183: 2150-2158, 2009.
10. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
11. Cintra JR, Teixeira MT, Diniz RW, Gonçalves Junior H, Florentino TM, Freitas GF, Oliveira LR, Neves MT, Pereira T and Guerra MR: Immunohistochemical profile and clinical-pathological variables in breast cancer. Rev Assoc Med Bras 58: 178-187, 2012.
12. Xiao ZS, Li Y, Guan YL and Li JG: GSTT1 polymorphism and breast cancer risk in the Chinese population: an updated meta-analysis and review. Int J Clin Exp Med 8: 6650-6657, 2015.
13. Hong W and Dong E: The past, present and future of breast cancer research in China. Cancer Lett 51: 1-5, 2014.
14. Arjonen A, Kaukonen R, Mattila E, Rouhi P, Högnäs G, Sihto H, Miller BW, Morton JP, Bucher E, Taimen P, *et al*: Mutant p53-associated myosin-X upregulation promotes breast cancer invasion and metastasis. J Clin Invest 124: 1069-1082, 2014.
15. Macpherson IR, Rainero E, Mitchell LE, van den Berghe PV, Speirs C, Dozynkiewicz MA, Chaudhary S, Kalna G, Edwards J, Timpson P, *et al*: CLIC3 controls recycling of late endosomal MT1-MMP and dictates invasion and metastasis in breast cancer. J Cell Sci 127: 3893-3901, 2014.
16. Beresford MJ, Wilson GD and Makris A: Measuring proliferation in breast cancer: Practicalities and applications. Breast Cancer Res 8: 216, 2006.
17. Miller HC, Drymousis P, Flora R, Goldin R, Spalding D and Frilling A: Role of Ki-67 proliferation index in the assessment of patients with neuroendocrine neoplasias regarding the stage of disease. World J Surg 38: 1353-1361, 2014.
18. Rademakers SE, Hoogsteen IJ, Rijken PF, Terhaard CH, Doornaert PA, Langendijk JA, van den Ende P, van der Kogel AJ, Bussink J and Kaanders JH: Prognostic value of the proliferation marker Ki-67 in laryngeal carcinoma: results of the accelerated radiotherapy with carbogen breathing and nicotinamide phase III randomized trial. Head Neck 37: 171-176, 2015.
19. Warth A, Cortis J, Soltermann A, Meister M, Budczies J, Stenzinger A, Goeppert B, Thomas M, Herth FJ, Schirmacher P, *et al*: Tumour cell proliferation (Ki-67) in non-small cell lung cancer: A critical reappraisal of its prognostic role. Br J Cancer 111: 1222-1229, 2014.

20. Pollack A, DeSilvio M, Khor LY, Li R, Al-Saleem TI, Hammond ME, Venkatesan V, Lawton CA, Roach M III, Shipley WU, *et al*: Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02. J Clin Oncol 22: 2133-2140, 2004.
21. Khor LY, Bae K, Paulus R, Al-Saleem T, Hammond ME, Grignon DJ, Che M, Venkatesan V, Byhardt RW, Rotman M, *et al*: MDM2 and Ki-67 predict for distant metastasis and mortality in men treated with radiotherapy and androgen deprivation for prostate cancer: RTOG 92-02. J Clin Oncol 27: 3177-3184, 2009.
22. Inwald EC, Klinkhammer-Schalke M, Hofstädter F, Zeman F, Koller M, Gerstenhauer M and Ortmann O: Ki-67 is a prognostic parameter in breast cancer patients: Results of a large population-based cohort of a cancer registry. Breast Cancer Res Treat 139: 539-552, 2013.
23. Zheng Z, Li HZ and Li YQ: Prognostic factors and associated models for metastatic renal cell carcinoma treated with targeted therapy. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 36: 450-453, 2014 (In Chinese).
24. Pichu S, Krishnamoorthy S, Shishkov A, Zhang B, McCue P and Ponnappa BC: Knockdown of Ki-67 by dicer-substrate small interfering RNA sensitizes bladder cancer cells to curcumin-induced tumor inhibition. PLoS One 7: e48567, 2012.