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Original Article

Over-expression of *HER2* promotes cell metastasis and is associated with the prognosis of osteosarcoma

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Abstract: Purpose: The objective aimed to detect the associations between *HER2* expression and osteosarcoma (OS) metastasis as well as its effects on the prognosis of OS. Methods: Immunohistochemistry was adapted to test *HER2* expression in 61 OS patients. The metastasis of cells was detected by transwell assays and the expression of *HER2* in cell lines was measured by western blot. Results: The positive rate of *HER2* expression in OS tissues was 62.1% according to immunohistochemistry. There were significance differences among osteosarcoma tissues, adjacent tissues and healthy tissues ($P<0.01$). Besides, the expression of *HER2* was closely associated with enneking stage. Kaplan-Meier showed the overall survival time of patients with positive-*HER2* expression was shorter than those with negative-*HER2* expression. Log rank test proved the results was statistical significance. And positive *HER2* expression as well as ennking were also important factors in the prognosis of osteosarcoma via Cox regression analysis and they could be independent prognostic markers. Western blot and transwell assays demonstrated that the over-expression of *HER2* was promotor for the metastasis of osteosarcoma cells. Conclusion: *HER2* was over-expressed in osteosarcoma patients as well as in cell lines and its expression was impacted by enneking. The over-expression of *HER2* promoted the metastasis and could be an independent prognostic marker in osteosarcoma.

Keywords: *HER2,* osteosarcoma, metastasis, prognosis

Introduction

Osteosarcoma (OS), a common primary bone tumor, often occurs in the long bones (inferior femora and superior shinbones) of children and adolescents [1]. Most patients are at II B stages when they are initially diagnosed. It has been reported that 50% patients with osteosarcoma suffer metastasize especially lung metastasis which bringing a low cure rate and 5-years' survival rate [2-4]. As the development of comprehensive therapy, the 5-years survival rate rises from 30% to 60-70% [2, 5]. However, the prognosis of OS is still poor and it is important to study and detect new prognosis factors for OS patients.

HER2, a receptor tyrosine kinase, is a member of the ErbB family which take effect on the regulation of many essential cellular functions such as cell proliferation, differentiation, and apoptosis [6]. In previous studies, the expression of *HER2* was up-regulated in 15%-40% breast cancer patients and lead to a poor prognosis [7]. There are two completely opposite opinion about *HER2* expression in OS patients. Gorlick et al., found that high *HER2* expression was associated with lung metastasis, and insensitive preoperative chemotherapy as well as shortened survival time of OS patients [8]. Nevertheless, high *HER2* expression was considered to prolong patients' survival time in OS patients in the study of Akatsuka et al. [9]. So the expression pattern of *HER2* was uncertain. Besides, the other function of *HER2* in osteosarcoma is not clear.

In this study, our purpose was to further estimate the association between *HER2* expression and OS prognosis as well as metastasis. The expression of *HER2* was detected in tissues and cell lines by immunohistochemistry and western blot, respectively. Meanwhile, we analyzed the relationship between *HER2* expression and clinicopathologic characteristics. Moreover, the function of *HER2* in cell

metastasis and prognosis of OS were evaluated.

Materials and methods

Patients and samples

A total of 61 OS patients (33 men and 28 women, aged from 8 to 46 years) were recruited in this study. The study was conducted in Shaanxi Provincial Forestry Hospital and permitted by the Ethical Commission of the hospital. All patients involved hadn't received any radiotherapy and chemotherapy. Besides, 61 healthy people without osteosarcoma were collected as controls. Written informed consent had been signed by every participator in advance.

The tissues and adjacent tissues from OS patients as well as the healthy tissues from controls were obtained and frozen by liquid nitrogen. Then the samples were stored at -80°C for using. A 5-years' follow-up was performed to estimate the prognosis of OS. The clinicopathologic characteristics including age, gender, tumor size, enneking, price grading and relapses were recorded in a database. Patients who died from unexpected events or other diseases were excluded from our study.

Immunohistochemistry

Osteosarcoma tissues, adjacent tissues and healthy tissues were initially fixed by formalin, then dehydrated and embedded in paraffin. All samples were cut into a section with thickness of 4 μm. Deparaffinage and rehydration were made to the sections. Then washing the sections with PBS. The sections were hatched for 10 min with 3% H_2O_2 in room temperature and washed with PBS. Antigen retrieval reaction was implemented and the primary antibody was added and incubated at 4°C for overnight. Rewarming for 45 min at 37°C and washing with PBS. After that, the secondary antibody was put into and incubated for 1 h at 37°C. Then the sections were washed with PBS, colored with DAB coloration for 5-10 min, and redyed with hematoxylin. Finally, the sections were examined by light microscope after routine dehydration and lucency. Positive staining slices were regarded as positive controls and PBS solution were brought in as negative controls. Every section was randomly divided into three fields (100 cells in every field) to calculate the percent of positive stained cells in all of the cells. The positive expression of *HER2* was stained as brown and the results were divided as follows: negative ("-", the percentage of stained cells were less than 10%), weak positive ("+", the percentage of stained cells were 10%-50%), strong positive ("++", the percentage of stained cells were more than 50%). For further study, staining results were also grouped into negative and positive (including weak positive, and strong positive).

Cell culture and transfection

Human osteosarcoma cell lines U2OS were obtained from the Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Non-silencing (NS) control, and *HER2* siRNAs were purchased from Qiagen. When the cells concentration reached to 50-60% (GenePharma, Shanghai, China), U2OS cell lines were transfected with *HER2* siRNAs or NS-siRNA using the Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer.

Cell metastasis assay

Transwell migration assays (Millipore, Billerica, MA, USA) were used to detect the metastasis of osteosarcoma cells. Transfected cells were resuspended in serum-free medium. Then, the cell suspensions were seeded into the bottom wells of the chambers filling with complete medium. 24 h later, the cell numbers were counted from five nonoverlapping fields of each membrane. Three independent assays were performed.

Western blot

Protein samples extracted from transfected cells were separated by SDS-PAGE. The brands were transfered into PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% non-fat milk (Bio-Rad) and incubated with primary antibody at 4°C overnight. Then washing the membrane and adding into horseradish peroxidase-conjugated secondary antibody.

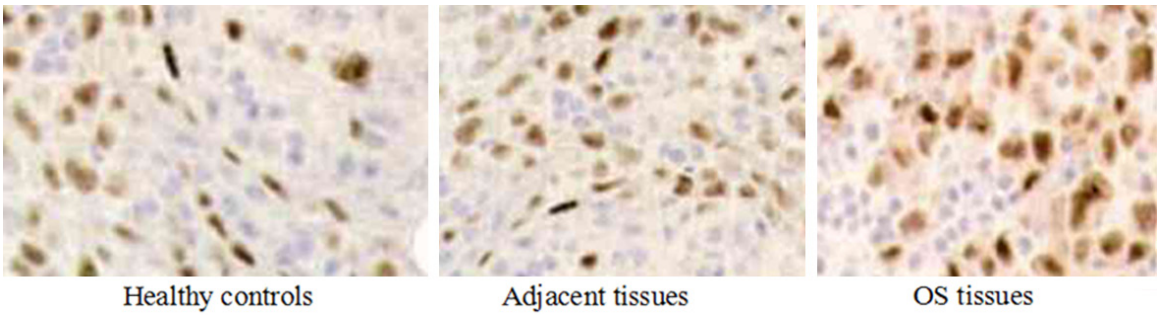

Figure 1. The immunohistochemistry results. The positive stained cells was 62.1%.

Table 1. Association between *HER2* expression and clinicopathologic characteristics of OS patients

Clinicopathologic characteristics	n	*HER2* expression		*P*
		positive	negative	
Age				0.660
≤18	33	15	18	
>18	28	14	14	
Gender				0.406
female	27	13	14	
male	34	16	18	
Tumor size				0.326
<3 cm	13	7	6	
3-5 cm	20	9	11	
≥5 cm	28	13	15	
Enneking stage				0.003
I	15	2	13	
II	28	13	15	
III	18	14	4	
Price grading				0.516
I	21	11	10	
II	30	14	16	
III	10	4	6	

The expression level of *HER2* proteins normalized to β-actin. The bands were visualized using a chemiluminescence reagent (New England Nuclear, Boston, MA, USA).

Statistic analysis

All data were stated at Mean ± SD and handled by SPSS13.0 software. The differences of *HER2* expression among groups as well as the associations between *HER2* and clinicopathologic characteristics were analyzed by chi-square test. The relationship between *HER2* and overall survival time was estimated via Kaplan-Meier and Cox regression analysis. It was considered to be significant difference when $P<0.05$.

Results

Immunohistochemistry identification of HER2 in osteosarcoma patients

The expression of *HER2* in the tissues and adjacent tissues of 61 OS patients as well as the tissues of 61 healthy controls were detected by immunohistochemistry. The reaction products are shown as brownish yellow. The positive-stained *HER2* was 62.1% (32/61) which indicated that the expression of *HER2* was higher in OS tissues than in adjacent tissues and healthy tissues (**Figure 1**, $P<0.05$).

Association between HER2 and clinicopathologic characteristics

The clinicopathologic characteristics were important influential factors for gene expression. In our study, enneking stage ($P<0.05$) were considered to be associated with the expression of *HER2* while age, gender, tumor size and price pathology grading had no significant relationship with *HER2* expression ($P>0.05$, **Table 1**). These result showed that *HER2* might participate in the development of OS.

Relationship between HER2 expression and overall survival time of OS patients

During the follow-up, there were patients died and the follow-up rate was. Kaplan-Meier analysis revealed the overall survival time of patients with positive *HER2* expression was shorter than those with negative *HER2* expression (**Figure 2**, log rank test, P<0.001). As respect to

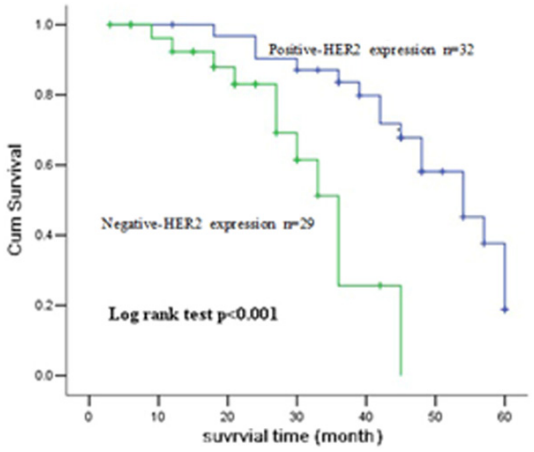

Figure 2. Kaplan-Meier analysis which was used to estimated the relationship between *HER2* and overall survival time.

Table 2. The prognostic value of *HER2* according to Cox regression analysis

Parameter	Risk ratio	95% confidence interval	*P* value
Ennking	0.110	0.024-0.498	0.014
High-*HER2* expression	0.265	0.097-0.720	0.009
Low-*HER2* expression	-	-	-

the prognostic value of *HER2,* Cox regression analysis was adopted. The outcome was displayed in **Table 2**, enneking stage (HR=0.110, 95% CI=0.024-0.498, *P*=0.014) and *HER2* expression (HR=0.265, 95% CI=0.097-0.720, *P*=0.009) were related to the prognosis of OS and they might be independent prognostic markers in OS (**Table 2**).

Over-expression of HER2 promoted metastasis of osteosarcoma cells

At the 24 h after gene transfection, the expression of *HER2* was increased in *HER2*-siRNA group compared with in NS-siRNA group (**Figure 3**). Meanwhile, the cell metastasis was promoted in cells transfected with siRNAs liken to the cell s transfected with NS-siRNA (**Figure 4**).

Discussion

OS is a differentiation disease caused by genetic changes and accounts for approximately 5% of all pediatric tumors and 8.9% of cancer-related deaths in children [10, 11]. It is characterized as the pain of tumor site which was induced by the erosion and dissolution of bone cortex. Now, many clinical and basic researches about the progress of osteosarcomas have been made [12], but the molecular genetic mechanism involved in osteosarcoma is still unclear. Many genes have been confirmed to be related to the development of OS such as RB1, p53, *HER2*, RECQL4 genes and so on [13-17].

HER2, also called *c-erbB-2* or *Her-2*, is located in 17q21 chromosome and encodes a kind of transmembrane protein with 185000 relative molecular mass. Its structure is similar with EGFR and its cytoplasmic region possesses with tyrosine protein kinase (TPK) activity. Normally, *HER2* is in inactive state and it can regulate the fission, differentiation and proliferation of cells combining with its specific ligands. The structure or expression disorder of *HER2* will be activated by some carcinogenic factors which make *HER2* owns tumor transformation activity so that it can promote malignant transformation of cells [18]. At present, amounts of studies have discovered that the abnormal expression of *HER2* in different kinds of malignant tumors such as ovarian cancer, colorectal cancer, lung cancer and stomach cancer [19-22]. People have confirmed that there were amplification and over expression of *HER2* in 20%-40% breast cancers and *HER2* could act as the independent factor of prognosis [23]. In our study, according to immunohistochemistry and western blot, the expression of *HER2* was verified to be over-expressed both in osteosarcoma tissues and cell lines which revealed *HER2* might be an oncogene in OS.

Metastasis often occurs in OS which has become the main reason of death caused by OS [2]. If patients suffered from tumor metastasis before long clinical symptoms occurred, it often suggests typing of cases with higher malignant grade. Owing to the abnormal expression of *HER2* in tumor cells, the adhesive force is weakened, mobility is strengthened and the cell differentiation is lowered among cells, then its invasiveness was strengthened. The tumor cells were easy to be broke away from the primary tumors and resulted in lymphatic or even distant metastasis. The metastatic potentials of OS cells might be related to the amount of *HER2* expression. Therefore, transwell assay was performed to examine the metastasis of OS cells in current study. The result indicated

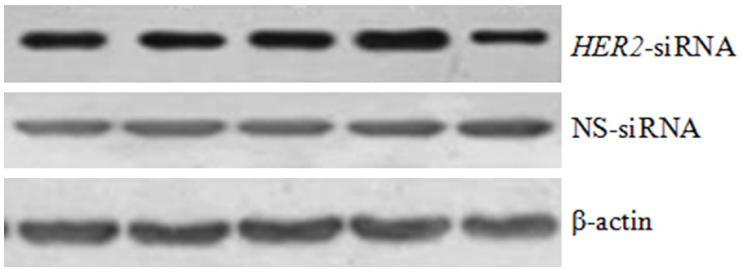

Figure 3. The expression of *HER2* in osteosarcoma cells transfected with *HER2*-siRNA and NS-siRNA normalized to β-actin.

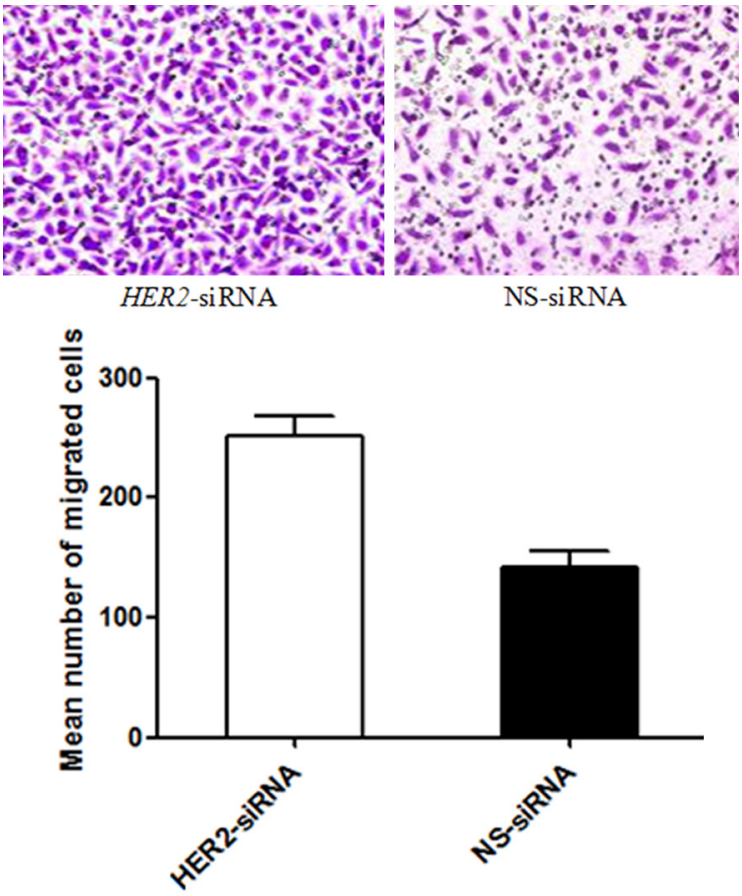

Figure 4. The cell metastasis was influenced by *HER2*.

the acceleration effects of *HER2* in the metastasis of OS cells. *HER2* expression affected the metastasis abilities of OS cells in patients that was to say, *HER2* expression could reflect the metastasis potentials of cancer cells and it could be regarded as one of the predicated markers for OS metastasis.

Relevant literatures have pointed out that OS prognosis is influenced by many factors including tumor size, local necrosis rate after chemotherapy, reaction to chemotherapy, and concentration of ALP in serum [24-26]. The prognostic value of *HER2* had been reported in various cancers like breast cancer, gastric cancer, ovarian cancer, and bladder cancer [27-30]. Patients with positive *HER2* expression lived shorter than those with negative *HER2* expression via Kaplan-Meier analysis. The outcome showed that ennking and *HER2* expression were the candidate factors for the prognosis of OS. Besides, this study also tested the role of *HER2* and clinicopathologic characteristics in the prognosis of OS using Cox regression analysis which manifested ennking and *HER2* might be independent prognostic marker in OS.

To conclude, *HER2* is up-regulated in OS tissues and cell lines. It also plays an important part in the metastasis of OS cells as well as its prognostic value may be good. *HER2* may be an independent prognostic marker in OS and it will supply molecular biology references for the treatment options of OS.

Disclosure of conflict of interest

None.

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References

[1] Siegel HJ and Pressey JG. Current concepts on the surgical and medical management of osteosarcoma. Expert Rev Anticancer Ther 2008; 8: 1257-1269.

[2] Tsuchiya H, Tomita K, Mori Y, Asada N and Yamamoto N. Marginal excision for osteosar-

coma with caffeine assisted chemotherapy. Clin Orthop Relat Res 1999; 27-35.
[3] Lamoureux F, Richard P, Wittrant Y, Battaglia S, Pilet P, Trichet V, Blanchard F, Gouin F, Pitard B, Heymann D and Redini F. Therapeutic relevance of osteoprotegerin gene therapy in osteosarcoma: blockade of the vicious cycle between tumor cell proliferation and bone resorption. Cancer Res 2007; 67: 7308-7318.
[4] Chen L, Wang Q, Wang GD, Wang HS, Huang Y, Liu XM and Cai XH. miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. FEBS Lett 2013; 587: 1366-1372.
[5] Mirabello L, Troisi RJ and Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 2009; 115: 1531-1543.
[6] Yarden Y and Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001; 2: 127-137.
[7] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235: 177-182.
[8] Gorlick R, Huvos AG, Heller G, Aledo A, Beardsley GP, Healey JH and Meyers PA. Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 1999; 17: 2781-2788.
[9] Akatsuka T, Wada T, Kokai Y, Kawaguchi S, Isu K, Yamashiro K, Yamashita T, Sawada N, Yamawaki S and Ishii S. ErbB2 expression is correlated with increased survival of patients with osteosarcoma. Cancer 2002; 94: 1397-1404.
[10] Tang N, Song WX, Luo J, Haydon RC and He TC. Osteosarcoma development and stem cell differentiation. Clin Orthop Relat Res 2008; 466: 2114-2130.
[11] Yang J and Zhang W. New molecular insights into osteosarcoma targeted therapy. Curr Opin Oncol 2013; 25: 398-406.
[12] Akiyama T, Dass CR and Choong PF. Novel therapeutic strategy for osteosarcoma targeting osteoclast differentiation, bone-resorbing activity, and apoptosis pathway. Mol Cancer Ther 2008; 7: 3461-3469.
[13] Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y and Yamamuro T. Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer Res 1994; 54: 3042-3048.
[14] Scholz RB, Kabisch H, Weber B, Roser K, Delling G and Winkler K. Studies of the RB1 gene and the p53 gene in human osteosarcomas. Pediatr Hematol Oncol 1992; 9: 125-137.
[15] Schreck RR. Tumor suppressor gene (Rb and p53) mutations in osteosarcoma. Pediatr Hematol Oncol 1992; 9: ix-x.
[16] Nellissery MJ, Padalecki SS, Brkanac Z, Singer FR, Roodman GD, Unni KK, Leach RJ and Hansen MF. Evidence for a novel osteosarcoma tumor-suppressor gene in the chromosome 18 region genetically linked with Paget disease of bone. Am J Hum Genet 1998; 63: 817-824.
[17] Onda M, Matsuda S, Higaki S, Iijima T, Fukushima J, Yokokura A, Kojima T, Horiuchi H, Kurokawa T and Yamamoto T. ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. Cancer 1996; 77: 71-78.
[18] Schechter AL, Hung MC, Vaidyanathan L, Weinberg RA, Yang-Feng TL, Francke U, Ullrich A and Coussens L. The neu gene: an erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 1985; 229: 976-978.
[19] Stahl P, Kissau L, Mazitschek R, Huwe A, Furet P, Giannis A and Waldmann H. Total synthesis and biological evaluation of the nakijiquinones. J Am Chem Soc 2001; 123: 11586-11593.
[20] Potti A, Ganti AK, Koch M, Mehdi SA and Levitt R. Identification of HER-2/neu overexpression and the clinical course of lung carcinoma in non-smokers with chronic lymphocytic leukemia. Lung Cancer 2001; 34: 227-232.
[21] Delektorskaya VV, Perevoshchikov AG and Kushlinskii NE. Immunohistological study of NM 23 and C-erbB-2 expression in primary tumor and metastases of colorectal adenocarcinoma. Bull Exp Biol Med 2003; 135: 489-494.
[22] Orr MS, O'Connor PM and Kohn KW. Effects of c-erbB2 overexpression on the drug sensitivities of normal human mammary epithelial cells. J Natl Cancer Inst 2000; 92: 987-994.
[23] Sugano K, Ushiama M, Fukutomi T, Tsuda H, Kitoh T and Ohkura H. Combined measurement of the c-erbB-2 protein in breast carcinoma tissues and sera is useful as a sensitive tumor marker for monitoring tumor relapse. Int J Cancer 2000; 89: 329-336.
[24] Cai LS, Zhang JX, Shen Q, Situ HL, Song GH and Xie YC. [Features of preoperative contrast-enhanced ultrasound of breast cancer: relationship with the syndrome classification in traditional Chinese medicine]. Nan Fang Yi Ke Da Xue Xue Bao 2010; 30: 1404-1406.
[25] Yao Y, Zhao H, Sun Y, Lin F, Tang L and Chen P. Combined chemotherapy of hydroxycamptothecin with oxaliplatin as an adjuvant treatment for human colorectal cancer. Tohoku J Exp Med 2008; 215: 267-278.
[26] van Maldegem AM, Bhosale A, Gelderblom HJ, Hogendoorn PC and Hassan AB. Com-

prehensive analysis of published phase I/II clinical trials between 1990-2010 in osteosarcoma and Ewing sarcoma confirms limited outcomes and need for translational investment. Clin Sarcoma Res 2012; 2: 5.

[27] Cuello-Carrion FD, Shortrede JE, Alvarez-Olmedo D, Cayado-Gutierrez N, Castro GN, Zoppino FC, Guerrero M, Martinis E, Wuilloud R, Gomez NN, Biaggio V, Orozco J, Gago FE, Ciocca LA, Fanelli MA and Ciocca DR. HER2 and beta-catenin protein location: importance in the prognosis of breast cancer patients and their correlation when breast cancer cells suffer stressful situations. Clin Exp Metastasis 2015; 32: 151-168.

[28] Qiu M, Zhou Y, Zhang X, Wang Z, Wang F, Shao J, Lu J, Jin Y, Wei X, Zhang D, Li Y, Yang D and Xu R. Lauren classification combined with HER2 status is a better prognostic factor in Chinese gastric cancer patients. BMC Cancer 2014; 14: 823.

[29] Cai Y, Wang J, Zhang L, Wu D, Yu D, Tian X, Liu J, Jiang X, Shen Y, Ren M and Huang P. Expressions of fatty acid synthase and HER2 are correlated with poor prognosis of ovarian cancer. Med Oncol 2015; 32: 391.

[30] Zhao J, Xu W, Zhang Z, Song R, Zeng S, Sun Y and Xu C. Prognostic role of HER2 expression in bladder cancer: a systematic review and meta-analysis. Int Urol Nephrol 2015; 47: 87-94.