



EFFECT OF HER-2 EXPRESSION VECTOR TRANSFECTION ON THE DISTRIBUTION OF β -CATENIN AND THE INVASION/MIGRATION IN HUMAN GASTRIC CANCER CELLS

Bibek kandel¹, Zhiqiang Cai² and Jiyuan Yang^{2*}

¹Clinical school of medicine of The Yangtze university, Jingzhou 434000, Hubei Province, China.

²Department of Oncology, the First Hospital Affiliated to Yangtze University, Jingzhou 434000, Hubei Province, China.

ABSTRACT

Objective: To investigate the change of invasion and migration when Perbb2 - EGFP was transfected into gastric cancer cell line BGC823, with a focus on the protein distribution of β -catenin. To explore the relationship between Her-2 and β -catenin. **Methods:** Perbb2-EGFP and p-EGFP were transfected into BGC823 cells through lipofectamine. The inverted fluorescence microscope was employed to observe the transfection condition. Realtime PCR was employed to identify the mRNA expression of Her-2 in three groups of gastric cancer cells. Western blotting was employed to identify the protein concentration of Her-2 and β -catenin in three groups of gastric cancer cells. Transwell model was employed to observe the invasion and migration of three groups of gastric cancer cells. **Results:** EGFP was expression in BGC823 successfully transfected with perbb2-EGFP and p-EGFP. The mRNA expression of Her-2 and the protein expression of Her-2 was increased significantly in BGC823 successfully transfected with perbb2-EGFP ($p < 0.05$). The concentration of β -catenin was increased significantly in the nucleus of BGC823 successfully transfected with perbb2-EGFP ($p < 0.05$). The Invasion and migration ability of BGC823 successfully transfected with perbb2-EGFP had improved significantly ($p < 0.05$). **Conclusion:** The invasion and migration ability of human gastric cancer cells can enhance significantly when overexpression of Her-2 mRNA and protein. The mechanism may be its interaction between Her-2 and beta - catenin. It lead to disintegration of E-cadherin/ β -catenin complex, and at the same time WNT signaling pathways are activated.

Keywords: Human gastric cancer; Her-2; β -catenin; transfection; invasion; migration

INTRODUCTION

Gastric cancer is one of the most common malignant tumor in the world, the incidence of Asian countries is higher than Europe and the United States, the incidence of gastric cancer in China is 6 times as large as Europe and the United States, The gene for the c-erb-B2 protein (also known as ErbB-2, HER2 or Her2/neu) is a proto-oncogene located on the chromosome 17. The c-erb-B2 gene encodes a 185-kDa transmembrane tyrosine kinase receptor protein, which is a member of the HER-family(HER1-4)^[1, 2], Her-2 gene over expression of gastric cancer patients tend to have a poor prognosis. β -catenin, as a kind of connecting protein, is an important component of WNT signaling pathway^[3], and it also mediates the adhesion between cells of the same kind. Its abnormal expression is closely related to the occurrence and development of tumor.

In this study, we will transfect recombinant plasmid perbb2-EGFP carrying Her-2 gene into gastric cancer cell line BGC823, and observe the change of invasion, migration of gastric cancer cells, and distribution change of β -catenin. Also we will explore relationship between Her-2 protein and β -catenin, the possible mechanism of Her-2 gene effect on biological characteristics of invasion and migration of gastric cancer cells.

MATERIALS AND METHODS

experimental materials:

Human gastric cancer cell line BGC823.

experimental methods:

cell culture:

The human gastric cancer cell line BGC823 was placed in the incubator of 37 degrees Celsius and 5% carbon dioxide, and the culture medium was subcultured with 10% fetal bovine serum 1640.

Plasmid Extraction:

10 μ l perbb2-EGFP and 10 μ l p-EGFP were transformed into escherichia coli respectively, the transfected Escherichia coli placed in ampicillin medium for culture, selected colonies shake from 8 to 12 hours in cultured medium. Plasmid Extraction Kit was used for plasmid extraction, the extracted perbb2-EGFP was sequenced by Nanjing Kingsy biological technology company, if they confirmed that the Her-2 gene fragment was exist, we will do the further test.

cell transfection and grouping:

The human gastric cancer cell line BGC823 was inoculated into 6 hole plates at 105×2 per hole, and the human gastric cancer cell line was placed in constant temperature chamber RPMI-1640 culture medium with 10% fetal calf serum. When the cells grew to 70% or so, the cell fusion was carried out by liposome mediated transfection. The amount of each plasmid per orifice 5.63 L (concentration of $2.71 \mu\text{g}/\mu\text{L}$), 2000 Lip dosage of 11.2 L, empty plasmid dosage per orifice 4 L (concentration of $2.27 \mu\text{g}/\mu\text{L}$), 2000 Lipo dosage of 8 L. Each unit diluted with 200 LoptiMEM for 25 minutes. The morphology of cells and the condition of transfection were observed by fluorescence inverted microscope. Perbb2-EGFP group cells were transfected with perbb2-EGFP, p-EGFP group cells were transfected with p-EGFP, and the BGC823 group cells did not add the plasmid.

PCR realtime (real time fluorescent quantitative PCR) to detect the expression of HER2 gene mRNA:

Cells were cultured for 48 hours, using RNA's Invitrogen extraction kit to extract total RNA of human gastric cancer cell line BGC823, and the concentration and purity of RNA were determined by spectrophotometer. Each group took the same amount of RNA to reverse to format the cDNA template. Her-2 upstream primer 5' -3' CATCATCTCTGCGGTGGTTG', the downstream primer 5' -3' TCCGTCTTTTCAGGATCCG', amplified fragment length 198bp. Reference β -actin primer: 5' -3' agcgagcatccccaaagt-3', 'gggcacgaaggctcatt-3' - 5 primers, amplified fragment length 284bp. Realtime PCR reaction system as follows: cDNA 4ul, the upstream primer 0.4ul, primer 0.4ul, 2 x green fluorescent qPCRmaster10ul, add distilled water to 20ul. The amplification and melting curves were obtained, and three experiments were repeated. Using β -actin as reference, calculates the relative amount of Her-2 mRNA compared with β -actin by the Delta ct method.

The expression of β -catenin and Her-2 was detected by western blotting method:

The total protein and nuclear protein, were extracted for each groups of human gastric cancer cells BGC823, test the concentration of protein in each group. Her-2 detected from the total protein, β -catenin was detected from the cytoplasmic nuclear protein. 50ug protein was taken from each group for western-blotting, and 10% PAGE gel electrophoresis was applied based on protein molecular weight. According to the pre dyed marker display, the target protein was judged to be fully separated and then stopped. After the nuclear protein was transferred to 200mA for 120min 300mA was transferred for 10min, total protein was transferred to 200mA after 120min, and 300mA was transferred to 50min. Both were shaken at room temperature with 5% skim milk TBST (closed liquid) for 2h. Then the color exposure, the experiment was repeated 3 times and calculate the relative content of protein by using BandScan software

cell migration test:

We get gastric carcinoma cells from BGC823 group, p-EGFR group, perbb2-EGFP group ,drained culture medium, 3ml PBS cleaning, 0.25% trypsin, centrifuged 1000rpm for 5min, washed two times by PBS, wash away the residual serum.No serum 1640 culture medium suspension cell, cell count plate count, no serum 1640 culture medium dilution cell concentration to 2×10^4 /ml. 10% 800ul fetal bovine serum PRMI-1640 pre added in 24 well plate medium (including double antibody), and then be taken into the Transwell chamber, 1H later added 150ul cell suspension from each group, 37 C, 5% CO2 incubator training for 24h. After the cell suspension was diluted, the cell suspension was inoculated at 2×10^5 /ml, and the cell suspension was cultured for 24 hours. Remove the Transwell, careful cleaning chamber again with PBS, with 10% methanol fixed cells for 30 seconds. Carefully cut the film, a drop of 5% crystal violet dye, at room temperature for 20 minutes, PBS cleaning, observed and photographed under the microscope.

cell invasion test:

Matrigel be melted overnight in 4°C and diluted with no serum RPMI-1640 culture medium, final concentration is 1mg/ml. Adding Matrigel 100ul (concentration of 1mg/ml), 37 degrees Celsius incubation 5h. 10% 800ul fetal bovine serum PRMI-1640 pre added in 24 well plate medium (including double antibody), and then be taken into the Transwell chamber, 1H later added 150ul cell suspension from each group, 37 C, 5% CO2 incubator training for 24h. After the cell suspension was diluted, the cell suspension was inoculated at 2×10^5 /ml, and the cell suspension was cultured for 24 hours. Remove the Transwell, careful cleaning chamber again with PBS, with 10% methanol fixed cells for 30 seconds. Carefully cut the film, a drop of 5% crystal violet dye, at room temperature for 20 minutes, PBS cleaning, observed and photographed under the microscope.

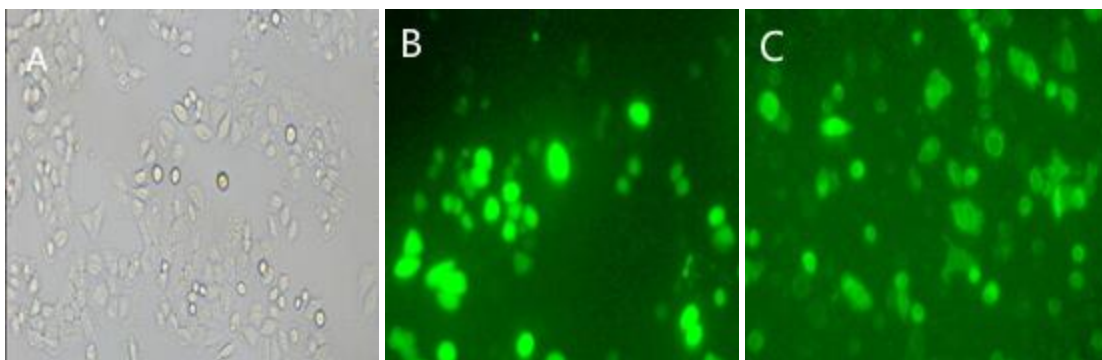
STATISTICAL ANALYSIS

Using SPSS17.0 software for data analysis and processing, taking the T test for the group comparison, the test level $\alpha=0.05$.

RESULT

The morphology of human gastric cancer cell BGC823:

After transfection, the expression of perbb2-EGFP and p-EGFP in gastric carcinoma cells was observed under fluorescence microscope, and the expression of fluorescence was proved to be successful in the expression of BGC823 and perbb2-EGFP in gastric cancer cells. figure 1.



A: BGC823 group ; B: perbb2-EGFP group ; C: p-EGFP group

Figure 1: Observation of perbb2-EGFP group and p-EGFP group of gastric cancer cell line BGC823 under fluorescent microscope

Her-2 mRNA expression after transfection by Real time PCR:

In BGC823 group, p-EGFP group, perbb2-EGFP group of gastric cancer cell Her-2, the amplification curve of her2 gene shows a smooth S curve, including the baseline period, the index of the initial period, the exponential period and the platform period. The melting curve showed only one peak, no peak appeared, which indicated that there was no other amplification product. The results showed that the relative expression of Her-2 gene in group BGC823 was 0.991 ± 0.044 , and in the p-EGFP group was 1.007 ± 0.022 , and in the perbb2-EGFP group was 1.817 ± 0.036 . MRNA Her-2 expression levels were significantly higher in the perbb2-EGFP group than in the other two groups ($P < 0.05$). There was no significant difference between the BGC823 group and the p-EGFP group ($p=0.61 > 0.05$). Figure 2-4.

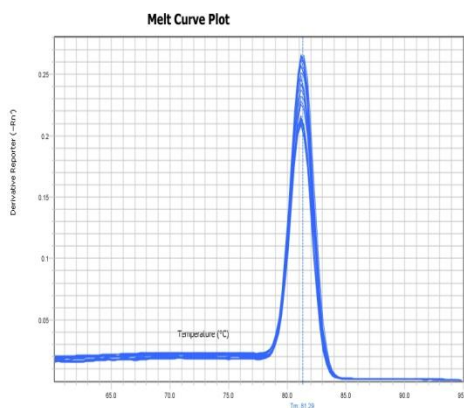


Figure 2: Her-2 gene fluorescence quantitative PCR melting curve

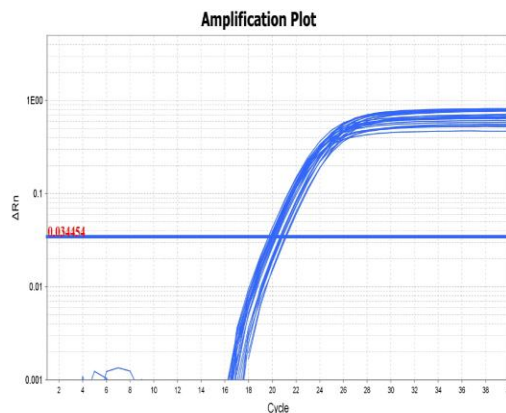


Figure 3: Her-2 gene fluorescence quantitative PCR amplification curve

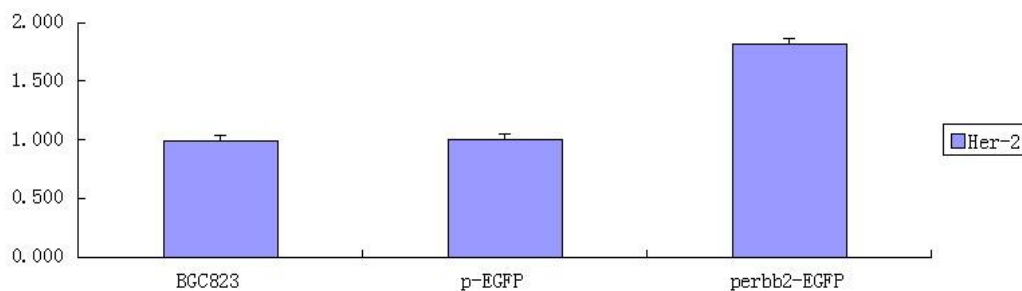
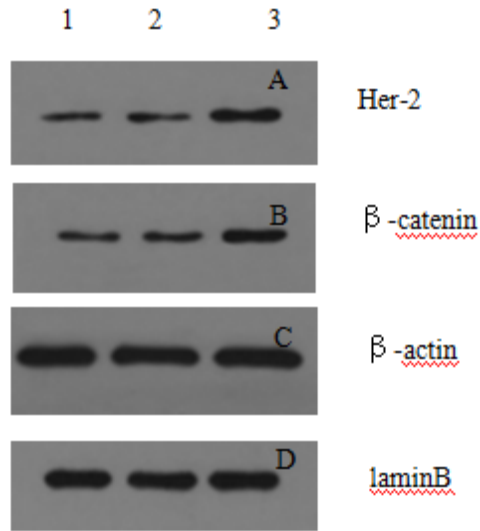


Figure 4: fluorescent quantitative PCR relative expression of Her-2 mRNA in BGC823 group, p-EGFP group, perbb2-EGFP group.

The content of Her-2 and β -catenin protein was detected by western blotting method:

Her-2/ β -actin was 0.311 ± 0.040 , β -catenin/lamin was 0.329 ± 0.03 in BGC823 group; Her-2/ β -actin were 0.327 ± 0.007 , β -catenin/lamin was 0.332 ± 0.006 in p-EGFP group; Her-2/ β -actin were 0.582 ± 0.030 , β -catenin/lamin was 0.583 ± 0.022 in perbb2-EGFP group. The expression level of Her-2 protein in perbb2-EGFP group was significantly higher than that in other two groups ($P < 0.05$), and there was no significant difference between the BGC823 group and the p-EGFP group ($p=0.54 > 0.05$). The intracellular concentration of β -catenin in perbb2-EGFP group was significantly higher than that in the other two groups ($P < 0.05$). There was no significant difference between the BGC823 group and the p-EGFP group ($p=0.89 > 0.05$). Figure 5-6, Table 1.



A: Her-2, **B:** β -catenin, **C:** reference β -actin, **D:** reference laminB ;

1: BGC823 group, **2:** p-EGFP group, **3:** perbb2-EGF group ;

Figure 5: Her-2、 β -catenin protein detected by western blotting

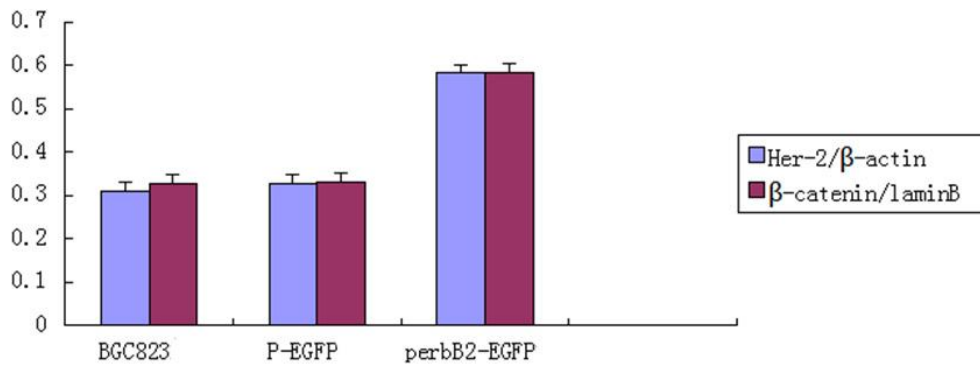


Figure 6: western blot detection of Her-2, β -catenin relative to the reference content

Group	Her-2/ β -actin	β -catenin/lamin B
BGC823	0.311 ± 0.040^a	0.329 ± 0.03^a
P-EGFP	0.327 ± 0.007^b	0.332 ± 0.006^b
perbB2-EGFP	0.582 ± 0.030^c	0.583 ± 0.022^c
P	< 0.05	< 0.05

a: Compared with P-EGFP cell group $p > 0.05$; **b:** Compared with perbB2-EGFP group $p < 0.05$, **c:** Compared with BGC823 group $p < 0.05$

Table 1: western blot detection of Her-2, β -catenin relative to the reference content

Cell migration:

The average number of cells in group BGC823 was $55 + 2.4$, the average number of cells in group p-EGFP was $57.8 + 2.2$, and the average number of cells in group perbb2-EGFP was $96.2 + 3$. Perbb2-EGFP group compared to other groups reach the number of cells in the lower layer of microporous membrane was significantly increased, the migration ability was significantly enhanced ($p=0 < 0.05$). There was no significant difference between the BGC823 group and the p-EGFP group ($p=0.092 > 0.05$). Figure 7.

Cell invasion test:

The average number of cells in group BGC823 was $24.4 + 3.5$, the average number of cells in group p-EGFP was $28 + 2.5$, and the average number of cells in group perbb2-EGFP was $65.2 + 3.8$. The invasion ability of perbb2-EGFP group was significantly increased compared to other groups ($p=0 < 0.05$). There was no significant difference between the BGC823 group and the p-EGFP group ($p=0.13 > 0.05$). figure 8.

DISCUSSION

Recent studies have shown that Her-2 over expression of gastric cancer patients tend to have a poor prognosis. The possible reason is that Her-2 over expression of gastric cancer cells showed a stronger invasion and migration ability, it is more likely to be transferred.H Yokoyama et al [4] isolated liver cancer cell from the advanced gastric cancer patients with liver metastasis and cultured in vitro, found the the her2 content of cancer cell lines was higher than the general cancer cell, which indicates that the overexpression of Her-2 is closely related with liver metastasis of gastric cancer.

Invasion and migration are the important biological characteristics of tumor cells, which are closely

related to the prognosis of tumor patients. The plasmid perbb2-EGFP with the Her-2 gene was transfected into gastric cancer cell BGC823 in the experiment, after transfection Her-2 gene was amplified than the other two groups detected by realtime-PCR and Her-2 protein was higher expressed than the other two groups detected by western blotting. Three groups were performed Transwell invasion and Transwell migration experiment, perbb2-EGFP group showed a stronger invasion and migration ability. It further showed that the high amplification of Her-2 gene and protein expression in gastric cancer cells showed a stronger invasion and migration ability.

β -catenin is a multifunctional protein, which is an important component of the WNT signaling pathway, and it is also a key component of the signaling pathway. In the normal tissue cells, β -catenin and E-cadherin on cell membranes formed by the combination of E-cadherin/ β -catenin adhesion complex, involved in similar adhesion between cells, little free state β -catenin exists only in the cytoplasm [5-6]. Usually, the WNT signaling pathway is in the closed state, β -catenin amino terminal 45 serine residues was phosphorylated, activated β -catenin is degraded by ubiquitin proteasome system [7]. A number of studies [8-9] have confirmed that in a variety of tumors, WNT signaling pathway is activated by E-cadherin/ β -catenin adhesion complex deletion and ectopic expression of β -catenin. When the β -catenin hydroxy stump 654 tyrosine phosphorylated by tyrosine kinase, β -catenin and E-cadherin activation binding capacity decreased, escape from the E-cadherin/ β -catenin adhesion complex, gathered in the cytoplasm, then transfer to the nucleus, combined with TCF/LEF, the expression of cyclinD1 C-mys, open the downstream gene transcription.

In this experiment, In gastric cancer cell BGC823 Her-2 gene relative expression was $0.991 + 0.044$, p-EGFP cell group (no load group) was $1.007 + 0.022$, perbb2-EGFP cell group was $1.817 + 0.036$. mRNA Her-2 levels were significantly higher in the perbb2-EGFP cell group than in the other two groups ($P < 0.05$). The expression of Her-2 and β -catenin protein in perbb2-EGFP group increased significantly than other two groups ($P < 0.05$); In perbb2-EGFP group β -catenin protein concentration in the nucleus increased significantly than other two groups of ($P < 0.05$), and invasion and migration ability in group perbb2-EGFP cell was significantly enhanced. The amplification and protein expression of Her-2 gene in gastric cancer cells may have a direct effect on the increase of the distribution of β -catenin in the nucleus. The cell invasion and migration ability of perbb2-EGFP group were significantly increased and there was a certain relationship with the increase of the distribution of β -catenin in the nucleus. In the construction of MMTV-C-Neu and MMTV-WNT-1 transgenic mice model, Schroeder et al [10] found that β -catenin 645 hydroxy terminal tyrosine can be phosphorylated by her2 protein, leading to E-cadherin/ β -catenin adhesion complex destruction, the nucleus of β -catenin distribution increased, the activation of the WNT signaling pathway, leading to tumor cell invasion and metastasis. Wang et al [11] applied geldanamycin (geldanamycin, GA) in Her-2 high expression breast cancer cell line SKBr3, leading the Her-2 protein inactivation, the nucleus of β -catenin distribution was decreased. The above two experiments confirm that there is a relationship between Her-2

and β -catenin.

This experiment also reached the same results, we hypothesized that perbb2-EGFP transfected gastric cancer cell lines BGC823, Her-2 gene amplification and protein expression increased, β -catenin beta hydroxy terminal 645 region tyrosine phosphorylated by cell membrane Her-2 protein tyrosine kinase, β -catenin free from cell membrane to cytoplasm, aggregation in cytoplasm stable. Then transfer to the nucleus, the nucleus increased β -catenin protein content, WNT signal is activated at the same time E-cadherin/ beta -catenin adhesion complex was damaged, allogenic cell adhesion between cells decreased, invasion and migration ability enhancement. It further confirmed that Her-2 plays an important role in the occurrence, development and metastasis of gastric cancer. It is an important prognostic indicator for gastric cancer.

REFERENCES

1. Kajitani T. The general rules for the gastric cancer study in surgery and pathology. Part I. Clinical classification. *Jpn J Surg* 1981; 11: 127-139.
2. Ross JS, Slodkowska EA, Symmans WF, et al. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist* 2009; 14:320-368.
3. Zulehner G, Mikula M. Nuclear β -catenin induces an early liver progenitor phenotype in hepatocellular carcinoma and promotes tumor recurrence [J]. *Am J Pathol*, 2010, 176(1):472-481.
4. Yokoyama H, Ikehara Y, Kodera Y, et al. Molecular basis for sensitivity and acquired resistance to gefitinib in Her-2 overexpressing human gastric cancer cell lines derived from liver metastasis[J]. *Br J Cancer*, 2006, 95(11):1504-1513.
5. Clevers H, Nusse R. Wnt/ β -catenin signaling and disease[J]. *Cell*, 2012, 149 (6) :1192-1205.
6. Bourboulia D, Han H, Jensen-Taubman S, et al. TIMP-2 modulates cancer cell transcriptional profile and enhances E-cadherin/ β -catenin complex expression in A549 lung cancer cells[J]. *Oncotarget*, 2013, 4(1):166-76.
7. Zeineldin M, Cunningham J, McGuinness W, et al. A knock-in mouse model reveals roles for nuclear APC in cell proliferation, Wnt signal inhibition and tumor suppression[J]. *Oncogene*, 2012, 31(19):2423-2437.
8. Berthon A, Drelon C, Ragazzon B, et al. WNT/ β -catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production[J]. *Hum Mol Genet*, 2014, 23(4):889-905.

9. Anatas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer[J]. Nat Rev Cancer. 2013 Jan;13(1):11-26.
10. Schroeder JA, Adriance MC, McConnell EJ, et al. ErbB- β -catenin complexes are associated with human infiltrating ductal breast and murine mammary tumor virus (MMTV)-Wnt-1 and MMTV-c-Neu transgenic carcinomas[J]. Biol Chem, 2002, 277 (25) :22692-8.
11. Wang K, Ma QY, Ren Y, et al. Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/ β -catenin signaling in HER2 overexpressing human breast cancer cells[J]. Oncology Reports, 2007, 17: 89-96.