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Original Article

IGF-1R promotes the expression of cyclin D1 protein and accelerates the G1/S transition by activating Ras/Raf/MEK/ERK signaling pathway

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Abstract: Objective: The objective of study was to detect the expression level of IGF-1R in breast cancer tissues and investigate the effect of IGF-1R expression on the proliferation and apoptosis of breast cancer cells. Methods: The expression of IGF-1R protein in breast cancer tissues and the adjacent normal tissues was detected by immunohistochemistry. By IGF-1R inhibitor mediated repression of IGF-1R expression in MCF-7 cells, the cell proliferation rate, cell cycle and the expression of Ras/Raf/MEK/ERK signaling pathway proteins as well as cyclin D1 protein were examined in the IGF-1R-inhibited cells. Results: The proportion of IGF-1R, cyclin D1, Ras and p-ERK1/2 positive cells in breast cancer tissues were (69.8±12.7)%, (38.0±6.2)%, (71.6±10.3)% and (3.1±5.7)%, respectively, which were all significantly higher than those in the adjacent normal tissues [(16.2±6.7)%, (0.5±0.7)%, (7.1±0.9)% and (12.2±5.4)%] ($P<0.05$). Compared to normal MCF-7 cells, the MCF-7 cells treated with IGF-1R inhibitor showed no expression of IGF-1R protein, increased apoptosis rate (370.3%), increased proportion of cells in G1 phase (81.1%), decreased proportion of cells in S phase (66.8%), decreased proportion of cells in G2/M phase (52.9%), decreased Ras protein expression (70.9%), decreased p-ERK1/2 protein expression (53.3%), decreased cyclin D1 protein expression (59.5%), and substantially unchanged expression of ERK1/2 protein ($P<0.05$). Conclusions: IGF-1R is overexpressed in breast cancer tissues. The overexpressed IGF-1R promotes the expression of cyclin D1 protein and accelerates the G1/S transition by activating Ras/Raf/MEK/ERK signaling pathway, thus further promoting cell proliferation and the carcinogenesis of breast cancer.

Keywords: IGF-1R, breast cancer, MCF-7 cells, proliferation

Introduction

Breast cancer is a malignant tumor that occurs in breast epithelial tissues, and the patients are generally females. In 2015, more than 260,000 cases of breast cancer patients were registered in the National Cancer Center of China, accounting for about 15% of all malignant tumor cases in women [1]. Moreover, breast cancer is with the highest morbidity of female malignant tumors worldwide, which is one of the most harmful diseases threatening the health of women in the world [2]. In recent years, with the influence of multiple factors, such as the ever-accelerating pace of social life, the deterioration of the environment, the changing concept of reproduction and the increased incidents of ionizing radiations, the morbidity of breast cancer in China has been increasing year by year [3]. Like any other type of tumors, the carcinogenesis and progression of breast cancer are closely related to the malignant transformation of normal cells and the abnormal proliferation of cancer cells.

Insulin like growth factor-1 receptor (IGF-1R) is an important member of insulin like growth factor/insulin family proteins, which is widely distributed in various tissues and organs such as ovary, prostate and heart and is involved in the regulation of cell proliferation and apoptosis by binding with IGF-I and IGF-II. In recent years, a great number of domestic and foreign studies have shown that IGF-1R is highly expressed in many types of malignant tumors including ovarian cancers [4], rectal cancers [5] and lung can-

cers [6, 7], and that the overexpression of IGF-1R is closely related to the proliferation/apoptosis as well as invasion/migration of tumor cells [8]. However, few studies have been reported about the expression of IGF-1R in breast cancer tissues and its effect on the proliferation of breast cancer cells both in China and abroad. In this study, by comparing the expression of IGF-1R in breast cancer tissues with that in the adjacent normal tissues and examining the effect of IGF-1R inhibitor on the proliferation/apoptosis and the cell cycle of MCF-7 cells, we demonstrated the significance of IGF-1R expression in breast cancer tissues as well as the mechanism of its effect on the proliferation of breast cancer cells.

Materials and methods

Clinical sample

28 cases of breast cancer tissues and the adjacent normal tissues were obtained from surgical sections in our hospital. After the surgical removal of the samples, a portion of each sample was washed by sterile water, routinely prepared as pathological sections and stored at -80°C, while other portions of the tissue samples were directly frozen in liquid nitrogen for reservation. All the 28 cases of patients were females diagnosed with breast cancer, among which 24 cases were diagnosed with infiltrating ductal breast cancer and the other 4 cases were mucinous breast cancer while no case of mixed breast cancer was diagnosed. The age of the patients was between 29-67 years old with the average age of (42.3±8.9). This research was approved by the ethics committee of the Affiliated Hospital of Southwest Medical University. And all of the patients signed the informed consent.

Reagents and cell lines

The human breast cancer derived MCF-7 cell line was purchased from American Type Culture Collection (ATCC). IGF-1R inhibitor (Picropodophyllin, PY99, Santa Cruz, USA). Tissue total protein extraction kit, tissue total RNA extraction kit, cell total protein extraction kit and BCA protein concentration detection kit were all purchased from Biyuntian Biotech Corporation. IGF-1R rabbit monoclonal antibody (ab182408, Abcam, Cambridge, MA), cyclin D1 (ab15196, Abcam, Cambridge, MA), Ras (ab221163, Abcam, Cambridge, MA), ERK1/2 (ab17942, Abcam, Cambridge, MA) and p-ERK1/2 rabbit polyclonal antibody (ab223500, Abcam, Cambridge, MA), goat anti-rabbit IgG-H&L (Alexa Fluor® 488) polyclonal antibody and goat anti-rabbit IgG H&L (HRP) polyclonal antibody.

Experimental methods

Cell experiments: MCF-7 cells were detached by trypsin treatment and plated into Petri dishes at the ratio of 1:4. 24 hours later, add IGF-1R inhibitor (Picropodophyllin dissolved in DMSO, prepared at the concentration of 60 mM and kept at 4°C as stock solution) to one of the Petri dishes at the final concentration of 60 nM and termed it as the PPP group. Another Petri dish of cells were treated with DMSO and termed as the control group. The two groups of cells were cultured regularly for 48 hours (for apoptosis assays) or 12 hours (for cell cycle assays) before trypsin digestion and washed twice by PBS to be used in the next procedures.

Immunohistochemistry detection of the protein expressions in the clinical samples: Paraffin sections were prepared from clinical samples, and incubated at 60°C for 1 h followed by H_2O_2 blockage, rinsing, antigen retrieval, PBS rinsing, non-fat milk blockage and incubation with the primary antibody (IGF-1R rabbit monoclonal antibody, PBS was used as negative control), the sections were rinsed and incubated with the secondary antibody before DAB coloration (coloration was terminated in due course). For each section, positive cells of IGF-1R, Ras, p-ERK1/2 and cyclin D1 from 5 different visual fields under 400× objective lens were counted respectively, and the mean value of the 5 visual fields was designated as the mean proportion of positive cells in a section.

Western Blot detection of protein expression: Tissue samples frozen in liquid nitrogen or cells collected from indicated experiments were subjected for protein extraction by tissue or cell total protein extraction kit and the protein concentrations were detected by BCA kit. 60 μg of total proteins in each group was loaded for SDS-PAGE and transferred to a wet PVDF membrane (fixed in methanol). The membrane was blocked with 5% no-fat milk in TBST for 2 h at room temperature and incubated with primary antibodies (cyclin D1, Ras, ERK1/2, and p-ERK1/2 rabbit monoclonal antibodies) at 4°C

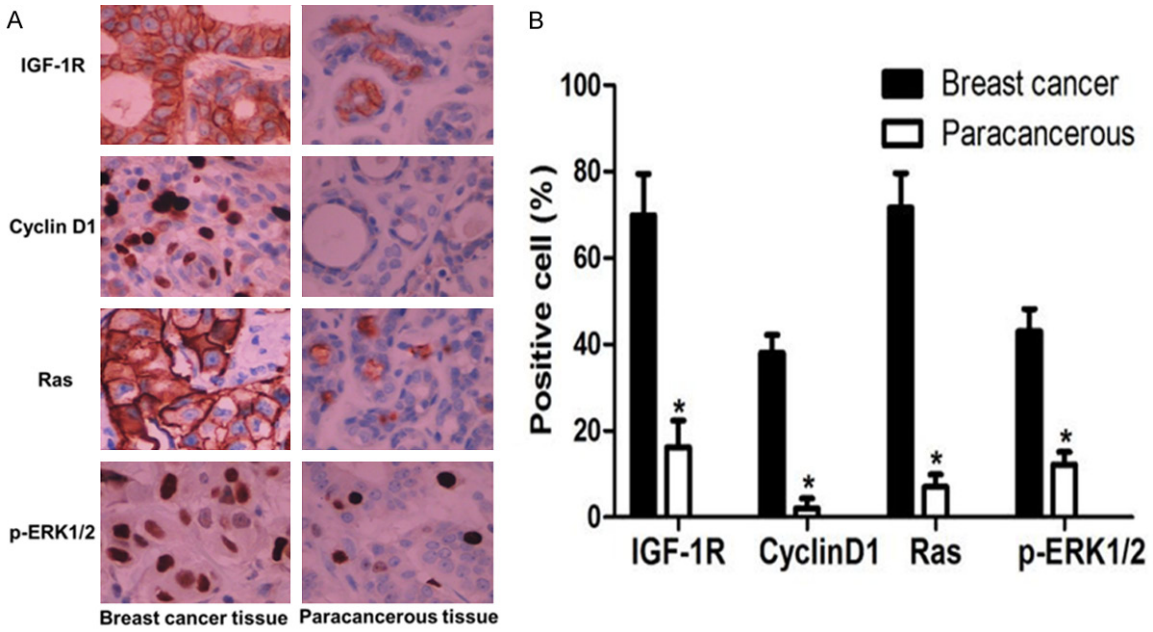

Figure 1. Immunohistochemistry detection of the protein expressions of IGF-1R, Ras, p-ERK1/2 and cyclin D1. A. IGF-1R was expressed in the cell membrane, and the positive cells were determined as those with brown stained cell membranes; Ras and p-ERK1/2 were both expressed in the cytoplasm, and the positive cells were determined as those with brown stained cell cytoplasm; Cyclin D1 was expressed in the cell nucleus, and the positive cells were determined as those with brown stained cell nuclei; B. The statistical analysis of positive cell in the two groups. *represented statistically significant differences compared with the breast cancer tissues.

overnight. After PBS rinsing and incubation with the secondary antibody (goat anti-rabbit IgG H&L (HRP)), the band density was analyzed by Image-J software and normalized against β-actin levels to be presented as the final results.

Immunofluorescence detection of intracellular protein expressions: Cells were seeded into culture dishes with pre-placed treated coverslips and cultured till the cells grow to 95%-100%. The coverslips were washed twice by PBS and fixed by paraformaldehyde, followed by PBS rinsing, Triton X-100 penetration, PBS rinsing, blockage, incubation with the primary antibody (IGF-1R rabbit monoclonal antibody) at 4°C overnight, PBS rinsing and incubation with the secondary antibody (goat anti-rabbit IgG-H&L (Alexa Fluor® 488) polyclonal antibody) for 2 h at room temperature. Finally, the coverslips were washed 3 times with PBS, dried, sealed and kept in the dark room.

Flow cytometry detection of apoptosis and cell cycle: The MCF-7 cells treated by different methods were collected and fixed by 70% pre-cooled ethanol (prepared by pre-cooled PBS and absolute ethanol) overnight at 4°C. After PBS rinsing, cells were stained with PI and analyzed by flow cytometry for apoptosis rate or cell cycle.

Statistical analysis

Statistical analysis was performed using the SPSS19.0 statistical program, t test was used to analyze the differences between groups and $P<0.05$ was considered statistically significant.

Results

Immunohistochemistry detection of the indicated protein expressions in different tissue samples

IGF-1R, Ras, p-ERK1/2 and cyclin D1 protein expressions were detected by immunohistochemistry and the percentage of positive cells stained with each antibody was used to represented the relative expression of the protein. The results showed that the proportions of IGF-1R, cyclin D1, Ras and p-ERK1/2 positive cells in the breast cancer tissues were (69.8±12.7)%, (38.0±6.2)%, (71.6±10.3)% and (43.1±5.7)%,

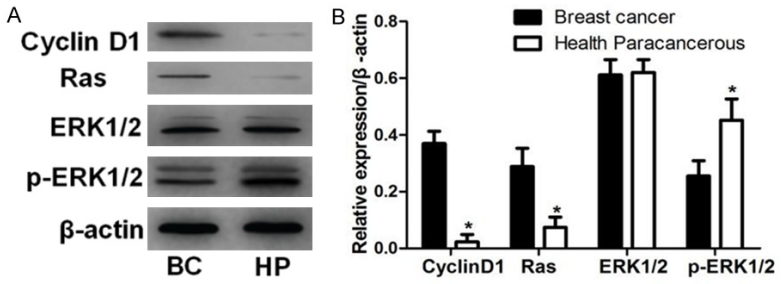

Figure 2. Western blot detection of the protein expressions of Cyclin D1, Ras, ERK1/2 and p-ERK1/2. A. Results of western blot assay. B. The statistical analysis of western blot. *presents: P<0.05.

respectively, which were all significantly higher than those in the adjacent normal tissues [(16.2±6.7)%, (0.5±0.7)%, (7.1±0.9)% and (12.2±5.4)%] (P<0.05) (**Figure 1**).

Western Blot detection of the indicated protein expressions in different tissue samples

Western Blots were performed to detect the protein expressions of cyclin D1, Ras, ERK1/2 and p-ERK1/2 in different tissue samples. The results showed that the relative expression of Cyclin D1, Ras and p-ERK1/2 in the breast cancer tissues were (0.37±0.04), (0.28±0.12) and (0.26±0.10), respectively, which were all significantly higher than those in the adjacent normal tissues [(0.023±0.03), (0.074±0.04) and (0.45±0.11)] (P<0.05) while the expression of ERK1/2 showed no significant differences in the breast cancer tissues and the adjacent normal tissues (**Figure 2**).

The effect of IGF-1R expression on the proliferation of MCF-7 cells

IGF-1R protein expression was suppressed by the treatment of IGF-1R inhibitor in MCF-7 cells and FACS was performed to examine the effect on cell apoptosis and cell cycle. The results showed that there was substantially no expression of IGF-1R in the IGF-1R inhibitor treated MCF-7 cells, and the proportions of S phase cells and G2/M phase cells were (29.7±11.3)% and (4.8+1.3)%, respectively, which were both significantly lower than those in the control group [(54.9±8.5)% and (10.2±2.9)%] (P<0.05). The proportions of apoptotic cells and G1 phase cells were (42.8±5.8)% and (64.3±7.7)%, respectively, in the IGF-1R inhibited group, which were both significantly lower than those in the control group [(9.1±3.8)% and (35.5±5.3)%%] (P<0.05) (**Figure 3**).

The effect of IGF-1R on the expressions of cyclin D1, Ras, ERK1/2 and p-ERK1/2

The IGF-1R protein expression was suppressed by IGF-1R inhibitor treatment for 48 hours in MCF-7 cells and then the cells were lysed and subjected for Western blots to detect the protein expressions of cyclin D1, Ras, ERK1/2 and p-ERK1/2. The results showed that after IGF-1R treatment, the expression levels of cyclin D1, Ras, and p-ERK1/2 proteins in MCF-7 cells were (0.42±0.05), (0.31±0.04) and (0.45±0.11), respectively, which were all significantly lower than those in the normal MCF-7 cells [(0.17±0.03), (0.09±0.03) and (0.21±0.12)] (P<0.05), while the expression level of ERK1/2 protein was substantially unchanged after IGF-1R inhibitor treatment (P>0.05) (**Figure 4**).

Discussion

IGF-1R is a kind of trans-membrane tyrosine kinase, which is synthesized by intracellular ribosomes and composed of 1332 amino acids [9]. Its coding gene is located in human chromosome 15q25-26. IGF-1R contains two subunits, α and β, the function of the α subunit is to combine with the extracellular insulin like growth factor while the β subunit exerts the function of a trans-membrane tyrosine kinase, which transfers the extracellular stimuli into the cells in order to activate the synthesis of relative proteins as well as other biological functions [10]. The IGF-1R binding protein IGF-I is a kind of growth factor, which has a similar structure with insulin and is synthesized and secreted by hepatocytes. The major function of IGF-1 is to regulate the proliferation and apoptosis of cells by binding to the extracellular IGF-1R [11]. Dale O T et al. [12] found that IGF-1R was highly expressed in head and neck squamous cell carcinoma tissues, which was closely related to the carcinogenesis, progression and prognosis of this malignant disease. Meanwhile, De Bruijn K et al. [13] found that the low-or-non expression of IGF-1R in the tumor tissues of patients with advanced esophageal adenocarcinoma

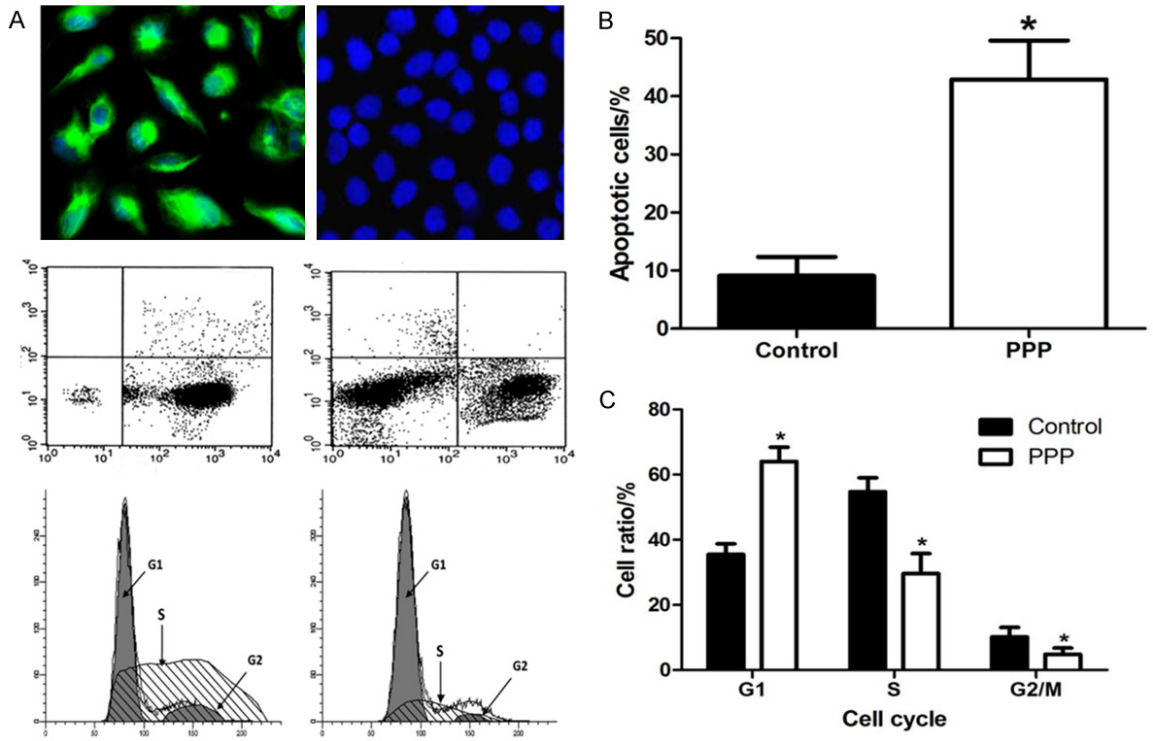

Figure 3. The effect of IGF-1R expression on the proliferation and apoptosis of MCF-7 cells. A. Immunofluorescence detection of IGF-1R protein expression, the stronger green fluorescence is associated with more expression of IGF-1R protein. There was no expression of IGF-1R protein in the PPP group; B. FACS analysis of cell apoptosis rate and the statistical chart; C. FACS analysis of cell cycle; *represented statistically significant differences compare to the Control group.

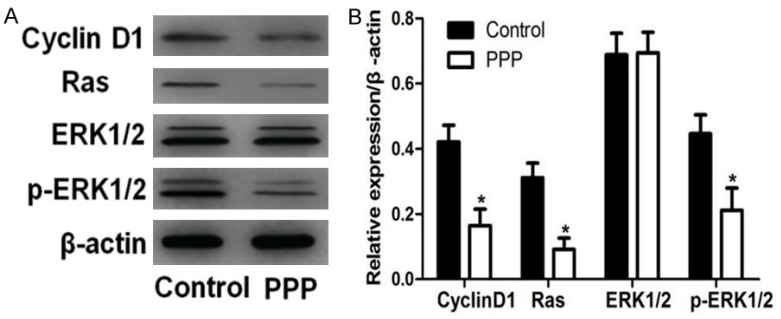

Figure 4. The effect of IGF-1R on the expression of cyclin D1, Ras, ERK1/2 and p-ERK1/2. A. Normal MCF-7 cells without any treatment were designated as the Control group; MCF-7 cells treated with IGF-1R inhibitor Picropodophyllin (PPP) for 48 hours were designated as the PPP group; B. Relative expression Cyclin D1, Ras, ERK1/2 and p-ERK1/2/β-actin. *represented statistically significant differences compare to the Control group (P<0.05).

was associated with better clinical prognosis, suggesting that IGF-1R may act as a tumor marker for the clinical prognosis of esophageal adenocarcinoma.

In this study, we found that the expressions of IGF-1R in the 28 cases of breast cancer tissues were significantly higher than those in the adjacent normal tissues (P<0.05). Under normal physiological conditions, IGF-1R signaling pathway regulates cell proliferation, apoptosis and differentiation. However, because there are multiple intersections between the IGF-1R signaling pathway and the oncogenes like Fos and Pals, IGF-1R is highly susceptible to be over expressed by the stimulation of oncogenes, which leads to an increased proportion of cells to exhibit malignant phenotypes and promotes the proliferation and differentiation as well as inhibits the apoptosis of cancer cells, thus accelerates the progression of malignancy [14]. Therefore, the high expression of IGF-1R in the breast cancer tissues may play an important role in the carcinogenesis and development of breast cancer.

Ras/Raf/MEK/ERK signaling pathway is an important pathway involved in the regulation of cell proliferation, apoptosis and differentiation, and it can function through the stimulation of trans-membrane proteins by extracellular growth factors [15]. Ras protein is a kind of GTP binding protein encoded by Ras gene, which has weak GTP enzyme activity and plays a role in signal transduction in Ras/Raf/MEK/ERK signaling pathway. When Ras protein is stimulated by the upstream signals, it activates a series of intracellular effector enzymes through self-activation, thus participating in the regulation of proliferation and apoptosis of cells [16]. ERK1/2 belongs to the family of MAPK proteins and is the downstream effector of Ras/Raf/MEK/ERK signaling pathway. The growth factors and the upstream Ras protein act as co-stimulatory signals to activate (phosphorylate) ERK1/2 in order to regulate the proliferation, apoptosis, metabolism and differentiation of cells. In this study, we suppressed the expression of IGF-1R in MCF-7 cells by IGF-1R inhibitor and found that the protein expressions of Ras and p-ERK1/2 were also down-regulated when IGF-1R was suppressed, which indicated that IGF-1R might exert its biological function through Ras/Raf/MEK/ERK signaling pathway.

Moreover, our studies indicated that when IGF-1R expression was inhibited in MCF-7 cells, the apoptosis rate and the proportion of cells in G0/G1 phase were both significantly higher than those of normal MCF-7 cells ($P<0.05$), and that the expression of Cyclin D1 protein was significantly down-regulated ($P<0.05$). The cell cycle is the process of the cell from the beginning of a split to the end of the next division, and the regulation of the cell cycle is mainly achieved by the retention in the G1 phase. There is an important node for cell cycle regulation in the G1 phase, which is the R point. When the cell cycle is prior to the R point, cells need the stimulations from extracellular growth factors to maintain normal operations of the cell cycle, while after the cycle passes the R point, the cell cycle becomes an autonomous-controlled process which is no longer dependent on the presence of extracellular cytokines. Cyclin D1 protein is encoded by CCND1 gene, and is also known as G1/S-specific Cyclin D1, which plays an important role in the cis-regulation of R point crossing in the cell cycle. The half-life of Cyclin D1 is only 25 min, and when cells enter the S phase in normal physiological state, the Cyclin D1 protein will immediately degrade, so it showed low-or-non expression in healthy tissue. Previous studies [17, 18] discovered that Cyclin D1 is highly expressed in breast cancer tissues, which accelerates the G1/S transition of tumor cells thus promoting the progression of malignancy. Margo et al. [19] detected the expressions of Cyclin D1 in the neuroblastoma tissues at different clinical stages by immunohistochemistry, and discovered that Cyclin D1 was highly expressed in neuroblastoma, which was closely associated with the progression and clinical stages of the patients. In addition, Liu et al. [20] showed that p-ERK1/2, which is activated by a variety of proteins including growth factors and Ras protein, enters the nucleus from cytoplasm and promotes the expression of Cyclin D1 via the NF-κB signaling pathway.

Collectively, our results demonstrated that IGF-1R was highly expressed in breast cancer tissues, and that the overexpressed IGF-1R promoted the expression of Cyclin D1 protein by activating Ras/Raf/MEK/ERK signaling pathway, thus promoting the proliferation and inhibiting the apoptosis of human breast cancer cells.

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Disclosure of conflict of interest

None.

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