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Transfer of interleukin 6 gene into MCF-7 human breast cancer cells enhances expression of tumor-associated antigens

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ABSTRACT **AIM:** To investigate the mechanism by which IL-6 is involved in cancer prognosis, and further to demonstrate the relationship between IL-6 and tumor-associated antigens such as CA15-3, CEA and CA125 in breast cancer. **METHODS:** In the present study, we transfected an exogenous IL-6 gene into the MCF-7 cells. Secretion of CA15-3, CEA and CA125 into the culture media were measured by Enzyme Linked Immunosorbent Assay (ELISA). **RESULTS:** After a 72 hours in culture, the amount of IL-6 in the media of pCI-neo-IL-6-transfected MCF-7 cells (338.5±22.6 pg/10^6 cells) was significantly higher than that of non-transfected MCF-7 cells (25.4±4.6 pg/10^6 cells, $P<0.01$, *paired t*-test), or pCI-neo-transfected MCF-7 cells (19.6±3.0 pg/10^6 cells, $P<0.01$, paired *t*-test). The levels of CA15-3, CEA and CA125 secreted by the pCI-neo-IL-6-transfected MCF-7 cells were significantly higher than that of the parental MCF-7 cells or pCI-neo-transfected MCF-7 cells. The specific IL-6 antibody could decrease the expression of CA15-3, CEA and CA125 in both the MCF-7 cells and the IL-6 cDNA-transfected MCF-7 cells. **CONCLUSION:** Transfer of IL-6 gene augments tumor-associated antigens of human breast cancer cells. The association of elevated IL-6 concentration with a poor prognosis in cancer patients may partially be the result of increased expression of tumor-associated antigens by IL-6.

KEY WORDS breast cancer; tumor-associated antigen; interleukin 6; gene transfection

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Interleukin 6 (IL-6) is a multipotent cytokine that plays an important role in immunology, inflammation, bone metabolism, reproduction, arthritis, aging and neoplasia[1]. It has been shown that serum levels of IL-6 are a prognostic factor in several cancers, including multiple myeloma, Hodgkin's lymphoma, renal cell carcinoma, bladder carcinoma, ovarian cancer, esophageal squamous cell carcinoma, and breast cancer[2－12]. A high concentration of serum IL-6 is associated with aggressive tumors, poor disease-free and overall survival. IL-6 is involved in cancer progression through a decrease in cancer cell adhesion, an increase in cancer cell motility, induction of thrombopoiesis, an increase in cancer cell proliferation, as well as stimulation of tumor specific antigen expression[13－18]. It has been reported that IL-6 increases carcinoembryonic antigen (CEA) and histocompatibility leukocyte antigen expression in human colorectal carcinoma cells[15－18]. Belluco, *et al* also reported that blood IL-6 concentration was associated with high circulating CEA and advanced disease with an IL-6 concentration of more than 10 pg/mL being an independent negative prognostic marker of survival in colorectal carcinoma[19]. Furthermore, Tsang reported that transfaction of IL-6 gene into a human colorectal carcinoma cell line resulted in enhanced CEA expression[20]. To date similar results in CEA have not been reported in breast cancer. In addition, such effects of IL-6 on other tumor-associated antigens (TAAs), such as the breast cancer antigen CA15-3 and the ovarian cancer antigen CA125, have not been re-

ported.

CEA expression is associated with metastatic disease in cancer, especially liver metastases of colorectal carcinomas[21—26]. CEA could promote liver colonisation through enhanced retention of human colorectal carcinoma cells[23]. Serum CA15-3 and CA125 are prognostic factors in a variety of cancers, such as breast cancer, cholangiocarcinoma, ovarian carcinoma and endometrial carcinoma[27—32], but their functions have not been elucidated.

In order to investigate the mechanism by which IL-6 is involved in cancer prognosis, and further to demonstrate the relationship between IL-6 and TAAs (CA15-3, CEA and CA125) in breast cancer, we introduced an exogenous IL-6 gene into the MCF-7 breast cancer cells. Secretion of CA15-3, CEA and CA125 was measured in the culture media. The results presented in this study are the first on the effect of IL-6 on the expression of tumor-associated antigens in breast cancer.

1 MATERIALS AND METHODS

1.1 Cell culture The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD); IL-6 monoclonal antibody (IL-6Ab) was purchased from Sigma (St. Louis, MO); MCF-7 cells were maintained in alpha-MEM supplemented with 10% fetal calf serum, 1 mmol/L glutamine and 0.05 mg/mL gentamicin (Life Technologies, Inc. Frederick, MD) in 5% CO_2 at 37 ℃. Cells were removed from the tissue culture flask or plate by trypsin-EDTA (0.05% trypsin and 0.53 mmol/L EDTA) digestion.

1.2 Transfection of IL-6 cDNA into MCF-7 cells IL-6 cDNA clone, zb85e08.r1, was purchased from the American Type Culture Collection (Rockville, MD). The zb85e08.r1 clone is a 1177 bp DNA sequence which contains a 535bp human IL-6 5'-coding sequence inserted in a pT7T3 vector (Pharmacia, Piscataway, NJ) at Not I and EcoR I sites. Bacteria containing the zb85e08.r1 clone were amplified in sterile LB medium containing 10 mg/mL tryptone, 5 mg/mL yeast extract, 10 mg/mL NaCl and 50 μg/mL ampicillin (Sigma, St. Louis, MO) with shaking at 37 ℃. Plasmid DNA was purified with a Wizard plus miniprep DNA purification system (Promega, Madison, WI). Plasmid DNA was dissolved in 50 μL of pH 7.4 TE buffer (10 mmol/L pH 7.4 Tris-HCl and 1mmol/L pH 8.0 EDTA). Plasmid DNA was digested with restriction enzymes Not I and EcoR I (Promega, Madison, WI) at 37 ℃, and the products of the digestion were run on 1% agarose electropheresis with ethidium bromide. The 1177 bp DNA band in the agarose gel was cut with a clean blade. The DNA fragment was purified with a Wizard PCR prep DNA purification system (Promega, Madison, WI) and quantitated by measuring its optical density at A_{260}. One μg DNA of pCI-neo mammalian expression vector with the G418 drug resistance marker (Promega, Madison, WI) was digested with Not I and EcoR I at 37 oC and purified with Wizard DNA clean purification system (Promega, Madison, WI). The optimal ratio of 1177 bp fragment and digested pCI-neo vector DNA was calculated according to the length of the DNA. Both of the DNA fragments were added to a reaction containing T4 ligase and buffer (Promega, Madison, WI) and incubated for 16 h at 22 ℃. JM109 E. Coli competent cells (Promega, Madison, WI) were transfected with the above ligated DNA and incubated overnight on LB plates with 100 μg/mL ampicillin, 0.5 mmol/L IPTG and 40 μg/mL X-gal (Sigma, St. Louis, MO) at 37 ℃. The clones were subcultured in LB media containing 50 μg/mL ampicillin with shaking at 37 ℃. Plasmid DNA was purified, digested with Not I and EcoR I, and run on 1% agarose electropheresis. Clones with 1177 bp DNA Not I-EcoR I fragment were positive clones, contained IL-6 cDNA. MCF-7 cells (2.5×10^5/well) were placed in 6-well plates and grown overnight at 37 ℃. Culture medium was aspirated, and the MCF-7 cells were transfected with pCI-neo-IL-6 recombinant DNA or pCI-neo DNA with TransFast™ Transfection Reagent (Promega, Madison, WI). After incubation for 48 h in alpha-MEM supplemented media, the cells were removed with trypsin-EDTA and then plated in 24-well plates (2×10^4 cells/well) in selective media containing 150, 300, 600 and 1200 μg/mL G418 (Life Technologies, Inc. Frederick, MD), respectively. Non-transfected MCF-7 cells were used as the control. The selective media were replaced every 3 to 4 days for the next 2—3 weeks. The optimal G418 concentration was generally the amount that induced cell death in > 90% of nontransfected cells within 5—7 days. Pools of stable transfectants were maintained in the presence of the optimal concentration of G418.

1.3 Enzyme linked immunosorbent assay (ELISA) We used ELISA to determine the secretion into the media of IL-6 and TAAs (CA15-3, CEA and CA125) from non-transfected MCF-7 cells and stable transfected MCF-7 cells. Non-transfected and stable transfected MCF-7 cells (2×10^5 cells/well) were subcultured in 6-well cell cul-

ture plates and incubated for 72 h. Culture media was collected to quantitate IL-6 and TAAs. The concentrations of IL-6 and CA15-3 in culture media were examined with CytElisa™ IL-6 ELISA kit (SYTImmune Science, Inc., College Park, MD) and CA15-3 ELISA test kit (Endocrine Technologies, Inc, San Francisco, CA), respectively. For CEA and CA125 determination by ELISA, murine anti-human monoclonal CEA or CA125 antibodies (Fitzgerald Industries, Concord, MA) were diluted in 0.05 mol/L, pH 9.6, carbonate-bicarbonate buffer and placed at 96-well plate overnight at room temperature for coating protein. The rabbit anti-human CEA and CA125 antibodies were purchased from Fitzgerald Industries (Concord, MA). Cell culture supernatants were added to wells which were pre-coated with murine monoclonal IL-6, CA15-3, CEA or CA125 antibodies, and followed by addition of specific rabbit anti-human polyclonal IL-6, CA15-3, CEA or CA125 antibodies, respectively. The 96-well plates were incubated for 3 h at room temperature and washed. Then the wells were covered with goat anti-rabbit polyclonal antibody conjugated with alkaline phosphatase and incubated for another 45 min at room temperature and washed again. Finally the mixed Color Reagent A and B was added to the plate and the optical density (*OD*) was measured. The standard curves demonstrated a direct relationship between *OD* and IL-6, CA15-3, CEA and CA125 concentrations. The concentrations of IL-6, CA15-3, CEA and CA125 in media were determined from the standard curve, and the amount of IL-6, CA15-3, CEA and CA125 per 10^6 cells was calculated. The experiments were repeated three times independently.

1.4 Statistics All data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Differences in cell growth and protein secretion were evaluated for statistical significance by paired *t*-test. Statistical significance was considered as $P < 0.05$.

2 RESULTS

2.1 IL-6 cDNA-transfected MCF-7 cells secrete increased levels of IL-6 MCF-7 cells were transfected with pCI-neo or pCI-neo-IL-6 DNA. Non-transfected and transfected MCF-7 cells were incubated in media containing a series of concentrations of G418. After three weeks, >95% of the nontransfected MCF-7 cells were killed by 600 μg/mL of G418. However, MCF-7 cells containing pCI-neo or pCI-neo-IL-6 DNA grew well in 600 μg/mL or even higher concentrations of G418. Pools of these transfected cell clones were subcultured in flasks and maintained in medium containing 600 μg/mL of G418. We found that secretion of IL-6 in media of pCI-neo-transfected MCF-7 cells (19.6±3.0 pg/10^6 cells) was similar to that of non-transfected MCF-7 cells (25.4±4.6 pg/10^6 cells) at 72 h incubation ($P > 0.05$, paired *t*-test). However, the amount of IL-6 in media of pCI-neo-IL-6-transfected MCF-7 cells (338.5±22.6 pg/10^6 cells) was significantly higher than that of non-transfected and pCI-neo-transfected MCF-7 cells ($P < 0.01$, respectively, paired *t*-tested). Furthermore, pCI-neo-IL-6-transfected MCF-7 cells were continuously cultured in medium containing 600 μg/mL of G418 for 10 weeks and these cells still maintained high levels of IL-6 secretion (276.7±25.2 pg/10^6 cells).

2.2 IL-6 cDNA transfection increased the expression of TAAs by MCF-7 cells After 72 h incubation, the level of CA15-3 secreted by pCI-neo-IL-6-transfected MCF-7 cells (14.90±2.31 U/10^6 cells) was significantly higher than that of the parental MCF-7 cells (6.16±0.35 U/10^6 cells) and pCI-neo-transfected MCF-7 cells (3.40±0.70 U/10^6 cells) ($P < 0.05$ and $P < 0.05$, respectively, paired *t*-test). Also, pCI-neo-IL-6-transfected MCF-7 cells produced significantly increased amounts of CEA (1.84±0.20 ng/10^6 cells) compared with parental MCF-7 cells (0.60±0.05 ng/10^6 cells) and pCI-neo-transfected MCF-7 cells (0.67±0.21 ng/10^6 cells) ($P < 0.05$, respectively, paired *t*-test) (Table 1). Table 1 also showed that CA125 secretion of pCI-neo-IL-6-transfected MCF-7 cells (194.0±24.6 U/10^6 cells) was significantly higher than that of parental MCF-7 cells (93.0±11.8 U/10^6 cells) and pCI-neo-transfected MCF-7 cells (129.3±27.5 U/10^6 cells) ($P < 0.05$, respectively, paired *t*-test).

Table 1 Effect of exogenous IL-6 cDNA-transfection on secretions of CA15-3, CEA and CA125 of MCF-7 cells ($\bar{x} \pm s$, $n = 3$)

Cells	CA15-3 (U/10^6 cells)	CEA (ng/10^6 cells)	CA125 (U/10^6 cells)
MCF-7	6.2±0.4	0.60±0.05	93±12
MCF-7(pCI-neo)	3.4±0.7[b]	0.67±0.21	129±28
MCF-7(pCI-neo-IL-6)	14.9±2.3[bc]	1.84±0.20[bc]	194±25[bc]

Cells were incubated for 72 h, and tumor-associated antigens in media were measured with ELISA. [b]$P < 0.05$, compared with MCF-7 cells; [c]$P < 0.05$, compared with MCF-7 cells transfected with vector pCI-neo.

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2.3 IL-6Ab inhibited the expression of TAAs in breast cancer cells IL-6Ab suppressed the growth of MCF-7 cells and pCI-neo-IL-6-transfected MCF-7 cells in a dose-dependent manner (Fig 1). After MCF-7 cells and IL-6-transfected MCF-7 cells were incubated in media containing specific IL-6Ab for 72 h, the media was collected and CA15-3, CEA and CA125 were measured in the media by ELISA. We found that secretions of CA15-3, CEA and CA125 were significantly inhibited by the IL-6Ab in a dose-dependent manner (Fig 2 and Table 2).

Table 2 Effect of IL-6Ab on secretion of CA15-3, CEA and CA125 of non-transfected and IL-6-transfected MCF-7 cells($\bar{x}\pm s$, $n=3$)

IL-6Ab (μg/mL)	MCF-7 cells			MCF-7 cells(IL-6-transfected)		
	CA15-3 (U/10^6 cells)	CEA (ng/10^6 cells)	CA125 (U/10^6 cells)	CA15-3 (U/10^6 cells)	CEA (ng/10^6 cells)	CA125 (U/10^6 cells)
Control	6.17±0.35	0.60±0.05	93.0±11.8	14.90±2.31	1.84±0.20	194.0±24.6
4.7	5.87±0.51	0.52±0.09	88.0±2.7	13.83±1.26	1.81±0.18	181.3±14.0
18.8	4.57±1.11	0.44±0.04	81.0±3.6	13.17±0.76	1.77±0.16	172.7±9.5
75	3.87±0.51[b]	0.35±0.09	53.3±7.6[b]	10.17±1.26	1.38±0.13	151.3±10.3
300	2.83±0.47[b]	0.24±0.05[c]	28.0±7.0[c]	7.47±1.50[b]	1.03±0.18[b]	100.7±5.1[b]

Cells were cultured for 72 hours and CA15-3, CEA and CA125 in media were measured with ELISA. [b]$P<0.05$, [c]$P<0.01$, Compared with control

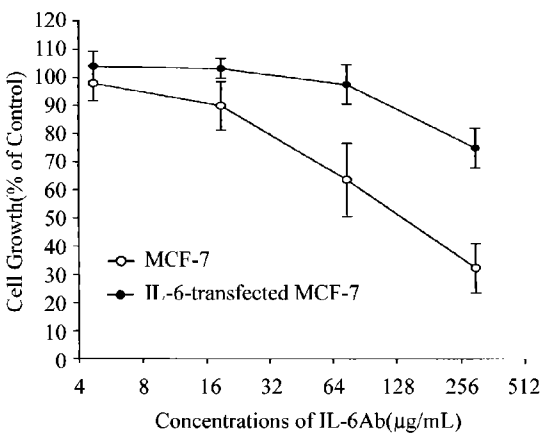

Fig 1 Effect of IL-6 Ab on the growth of non-transfected and IL-6-transfected MCF-7 cells

Cells were incubated for 72 hours and cell number was counted with trypan blue staining.

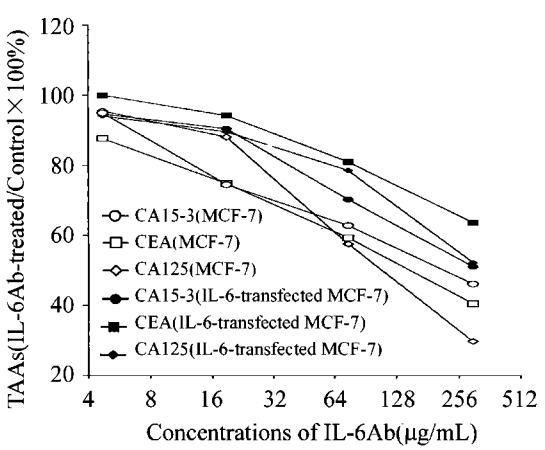

Fig 2 Effect of IL-6 Ab on secretion of TAAs of non-transfected and IL-6-transfected MCF-7 cells

Cells were incubated for 72 hours and TAAs in media were measured with ELISA.

3 DISCUSSION

Tumor cells secrete many different growth factors. The secreted growth factors may create paracrine or autocrine loops in the local microenvironment of the tumor to drive expansion of the tumor mass and/or may be released into the circulatory system to function systemically. Increased serum IL-6 levels have been found in patients with a variety of human carcinomas. A high concentration of serum IL-6 is associated with aggressive tumors, poor disease-free and overall survival[2-12,33]. We have previously reported that the level of serum IL-6 in breast cancer patients was reduced after vaccination with tumor-associated antigens[33]. IL-6 is involved in cancer progression through a variety of mechanisms, such as decrease in cancer cell adhesion, increase in cancer cell proliferation and motility, induction of thrombopoiesis, and stimulation of tumor specific antigen expression[13-18]. Badache reported that IL-6 inhibited the proliferation of the T47D breast cancer cells but increased migration of T47D cells[34]. In the present study, although transfection with exogenous IL-6 gene could not stimulate MCF-7 cell growth, neutralization of IL-6 by specific IL-6Ab resulted in inhibition of MCF-7 cell growth (Fig 1). These results suggest that IL-6 is a growth factor that maintains the growth and proliferation of MCF-7 breast cancer cells. Numerous studies have shown that interferon-gamma can enhance the expression of CEA and HLA on tumor cell surfaces[35-38], and in a similar manner IL-6 can increase CEA and HLA

expression in human colorectal carcinoma cells[15-18]. Furthermore, Tsang reported that transfer of the IL-6 gene into a human colorectal carcinoma cell line resulted in enhancement of CEA expression[20]. We preciously found addition of IL-6 in culture media increased the expression of CA153, CEA and CA125 of MCF-7 cells (data not shown). In the present study, transfection of IL-6 gene into MCF-7 cells significantly enhanced the secretion of CEA, and further that neutralization of IL-6 by specific IL-6 antibody reduced CEA expression. The augmentation of CEA and HLA expression could improve the immunogenic of tumor cells, and thus IL-6 might be a useful adjuvant in immunotherapy. However, the induction of CEA could also be associated with the promotion of metastasis, especially liver metastases in colorectal carcinoma[21-25]. In addition, clinical evidence has shown that a high concentration of serum IL-6 is associated with aggressive tumors, poor disease-free and overall survival[2-12, 19]. For example, Belluco et al reported that high blood concentrations of IL-6 were associated with high circulating CEA and advanced disease stage, and that an IL-6 concentration more than 10 pg/mL was an independent negative prognostic indication for survival in colorectal carcinoma[19]. Therefore, IL-6 would not be an appropriate adjuvant for immunotherapy, specifically in cancer vaccines. We have previously reported that vaccination with an autogenous and allogeneic breast cancer cell vaccines containing TAAs resulted in immune and clinical responses in breast cancer patients[33, 39]. To improve the immunogenicity of our whole cell cancer vaccine, we could transfer IL-6 gene into cancer cells to enhance CA15-3, CEA and CA125 expression of autogenous (patient) and allogeneic (MCF-7) breast cancer cells *in vitro* culture.

CA15-3 is a polymorphic epithelial mucin (MUC1) defined by the DF3 monoclonal antibody. Clinical studies have shown that elevated serum CA15-3 is a negative prognosis factor in a variety of cancers, such as breast cancer, cholangiocarcinoma and endometrial carcinomas[27-30]. MUC1 overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components and cytotoxic lymphocyte-target cell interaction[40, 41], but its exact function remains to be determined. At the molecular level, the MUC1 promoter contains a candidate binding site for transcription factors of the STAT family approximately 500 bp upstream of the transcription start site. Cytokines and/or growth factors such as IL-6 or interferon-gamma can activate STATs. In the human breast carcinoma cell line T47D, IL-6 and interferon-gamma are both able to stimulate transcription of a luciferase reporter gene under the control of a 750 bp MUC1 promoter fragment proximal to the transcription start site[42]. MUC1 expression in human normal breast epithelia and breast cancer cells is stimulated by interferon-gamma and tumor necrosis factor-alpha[43]. However, the effects of IL-6 on MUC1 or CA15-3 have not been reported previously. In the present study, transfection of exogenous IL-6 gene into MCF-7 cells resulted in significant increase in CA15-3 expression. Neutraliztion of IL-6 by specific IL-6 antibody significantly reduced the CA15-3 expression in both MCF-7 cells and IL-6 cDNA-transfected MCF-7 cells (Table 2).

Serum CA125 is a significant prognostic factor in a variety of carcinomas, especially ovarian cancer[31, 32, 44, 45]. Increased levels of serum CA125 and IL-6 are often associated in cancer patients[45, 46], but the effect of IL-6 on CA125 expression has not been demonstrated previously. In the current study, we observed that introduction of exogenous IL-6 gene into MCF-7 cells caused significant enhancement of CA125 expression.

In summary, transfer of IL-6 gene enhances the expression of CA15-3, CEA and CA125 of MCF-7 cells *in vitro*. Although augmentation of TAA expression may improve the immunogenicity of tumor cells, stimulation of CEA may be associated with the promotion of metastasis *in vivo*. Clinical evidence has also shown that elevated serum IL-6, CA15-3, CEA and CA125 are negative prognostic indicators in cancer patients. Therefore, IL-6 would not be an appropriate adjuvant for vaccine immunotherapy. However, we can use IL-6 gene or protein to augment the antigen expression of autogenous and allogeneic breast cancer cells *in vitro*, and therefore improve the immunogenicity of whole breast cancer cells used in cancer vaccines.

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转染 IL—6 基因增强 MCF-7 人乳腺癌细胞肿瘤相关抗原的表达

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目的: 研究 IL-6 影响癌症预后的机制和 IL-6 与乳腺癌肿瘤相关抗原 CA15-3, CEA 和 CA125 的关系。方法: 转染外源 IL-6 基因到 MCF-7 细胞株, 用酶联免疫吸附法(ELISA)定量培养液中细胞分泌的 CA15-3, CEA 和 CA125。结果: 在 72 h 培养后, 成功转染 pCI-neo-IL-6 质粒的 MCF-7 细胞分泌 IL-6 (338.5±22.6) pg/10^6细胞 明显高于没有转染的细胞(25.4±4.6) pg/10^6细胞 ($P<0.01$) 和无 IL-6 基因的质粒 pCI-neo 转染的细胞(19.6±3.0) pg/10^6 细胞 ($P<0.01$)。pCI-neo-IL-6 转染的 MCF-7 细胞分泌 CA15-3, CEA 和 CA125 同样明显高于没有转染的细胞和空载体 pCI-neo 转染的 MCF-7 细胞。特异性 IL-6 抗体降低 CA15-3, CEA 和 CA125 在 MCF-7 细胞和 IL-6 cDNA 转染的 MCF-7 细胞中表达。结论: 转染 IL-6 基因可增强人乳腺癌细胞肿瘤相关抗原的表达, 高 IL-6 血症的乳腺癌病人预后不良部分原因可能是高 IL-6 刺激癌细胞表达肿瘤相关抗原。

关键词 乳腺癌; 肿瘤相关抗原; 白介素-6; 基因转染

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