EGFR反义RNA的转染对人类鼻咽癌CNE-2细胞EGFR的表达下调及恶性表型的抑制

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with relative molecular weight of 17 000, consisting of extracellular ligand binding domain, transmembrane domain and intracellular tyrosine-kinase domain. A large number of ligands for EGFR have been characterized, including epidermal growth factor (EGF), transforming growth factorα(TGFα) and amphiregulin. Binding of these ligands to extracellular domain of EGFR initiates a variety of biological responses, resulting in the activation of mitogenic signal transduction pathway to maintain balanced proliferation[1][2].

Several investigations have shown that amplification and/or overexpression of EGFR gene may be associated with the growth rate and malignancy of the tumors, which in turn has prognostic significance in patients with these tumors[3], suggesting that receptor-directed therapies may be useful as anticancer strategies. Although EGFR down-regulation by antisense EGFR cDNA transfection has been demonstrated in human breast cancer[4][5], rhabdomyosarcoma[6], colon cancer[7], non-small-cell lung cancer[8], and glioma cells[9], no such efforts have been attempted in human nasopharyngeal carcinoma cells.

Nasopharyngeal carcinoma (NPC) is one of the most frequent cancers in south China. Although Epstein-Barr virus has been shown to be the likely etiologic agent on nasopharyngeal carcinogenesis, the molecular pathogenesis of NPC remains to be elucidated. The first insight into the potential role of EGFR in NPC progression came from the observations of a monoclonal antibody against EGFR, which significantly inhibited the growth and tumorigenesis of human NPC cells both in vitro and in vivo[10]. In this study, we transfected a plasmid containing a 1.35-kb gene fragment encoding the N-terminal fragment of human EGFR cDNA in the 3' to 5' orientation into NPC CNE-2 cell line with high EGFR expression and examined the effects of down-regulated EGFR expression on the growth behavior and tumorigenesis of the cells in nude mice.

MATERIAL AND METHODS

Cell lines and plasmids

Human nasopharyngeal carcinoma CNE-2 cells[11] were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO/BRL). A 1.35-kb BamH1 fragment containing the extracellular region for EGFR derived from a full-length 4.0-kb human cDNA was cloned

in the reverse 3' to 5' orientation into the pLXSN retrovirus vector as we described previously[4].

Plasmid transfection and production of stable cell lines

The antisense EGFR expression vector pLXSN- AS5 and pLXSN control vector were respectively transfected into CNE-2 cells via lipofectamine (GIBCO/ BRL) according to the manufacturer's instructions. Several G418-resistant clones were subsequently isolated and expanded. The stable integration and structural integrity of the plasmid DNA were examined by PCR as we described previously[4], and the selected clones of the cells with antisense EGFR expression vector transfection were designated as CNE-2/AS4 and CNE-2/AS8 respectively; pLXSN vector-transfected cells were designated as CNE-2/pLXSN.

^{125}I-EGF ligand binding assay

This assay was performed as described previously[12]. The parental cells or transfected cells (CNE-2/pLXSN,CNE-2/AS4, and CNE-2/AS8) were plated at 2×10^5/ well into 24-well Nunc dishes and allowed to grow overnight. The confluent cultures were placed the following day on ice and washed once with wash buffer (containing 0.01% Mg^{2+} and 0.005% Ca^{2+} plus 1 mg/ml BSA of PBS) pre-cooled to 4 ℃, prior to the addition of 1 ml unlabeled cold EGF (2.5-500 ng/ml) in wash buffer into the triplicate dishes and of ^{125}I-EGF (9×10^4 cpm) into each well. The culture dishes were then rocked gently on ice for 4 h, the cells washed 5 times with the wash buffer, lysed with RRA lysis buffer (HEPES pH7.3, 20 mmol/L, 10% glycerol, 1% TritonX-100) and counted in gamma counter. The results were underwent computer program LIGAND analysis.

In virto cell growth assay

The parental cells or transfected cells were seeded at 1×10^4 cells/well and grown in triplicate 35-mm dishes for 8 d. The cells were harvested and counted by trypan blue exclusion on day 0, 2, 4, 6, and 8, respectively.Anchorage-independent growth assay

Soft agarose assay was performed as described previously[7]. In brief, 1×10^3 cells/well were plated on 0.35% low-melting-point agarose in RPMI 1640 containing 15% fetal bovine serum. The colonies were scored under a magnifying glass after 10 days. Each soft agarose assay was done in triplicate.

Tumorigenicity and metastasis in nude mice

The parental cells or transfected cells were injected subcutaneously (1×10^6 cells) into 6 female nude mice (4-5 weeks old). Mice were examined twice a week and the tumor size was measured using a caliper. Tumor volumes (mm3) were calculated according to the formula: V=length×(width)2/2[13]. After 77 days, the mice were killed and the tumor, lung and lymph node were removed respectively, fixed in neutral buffer formalin, embedded in paraffin, and sectioned for histological analysis.

RESULTS

Effect of EGFR antisense RNA expression on EGFR expression levels and binding affinity

After transfection of CNE-2 cells with the EGFR antisense RNA expression vector or the empty vector, G418-resistant clones were identified and isolated. Genomic DNA was extracted from the 4 selected CNE-2/AS clones, 1 CNE-2/pLXSN clone and the parental CNE-2 cells. A 433-bp fragment of integrated neo gene sequence was detected in the transfected

CNE-2 cells, but not in the parental CNE-2 cells by PCR analysis (data not shown). Two representative CNE-2 clones CNE-2/AS4 and CNE-2/AS8 were reserved for later experimentation.

^{125}I-EGF binding and Scatchard analysis showed that the number of EGFR was reduced by 18% and 45% in the two selected clones (CNE-2/AS4, $P<0.05$; CNE-2/ AS8, $P<0.01$) respectively, whereas the clones transfected with the empty vector (CNE-2/pLXSN) had similar EGFR levels to those of the parental cells ($P>0.05$). However, the binding affinity of EGFR to the ligand was not significantly affected by EGFR antisense RNA expression (Tab 1).

Tab.1 Binding of ^{125}I-EGF to the parental and transfected CNE-2 clones (*Mean±SD*)

Cell line	Receptor No./cell	Binding affinity (Kd, nM)
CNE-2	67 658±2 700	2.50±0.29
CNE-2/pLXSN	62 273±1 900*	2.39±0.18*
CNE-2/AS4	55 790±620**	2.13±0.50*
CNE-2/AS8	37 512±430***	2.07±0.47*

*$P>0.05$, **$P<0.05$, ***$P<0.01$. The binding sites and binding affinity (ligand dissociation constant, Kd) were determined by Scatchard analyses. Student's t test was used for comparison between transfected clones and the parental cells.

Effect of EGFR antisense RNA on cell proliferation

In order to examine whether anti-sense EGFR RNA suppresses the growth of the tumor cells in vitro, we plated 1×10^4 CNE-2 cells in 35-mm plastic dishes containing RPMI 1640 supplemented with 10% FBS. The number of viable cells in each dish was counted every other day. As shown in Fig.1, the proliferation rate significantly decreased in the transfected clones of CNE-2 cells expressing EGFR antisense RNA, as compared with that of the parental cells or CNE-2 cells transfected with the empty vector. A reduction by 33% and 55% in the cell number occurred 8 d after the plating of the CNE-2/AS4 and CNE-2/AS8 cells respectively (Fig.1). These results suggested that the extent of growth inhibition in EGFR antisense RNA-tranfected clones as compared with the parental cells was in proportion to the degree of EGFR expression that was blocked.

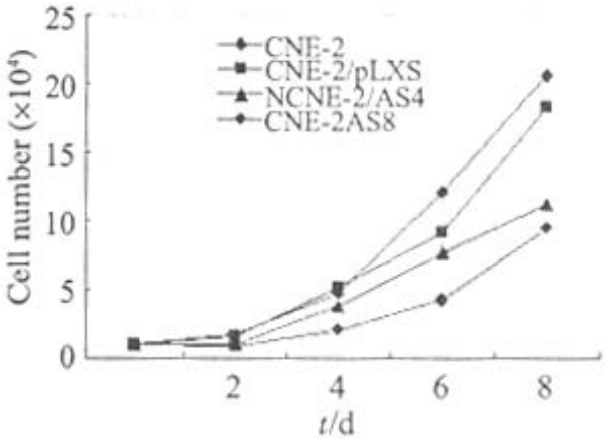

Fig.1 In vitro growth inhibition of CNE-2 cells transfected with EGFR antisense cDNA vector compared with the parental cells or the cells transfected with the empty vector

Effect of EGFR antisense RNA on CNE-2 cell growth on soft agarose

The parental and transfected clones were examined for their colony-forming ability in soft agarose medium, which was an accepted criterion for transformation capacity assessment. As shown in Fig.2, the growth of CNE-2/AS4 or CNE-2/AS8 clones was inhibited by 40%-50% in comparison with the parental and CNE-2/pLXSN control clones. In addition, as expected, CNE-2/AS4 and CNE-2Z/AS8 cells formed smaller and less colonies on soft agarose than the parental and empty vector- transfected cells (data not shown). These results suggest that EGFR antisense RNA can suppress in vitro the tumorigenicity of CNE-2 cells.

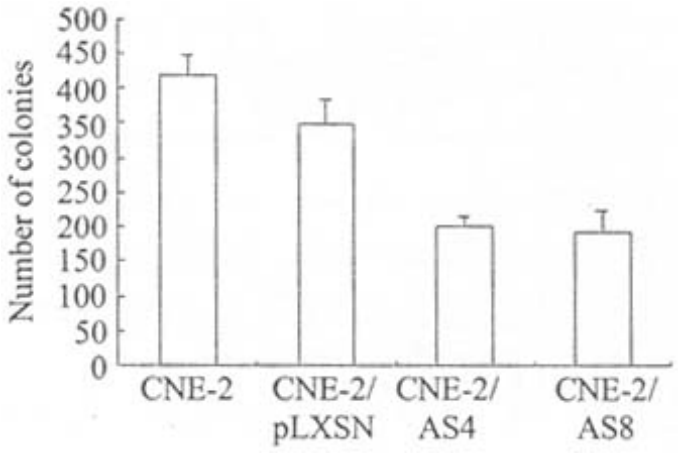

Fig.2 Growth inbibition of CNE-2/as4 and CNE-2/AS8 celle in soft agarose

Tumorigenicity and metastasis in nude mice

The tumorigenicity of the parental and transfected clones was examined in 6 female nude mice by subcutaneous injection. As shown in Tab.2, all mice injected with CNE-2 cells and CNE-2/pLXSN cells rapidly developed aggressively growing tumor nodules at the site of inoculation after 3-5 d. However, EGFR antisense RNA-expressing clones showed a longer latency period (9-11 d) and slower tumor growth rate compared with those of the parental cells and CNE-2/pLXSN clones 1 month after the inoculation (Fig.3).

Tab.2 Tumorigenicity and metastasis analysis of CNE-2 cell line transfected with antisense EGFR cDNA

Cell line	Nude mice	Tumor formation (days after inoculation)	Lung metastatsis/total lung lobes	Lymph node metastasis / total number of lymph nodes
CNE-2	6	3-5	4/25(16%)	28/39(71.8%)
CNE-2/pLXSN	6	4-5	3/25(12%)	22/44(50.0%)
CNE-2/AS4	6	9-11	0/30(0%)	19/43(44.2%)
CNE-2/AS8	6	10-11	2/30(6.6%)	24/42(57.1%)

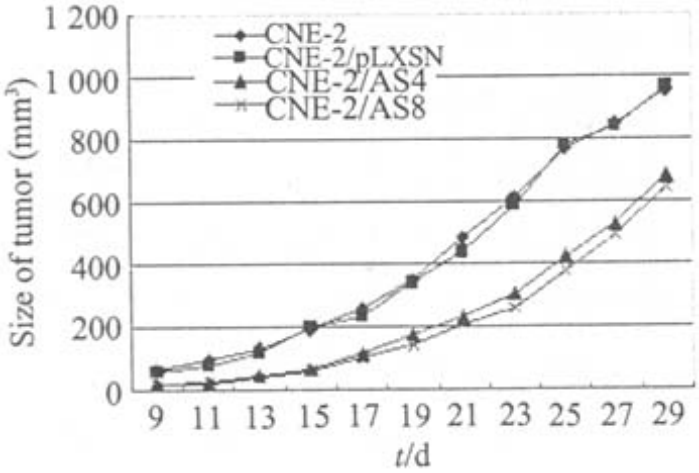

Fig. 3 Inhibitory effect of EGFR antisense RNA expression on tumor growth in nude mice

To investigate the effect of antisense EGFR RNA on the metastasis of the tumors in vivo, all mice were killed for histopathological examination on day 77 after injection, and the lungs and lymph nodes were removed. The sections obtained for histological analysis showed that CNE-2/AS4 cells had no lung metastasis with lower rate of lymph node metastasis than the parental and CNE-2/pLXSN cells, while CNE-2/AS8 cells had significantly different lymph node metastasis from that of the parental cells (Tab.2).

DISCUSSION

Overexpression of the epidermal growth factor receptor is frequent in human carcinoma, often accompanied by the expression of autocrine or paracrine transforming growth factor α, which results in the activation of EGFR tyrosine kinase and phenotype transformation [13]. CNE-2 cell line was established from biopsy of a poorly differentiated and moderately metastatic nasopharyngeal carcinoma[11]. Previous investigations have demonstrated that EGFR overexpression associates with the growth and tumorigenesis of CNE-2, which can be significantly inhibited by a monocolonal antibody against EGFR[10], and we consequently chose this cell line to study the effect of EGFR antisense RNA expression.

Previous therapeutic strategies designed to interfere with EGFR signal transduction have used antisense oligonucleotides[15], antisense RNA[16][9][8] or the antibodies against EGFR[17][18][19]. In this study, by an antisense RNA approach, we established CNE-

2/AS cells (CNE-2/AS4, CNE-2/AS8) in which antisense RNA was generated by a sequence that was stably incorporated into the genome for consistent expression of the transcript. EGFR antisense RNA expression could significantly down-regulate the number of EGFR on the surface of the two CNE-2 cells transfected with EGFR antisense cDNA vector, as compared with the parental and CNE-2/pLXSN cells. However, the binding affinity of the EGFR for ligand is not significantly affected by antisense EGFR RNA. These results are essentially in accordance with the previous finding of Chakrabarty et al[7], who demonstrated that a 31% reduction in the number of high-affinity cell surface EGFR and a 68% reduction in low-affinity cell-surface EGFR occurred in the human colon carcinoma Moser cells transfected with EGFR antisense cDNA vector, but the binding affinity of the EGFR for ligand remianed unchanged.

The growth rate in vitro and the ability to grow in soft agarose of these two EGFR antisense vector-transfected cells were also significantly reduced. After injection into nude mice, EGFR antisense transfectants showed a longer latency period, slower tumor growth and lower lymph node and lung metastasis rates in comparison with the parental cells. In addition, tumor growth and tumorigenesis were proportional to the degree of EGFR expression inhibition in cells transfected with EGFR antisense cDNA vectors. However, complete inhibition of the cell growth and tumorigenesis either in vitro or in vivo was not observed. A similar result was obtained by Moroni et al[16], who demonstrated that the inhibition of the phenotype was directly proportional to the residual amount of EGFR expressed in human epidermoid carcinoma KB cell with transfection of EGFR antisense RNA constructs.

Antisense RNA strategy have been successfully utilized to reduce the expression of a number of mammalian genes, including those for growth factors or related receptor genes [20][2][21]. This is the first report to address the effect of EGFR antisense plasmid transfection in inhibiting the tumoregenicity and metastasis of human NPC cells, thereby providing a valuable target for therapeutic intervention of tumors with EGFR overexpression.

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REFERENCES

[1] Khazaie K. EGFR in neoplasia and metastasis[J]. Cancer Metastasis Rev, 1993, 12 (3-4): 255-74.

[2] Mishima K, Asai A, Sugiyama A. Increased expression of schwannoma-derived growth factor (SDGF) mRNA in rat tumor cells: involvement of SDGF in the growth promotion of rat gliomas[J]. Int J Cancer, 1996, 66(3): 352-7.

[3] Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers[J]. Br Med Bull, 1991, 47(1): 87-98.

[4] Fan WH, Lu YL, Deng F, et al. EGFR antisense RNA blocks expression of the epidermal growth factor receptor and partially reverse the malignant phenotype of human breast cancer MDA-MB-231 cells[J]. Cell Research, 1998, 8(1): 63-71.

[5] Dixit M, Yang JL, Poirier MC, et al. Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA[J]. J Nat Cancer Inst, 1997, 89(5): 365-73.

[6] De Giovanni C, Landuzzi L, Frabetti F, et al. Antisense epidermal growth factor receptor transfection impairs the proliferative ability of human rhabdomyosarcoma cells [J]. Cancer Res, 1996, 56(17): 3898-901.

[7] Chakrabarty S, Rajagopal S, Huang S. Expression of antisense epidermal growth factor receptor RNA downmodulates the malignant behavior of human colon cancer cells[J]. Clin Exp Metastasis, 1995, 13(3): 191-5.

[8] Fang K, Chen MH. Transfection of anti-sense complementary DNA of human epidermal growth factor receptor attenuates the proliferation of human non small cell lung cancer cells[J]. Int J Cancer, 1999, 81(3): 471-8.

[9] Tian XX, Pang JC, Zheng J, et al. Antisense epidermal growth factor receptor RNA transfection in human glioblastoma cells down-regulates telomerase activity and telomere length[J]. Br J Cancer, 2002, 86(8): 1328-32.

[10] Guan EN, Zhou TC, Wang JH, et al. Effects of monoclonal antibody anti-EGF receptor on human nasopharyngeal carcinoma cell and other cells[J]. Sci China B(Chin), 1990, 33(11): 1334-40.

[11] Tang WP, Huang PG, Shen SJ, et al. Establishment and characteristics of transplantation model of human epithelial cell strain of poorly differentiated nasopharyngeal carcinoma[J]. Cancer (Chin.) 1989, 8(4): 247-50.

[12] Lichtner RB, Kaufmann AM, Kittmann A, et al. Ligand mediated activation of ectopic EGF receptor promotes matrix protein adhesion and lung colonization of rat mammary adenocarcinoma cells[J]. Oncogene, 1995, 10(9): 1823-32.

[13] Arteaga CL, Holt JT. Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer xenografts in nude mice[J]. Cancer Res, 1996, 56(5): 1098-1103.

[14] Di Fiore PP, Pierce JH, Hazan R, et al. Over-expression of the human epidermal-growth-factor receptor confers a EGF-dependent transformed phase -type to NIH3T3 cells[J]. Cell, 1987, 51(6): 1063- 70.

[15] Wang S, Lee RJ, Cauchon G, et al. Delivery of antisense oligode-oxyribonucleotides against the human epidermal growth factor Receptor into Cultured KB cells with liposomes conjugated to folate via polyethylene Glycol[J]. Proc Nat Acad Sci (Wash.), 1995, 92(8): 3318-22.

[16] Pu P, Liu X, Liu A, et al. Inhibitory effect of antisense epidermal growth factor receptor RNA on the proliferation of rat C6 glioma cells in vitro and in vivo[J]. J Neurosurg, 2000, 92(1): 132-9.

[17] Ma L, Gauville C, Berthois Y, et al. Calvo F. Role of epidermal growth factor receptor in tumor progression in transformed human mammary epithelial cells[J]. Int J cancer, 1998, 78(1): 112-9.

[18] Divgi CR, Welt S, Kris M, et al. Phase Ⅰ and imaging trial of indium 111-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma[J]. J Natl Cancer Inst, 1991, 83(2): 97-104.

[19] Baselga J, Norton L, Masui H, et al. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies[J]. J Natl Cancer Inst, 1993, 85(16): 1327- 33.

[20] Ueno H, Sasaki K, Miyagawa K, et al. Hirai H. Antisense repression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathway but not the ras pathway in epidermal growth factor receptor signaling[J]. J Biol Chem, 1997, 272(13): 8739-43.

[21] He Y, Zeng Q, Drenning SD, et al. Inhibition of human squamous cell carcinoma growth in vivo by epidermal growth factor receptor antisense RNA transcribed from the U6 promoter[J]. J Natl Cancer Inst, 1998, 90(14): 1080-7.

REFERENCES

[1] Khazaie K. EGFR in neoplasia and metastasis[J]. Cancer Metastasis Rev, 1993, 12 (3-4): 255-74.

[2] Mishima K, Asai A, Sugiyama A. Increased expression of schwannoma-derived growth factor (SDGF) mRNA in rat tumor cells: involvement of SDGF in the growth promotion of rat gliomas[J]. Int J Cancer, 1996, 66(3): 352-7.

[3] Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers[J]. Br Med Bull, 1991, 47(1): 87-98.

[4] Fan WH, Lu YL, Deng F, et al. EGFR antisense RNA blocks expression of the epidermal growth factor receptor and partially reverse the malignant phenotype of human breast cancer MDA-MB-231 cells[J]. Cell Research, 1998, 8(1): 63-71.

[5] Dixit M, Yang JL, Poirier MC, et al. Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA[J]. J Nat Cancer Inst, 1997, 89(5): 365-73.

[6] De Giovanni C, Landuzzi L, Frabetti F, et al. Antisense epidermal growth factor receptor transfection impairs the proliferative ability of human rhabdomyosarcoma cells [J]. Cancer Res, 1996, 56(17): 3898-901.

[7] Chakrabarty S, Rajagopal S, Huang S. Expression of antisense epidermal growth factor receptor RNA downmodulates the malignant behavior of human colon cancer cells[J]. Clin Exp Metastasis, 1995, 13(3): 191-5.

[8] Fang K, Chen MH. Transfection of anti-sense complementary DNA of human epidermal growth factor receptor attenuates the proliferation of human non small cell lung cancer cells[J]. Int J Cancer, 1999, 81(3): 471-8.

[9] Tian XX, Pang JC, Zheng J, et al. Antisense epidermal growth factor receptor RNA transfection in human glioblastoma cells down-regulates telomerase activity and telomere length[J]. Br J Cancer, 2002, 86(8): 1328-32.

[10] Guan EN, Zhou TC, Wang JH, et al. Effects of monoclonal antibody anti-EGF receptor on human nasopharyngeal carcinoma cell and other cells[J]. Sci China B(Chin), 1990, 33(11): 1334-40.

[11] Tang WP, Huang PG, Shen SJ, et al. Establishment and characteristics of transplantation model of human epithelial cell strain of poorly differentiated nasopharyngeal carcinoma[J]. Cancer (Chin.) 1989, 8(4): 247-50.

[12] Lichtner RB, Kaufmann AM, Kittmann A, et al. Ligand mediated activation of ectopic EGF receptor promotes matrix protein adhesion and lung colonization of rat mammary adenocarcinoma cells[J]. Oncogene, 1995, 10(9): 1823-32.

[13] Arteaga CL, Holt JT. Tissue-targeted antisense c-fos retroviral vector inhibits established breast cancer xenografts in nude mice[J]. Cancer Res, 1996, 56(5): 1098-1103.

[14] Di Fiore PP, Pierce JH, Hazan R, et al. Over-expression of the human epidermal-growth-factor receptor confers a EGF-dependent transformed phase -type to NIH3T3 cells[J]. Cell, 1987, 51(6): 1063- 70.

[15] Wang S, Lee RJ, Cauchon G, et al. Delivery of antisense oligode-oxyribonucleotides against the human epidermal growth factor Receptor into Cultured KB cells with liposomes conjugated to folate via polyethylene Glycol[J]. Proc Nat Acad Sci (Wash.), 1995, 92(8): 3318-22.

[16] Pu P, Liu X, Liu A, et al. Inhibitory effect of antisense epidermal growth factor receptor RNA on the proliferation of rat C6 glioma cells in vitro and in vivo[J]. J Neurosurg, 2000, 92(1): 132-9.

[17] Ma L, Gauville C, Berthois Y, et al. Calvo F. Role of epidermal growth factor receptor in tumor progression in transformed human mammary epithelial cells[J]. Int J cancer, 1998, 78(1): 112-9.

[18] Divgi CR, Welt S, Kris M, et al. Phase Ⅰ and imaging trial of indium 111-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma[J]. J Natl Cancer Inst, 1991, 83(2): 97-104.

[19] Baselga J, Norton L, Masui H, et al. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies[J]. J Natl Cancer Inst, 1993, 85(16): 1327- 33.

[20] Ueno H, Sasaki K, Miyagawa K, et al. Hirai H. Antisense repression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathway but not the ras pathway in epidermal growth factor receptor signaling[J]. J Biol Chem, 1997, 272(13): 8739-43.

[21] He Y, Zeng Q, Drenning SD, et al. Inhibition of human squamous cell carcinoma growth in vivo by epidermal growth factor receptor antisense RNA transcribed from the U6 promoter[J]. J Natl Cancer Inst, 1998, 90(14): 1080-7.

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