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Full Length Research Paper

Construction of a high-EGFR expression cell line and its biological properties comparing with A431 cell

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Targeted screening of EGFR compounds has become one of the medical research focuses for tumor therapy. A431, which naturally expresses high levels of EGFR, was compared with the stably high expressing EGFR cell line HEK293. Flow cytometry was used to analyze cell growth and Western blot was used to determine expression of EGFR, p-EGFR and p-ERK. Cellular membrane chromatography analysis revealed longer on-peak chromatographic and greater on-peak area of A431. Results demonstrated that the high-expression EGFR cell line A431 improved sensitivity of cellular membrane chromatography when screening drugs targeted to EGFR.

Key words: High-EGFR expression, drug-targeted screening, A431 cell line and Taspine.

INTRODUCTION

The human epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular domain that binds EGF, TGF-α, amphiregulin, betacellulin, heparin-binding EGF and epiregulin (EPR) (Pinaki et al., 2007). It is one of the best characterized targets of cancer cells and plays an important role in regulating cellular proliferation, differentiation and survival of normal epithelial tissues. EGFR belongs to the ErbB/HER family, along with HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4). EGFR has a cytoplasmic domain with intrinsic protein-tyrosine kinase activity. Ligand binding induces autophosphorylation and the formation of homo- or hetero-dimers with other members of the family. Receptor phosphorylation mediates activation of the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3 kinase)/AKT, phospholipase C gamma (PLC-γ)/protein kinase C (PKC), and the signal transducer and activator of transcription (STAT) pathways, which regulate cell transformation, proliferation, and survival (Cao et al., 2008; Yarden et al., 2001).

Previous studies demonstrated that EGFR over-expression in tumors often correlates with poor prognosis and resistance to therapy (Newby et al., 1997). Common cancer-related EGFR alterations include overexpression, mutation/truncation and activation by excessive autocrine growth factor expression or heterodimerization with other ErbB family members. EGFR deregulation contributes to proliferation, transformation, angiogenesis, invasion, metastasis and inhibition of apoptosis in cancer cells (Arteaga, 2002; Barker et al., 2001).

Treatments against EGFR are the most successful molecule-targeted drugs for cancer treatment. Pervious studies showed that the drug gefitinib (Iressa), which is an inhibitor of EGFR tyrosine kinase, has been shown to suppress activation of EGFR signaling for survival and proliferation in non-small cell lung cancer (NSCLC) cell lines. Some reports have demonstrated rapid down-

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Abbreviations: EGFR, Epidermal growth factor receptor; **EPR,** epiregulin; **MAPK,** mitogen-activated protein kinase; **PKC,** protein kinase C; **PLC-γ,** phospholipase C gamma; **STAT,** Signal transducer and activator of transcription; **NSCLC,** non-small cell lung cancer; **EDTA,** ethylene diamine tetraacetic acids; **RT-PCR,** reverse transcriptase- polymerase chain reaction; **FBS,** fetal bovine serum; **ECL,** enhanced chemiluminescence; **CMSP,** cell membrane stationary phase; **PBS,** phosphate buffer saline; **HEK293,** human embryonic kidney 293 cells.

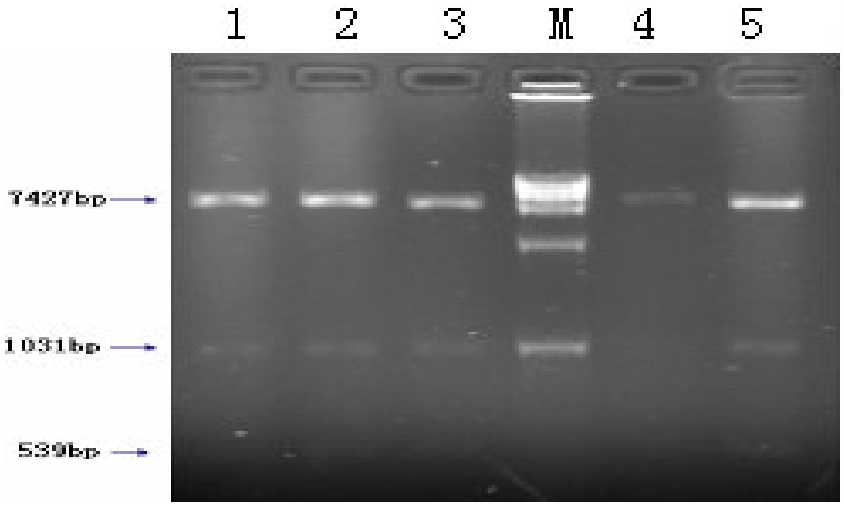

Figure 1. BamH1 digestion for plasmid detection.

regulation of ligand-induced EGFR in a gefitinib-sensitive cell line and inefficient down-regulation of EGFR in a gefitinib-resistant cell line during the exponential growth phase. This implies that each cell type employs a different down-regulation mechanism. However, the mechanisms of drug sensitivity to gefitinib remain unclear (Baselga et al., 2000; Arnoletti et al., 2004; Chang et al., 2008; Sordella et al., 2004; Paez et al., 2004; Lynch et al., 2004).

Although some drugs that inhibit EGFR have been used in the clinic, problems still remain. There is a need for more specific and sensitive methods to screen for drugs that target EGFR.

The present study established a human wildtype-EGFR eukaryotic vector and obtained cell lines with stable and high-expression of EGFR. Results demonstrated that EGF media induced faster proliferation with increasing EGFR expression, most likely due to influences of the intracellular MAPK pathways. The A431 cell line, which expresses the wildtype EGFR gene, is a naturally occurring, high expressing EGFR cell line, which was compared with the prepared high-expression HEK293-EGFR cell line. The gene sequence and encoding amino acids were basically identical and some of the biological characteristics were similar. Cellular membrane chromatography results, using the positive control drugs erlotinib, dasatinib, gefitinib, sorafenib, sunitinib, imatinnib and taspine, demonstrated that A431 improved specificity and sensitivity of target drug screening to EGFR. Accordingly, the A431 cell line could be a useful tool for targeted drug screening.

MATERIALS AND METHODS

Cell culture and reagents

HEK293 cells, a kind gift from Professor Yuan Bingxiang (School of Medicine, Xian Jiaotong University), were maintained in DMEM (Gibco/Invitrogen, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) and penicillin/streptomycin (Gibco/Invitrogen), and were incubated at 37℃ in a 5% CO_2 incubator with saturated humidity. The HEK293 and transformed EGFR cell lines were maintained in DMEM supplemented with G418 for screening and were incubated in identical conditions.

A431 cells (human epidermoid carcinoma) were a kind gift from Professor Li Xu, who obtained them from the ATCC (American Type Culture Collection, USA). The cells were maintained in DMEM (Gibco/Invitrogen) supplemented with 10% fetal calf serum and were incubated at 37℃ in a 5% CO_2 incubator.

The control drugs erlotinib, dasatinib, gefitinib, sorafenib, sunitinib and imatinib were purchased from AnGe Pharmaceutical Company, China and were diluted in fresh media prior to each experiment. The final DMSO concentration was < 0.1%.

Construction of a EGFR eukaryotic expression vector

The plasmid pBabe-Puro EGFR WT, which contains full-length EGFR cDNA, was a kind gift from Professor Heidi Greulich (Department of Medical Oncology, Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA). EGFR was amplified from a pBabe-Puro EGFR WT with PCR primers, 5'-CGGGGTACCA TGCGACCCTCCGGGAC-3' and 5'-GCTCTAGATCATGCTCCAAT AAATTC-3', (the underlined sequences contained restriction enzyme sites for KpnI and XbaI, respectively), and was digested with KpnI and XbaI. The thermocycling conditions were 35 cycles of denaturation at 98℃ for 10 s, annealing at 55℃ for 15 s and extension at 72℃ for 4 min, followed by a final 5-min extension at 72℃. The digested fragment was inserted into the same sites of pcDNA3.1 (+). The construction plasmid pcDNA3.1 (+)-EGFR was digested with BamH1 for detection (Figure 1). All sequences, with exception to two base pairs, were verified by DNA sequencing using the PRISM BigDye Terminator Sequencing Kit (Applied Biosystem Division, Perkin Elmer, Foster City, CA, USA) with wild-EGFR (Transcript variant 1). Furthermore, the sequences encoded the same amino acids as the wildtype EGFR transcript variant 1.

Transfection and infection

HEK293 cells infected with EGFR using Lipofectamine 2000

(Invitrogen) were plated on 24-well plates. After 24 h, a 1: 10 dilution was transferred to new plates. Two days after infection, G418 (200, 400, 600 and 800 ng/mL, respectively), was added to screen for a suitable condition. At 7 – 10 days after screening, 600 ng/mL was determined the most suitable condition. After 6 – 8 weeks in culture with 600 ng/mL G418, the aft-HEK293 cells were selected and screened. Subsequently, a clonal cell line was derived and termed HEK293-EGFR.

Cell cycle distribution by flow cytometric analysis of cell growth

HEK293-EGFR, A431 and EGF-treated cells were harvested by trypsinization, washed with PBS and then fixed in 95% ethanol. Following removal of ethanol by centrifugation, cells were then incubated with phosphate-citric acid buffer [0.2 M Na_2HPO_4 (pH 7.8)/4 mM citric acid] at room temperature for 45 min. After centrifugation, cells were then stained with a solution containing 33 µg/mL propidium iodide (PI), 0.13 mg/mL RNase A, 10 mM EDTA and 0.5% Triton X-100 at 4°C for 24 h. Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by ModFit (Verity Software House Inc., Topsham, ME, USA) for the proportion of cells in G_0/G_1, S and G_2-M cell cycle phases.

FCM analysis of cell surface EGFR expression

To analyze EGFR expression, 1×10^6 HEK293, HEK293-EGFR and A431 cells were resuspended in 200 µL PBS and incubated with fluorescein isothiocyanate-conjugated antibodies for 30 min at room temperature. EGFR monoclonal antibodies were used: (BD, PharMingen, San Diego, CA, USA). Following wash steps, the cells were analyzed with a flow cytometer.

RT-PCR for detection of EGFR expression

RNA from 1×10^7 cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed according to the manufacturer's instructions, (Invitrogen). EGFR full-length gene was amplified with primer pairs. The thermocycler conditions are described above.

Western blot analysis

Hek293, Hek293-EGFR and A431 cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS) v/v. Prior to incubation with monoclonal antibodies, the cells were starved for 18 h in DMEM supplemented with 1% FBS v/v. This low serum concentration was selected to reduce the amount of exogenous growth factors while ensuring cell survival, according to previously described methods (Fischel et al., 2005). Prior to growth factor stimulation, cells were incubated for four hours in serum-free medium (DMEM) with 10 ng/mL EGF, as previously described by Raben et al (1985). These three cell lines were incubated with gefitinib (0.5 µmol/L) for 4 h in serum-free medium (DMEM), followed by incubation with 10 ng/mL EGF. Membranes were incubated with the following antibodies: anti-phospho-EGFR residues (Tyr, 1197), anti-phospho-ERK 1/2 (Thr202/Tyr204), anti-EGFR, (Cell Signaling Technology, Beverly, MA, USA). GAPDH served as the control protein for Western blot analysis. Following incubation with secondary antibodies, immunoblots were detected using the enhanced chemiluminescence (ECL) reagent (GE Health Care, SP, Brazil) and bands were quantified with Labworks, version 4.6 (Bio-Rad, USA).

Cellular membrane chromatography preparation and analysis

HEK293-EGFR and A431 cells in log-phase growth were digested with trypsin. The cell suspension was centrifuged at 1000 × g/min for 10 min. The supernatant was removed. Sufficient hypotonic solution was added for 30 min and sonic oscillation was performed for 20 min, followed by centrifugation for 10 min at 1000 × g/min to remove cellular debris. The supernatant was centrifuged for 10 min at 12000 × g/min, and the remaining precipitate was the cell membrane. All above-mentioned steps were performed at 4°C.

Activated silica was placed into a reaction tube and the cell membrane suspension solution was added to it. Adsorption of the cell membrane on the silica surface was activated by oscillation until equilibrium was reached, followed by centrifugation at 2000 rpm to remove free cell membrane. The resulting solution served as the silica gel carrier cell membrane stationary phase (CMSP). CMSP was packed in the chromatography column and equilibrated with 50 $mmolL^{-1}$/pH 7.4 PBS buffer as a mobile phase for 3 – 4 h at a column temperature of 37°C. The positive controls erlotinib, dasatinib, gefitinib, sorafenib, sunitinib and imatinib were added to the mobile phase for analysis and were detected by ultraviolet 220 – 280 nm wavelength. Liquid chromatography constituted LC-20AD*2; SIL-20A; DAD: SPD-M20A; CTO-20A; CBM-20A and DGU-20A3.

RESULTS

Construct a high-EGFR expression HEK293-EGFR cell line

The target gene of human EGFR eukaryotic vector pcDNA3.1 (+)-EGFR was established in the present study and results from BamHI enzyme digestion are shown in Figure 1. The established vector was sequenced and compared with the gene bank of human EGFR transcript variant 1. Results demonstrated that both encoded amino acids were consistent (3637 base pairs were identical with exception of two bases). This did not affect EGFR functions, because the encoded amino acids of the eukaryotic vector pcDNA3.1 (+)-EGFR were not altered. Sequence data and amino acid encoding is not presented.

To investigate expression of full-length EGFR mRNA in the HEK293-EGFR cell line, RT-PCR analysis was performed, as shown in Figure 2.

According to flow cytometry results, EGFR expression was 4-5 times greater in the Aft-HEK293 cells following transfection. EGFR expression in the A431 cell lines is shown in Figure 3.

A431 compared with HEK293-EGFR cell line

According to the cell growth curve, there were no significant differences between HEK293 and HEK293-EGFR cells. However, A431 cells proliferated faster than HEK293 cells.

Immunofluorescence analysis was used to detect EGFR expression in Aft-HEK293 and A431 cells, revealing that A431 cells strongly expressed EGFR (Figure 4). Subsequently, under EGF stimulus, cell cycle before and after HEK293

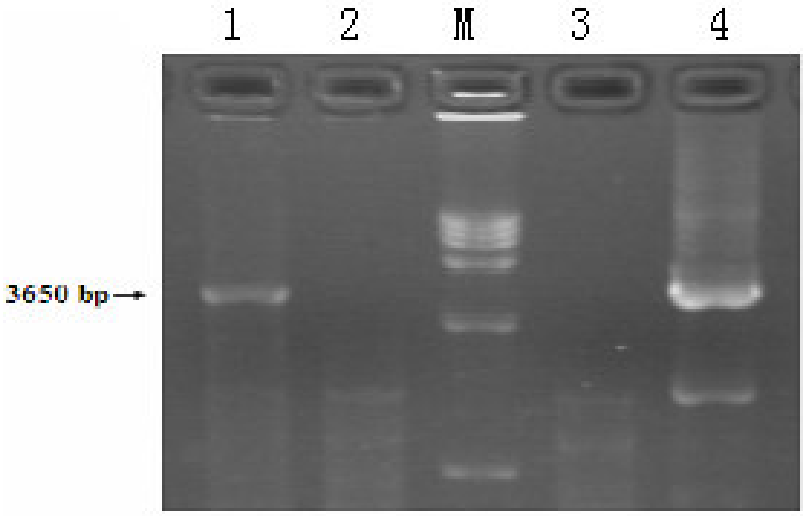

Figure 2. RT-PCR detection of EGFR mRNA in the HEK293-EGFR cell line. **Lane 1** - HEK293-EGFR cell line; **lane 2** - HEK293 cells; **lane 3** - HEK293 with empty vector; **lane 4** - plasmid pBabe-Puro EGFR. **M** - marker.

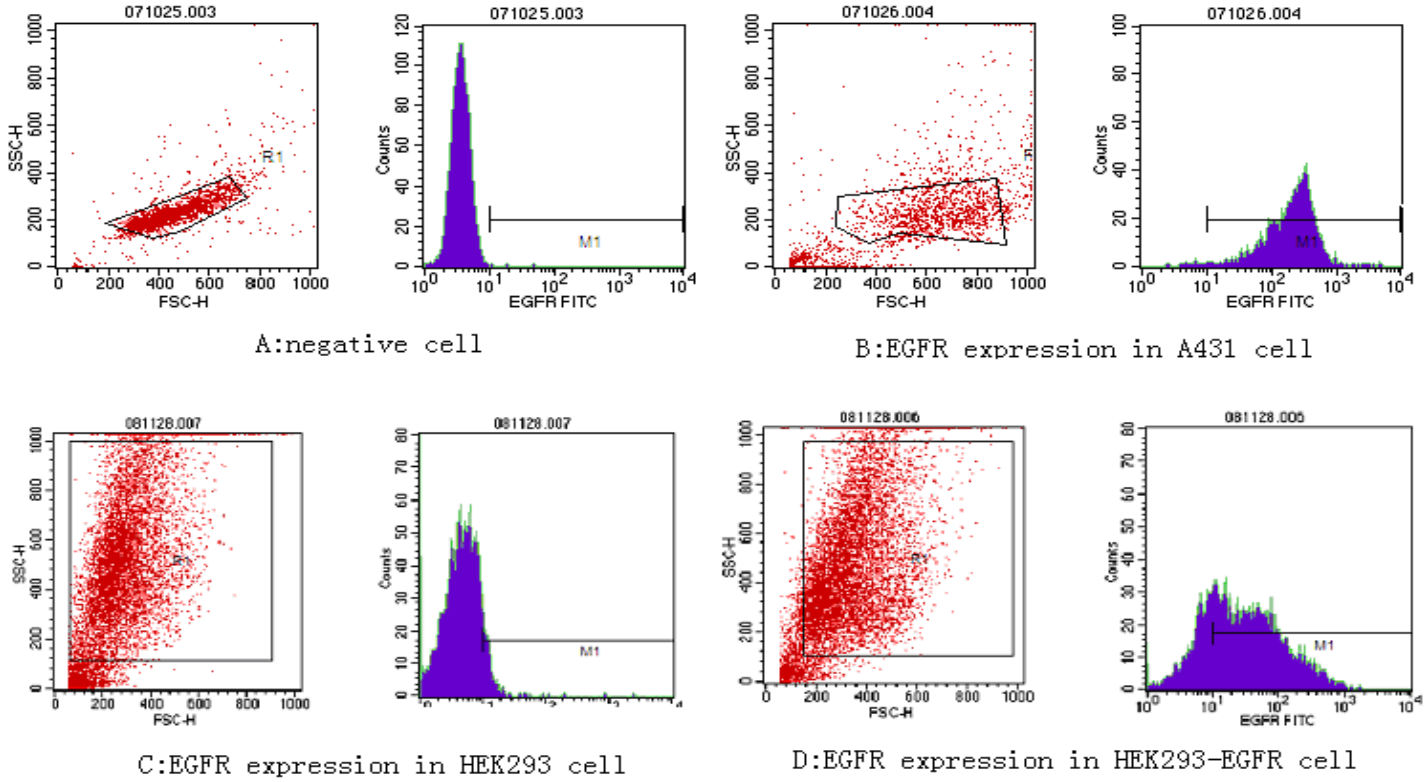

Figure 3. Detection of EGFR expression in various cell lines by flow cytometry. Cells were stained with anti-EGFR antibody. A: negative control; B: EGFR expression in A431 cell line; C: EGFR expression in fore-transfection HEK293 cell line; D: EGFR expression in aft-transfection HEK293 cell.

transfection, as well as in the A431 cell line, was analyzed by flow cytometry. Results showed no difference between the groups (data not shown).

Following EGF stimulation, Western blot analysis was used to screen fore-and-aft HEK293 and A431 cell lines for expression of EGFR, p-EGFR, and p-ERK. Gefitinib reduced phosphorylation of EGFR and Erk (Figure 5). Results demonstrated greater EGFR and p-EGFR

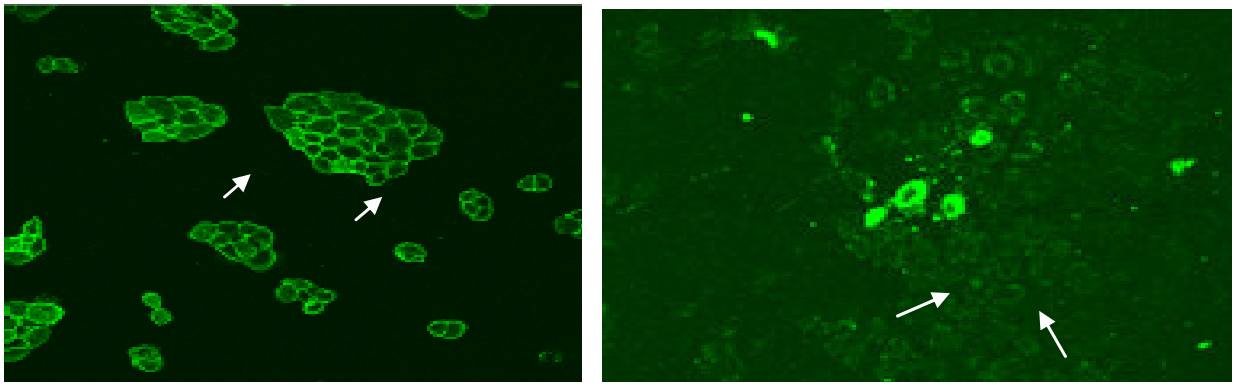

Figure 4. Immunofluorescence detection of EGFR expression. A: A431 cells exhibited strong EGFR expression; B: Aft-HEK293 cells.

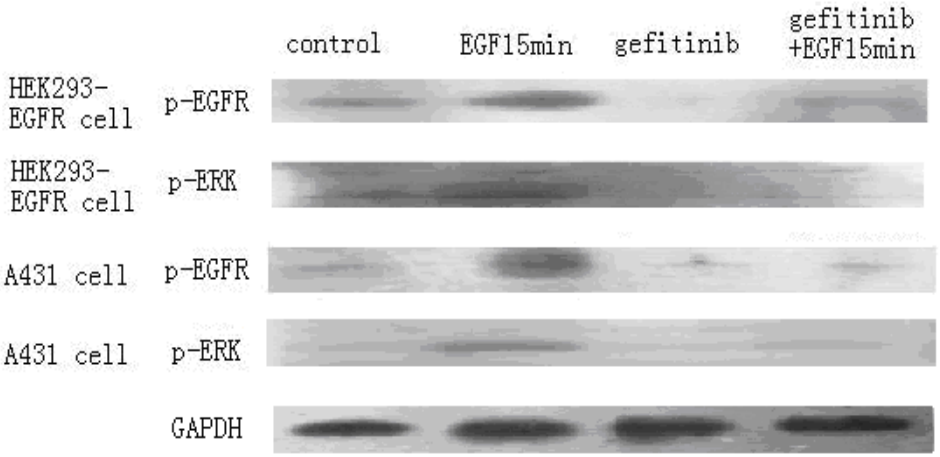

Figure 5. p-EGFR and p-ERK protein expression in HEK293-EGFR and A431 cell lines.

expression in the aft-transfection HEK293 cells, which demonstrated higher bioactivity of transfected EGFR. HEK293 cells expressed high expression and the A431 cells were thought to induce transcriptional up-regulation in the nucleus through the p-ERK pathway, resulting in cell proliferation.

Flow cytometry results demonstrated that the cell cycle was inhibited by gefitinib in the above-mentioned cells lines. Western blot analyses showed that p-EGFR and p-ERK expressions were also inhibited. These data further suggested that Hek293 and A431 cells exhibit similar signaling activation with regard to the EGFR/MAPK pathway.

EGFR-positive drugs detected using A431 cells by CMC

Following rinsing with phosphate buffer saline (PBS) of the HEK293-EGFR and A431 cell lines, ultraviolet wavelength detection by cell membrane chromatographic column was analyzed. Gefitinib was maintained on these two types of cell membrane and discharge curve behaved very similar. A431 retained longer on-peak chromatographic and greater on-peak area (data not shown). The other EGFR positive drugs, erlotinib, dasatinib, gefitinib, sorafenib, sunitinib, imatinnib and taspine were also detected. Results showed these drugs had remaining peaks in the A431 cell membrane. These results demonstrated that the A431 cell line could be used to screen drugs *via* cell membrane chromatographic analysis. Because EGFR was expressed at greater levels, the A431 cell line exhibited better affinity and higher sensitivity.

DISCUSSION

Despite large clinical trials with anti-EGFR and the clinical

use of gefitinib, etc, several questions remain. The screening of anti-EGFR compounds from natural compounds could assist the generation of synthetic compounds. The present study utilized the naturally high-expressing wild-type EGFR cell line (A431 cell line) and compared it to a stably, high-expressing HEK293-EGFR cells line. Results demonstrated 4 – 5 times higher expression following EGFR transfection.

HEK 293 cells were generated by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA in the laboratory of Alex Van der Eb in Leiden, Holland, in the early 1970s. Human embryonic kidney 293 cells, also referred to as HEK293, 293 cells, or less precisely, HEK cells, are easy to grow, transfect very readily and have been widely used in cell biology research for many years. They are also used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy.

In this study, the HEK293-EGFR cell line was established. EGFR expression in the HEK293-EGFR cell line was high expression receptor on the HEK293-EGFR cell membrane than compared to other cell lines. This characteristic could be beneficial for screening EGFR-targeted compounds. Previous studies indicated that A431 cells highly express EGFR (1.2×10^6 sites/cell) and largely depend on the EGFR/MAPK pathway for sustained proliferation (Janmaat et al., 2003; Meira et al., 2009).

The A431 cell line is a human epithelioma cell line, with high expression of EGFR in the coding region (GenBank accession number NM-005228; GI-4885198). Two base pairs are different between the EGFR expressed in the A431 cell line and wildtype EGFR (1620C/G and 2361G/A; GenBank, Transcript variant 1; NM-005228; GI-41327737). In the 1620C/G variant, the asparagine (N) is substituted by lysine (K) and the extracellular region is composed of 621 amino acid residues. The site 1620C/G is located in the extracellular region and is a non-reactive binding site. However, there are no amino acid exchanges in the 2361G/A variant. The Hek293-EGFR cell line, which stably expressed high levels of EGFR, did not contain any encoding amino acids changes compared with wildtype EGFR (GenBank, Transcript variant 1; NM-005228; GI-41327737).

Flow cytometric and Western blot analyses were used to confirm the above-mentioned results. Transfected EGFR exhibited the same biological activity; EGFR, p-EGFR and p-ERK expression increased with increasing EGFR gene expression in the cells. EGFR expression was induced by EGF and triggered bioactivity functions, which promoted proliferation and activated the MAPK signal pathway.

The cell membrane receptor acts as the “start” signal for target cells to transfer signals along a pathway. The receptor recognizes the combination and interaction of particular characteristics on the corresponding receptor in the cytoplasm of the target cell, thereby activating biochemical changes in cells, or second messengers. The receptor also transfers signals into the nucleolus, which leads to gene expression changes and relevant biological effects. The cell surface receptor exhibits greater sensitivity to new drugs when its expression is high (Oh et al., 2004; Okuse et al., 2002).

The A431 and HEK293-EGFR cell lines exhibited similar biological characteristics. Because the gene sequence and encoding amino acids were basically identical, the cell cycle and signal pathways were important factors of greater bioactivity with increasing EGFR expression. Moreover, the A431 cell line proliferated faster, which could be beneficial to cellular membrane chromatography preparation and analysis.

The two cell lines exhibited similarity with regard to gefitinib remaining in the cellular membrane chromatography analysis. Other drugs, such as erlotinib, dasatinib, gefitinib, sorafenib, sunitinib, imatinnib and taspine exhibited better maintained in the A431 cell membrane. The A431 cell line improved specificity and sensitivity for screening drugs targeted to EGFR, based on cellular membrane chroma-tography analysis. Because the A431 cell line is a tumor cell line, it also expresses many other receptors at high levels. Other compounds could target A431 surface receptors. However, the HEK293-EGFR established cell line only expresses little receptors and could, therefore, be used for further drug screening.

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