Lab Note

Construction of Y367C-FGFR4 eukaryotic expression plasmid and its biological activity in HEK293 cell

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Fibroblast growth factor receptor (FGFR), a member of tyrosine kinase family, is composed of three domains, including a three-extracellular Ig-like domain, a transmembrane domain, and a tyrosine kinase domain. FGFR4 is a member of the FGFR family, which plays a pivotal role in tumorigenesis. It has been found that FGFR4 plays an important role in melanoma, prostate cancer, head and neck cancer, and primary liver cancer malignant development [1,2]. The poor response of FGFR4 to chemotherapy has been associated with its over-expression [3].

Mutations of receptor tyrosine kinases have major pathological consequences. In the FGFR subfamily, various mutations have been identified to have a dominant role in the development of diverse diseases such as bone disorder and cancer [4,5]. Dysregulation of FGFR activity contributes to cancer development by increasing cell proliferation and angiogenesis, and inhibiting apoptosis. Recently, mutations occurring in FGFR4 have been extensively investigated [6,7]. Previous studies focused on Gly388Arg mutation which is associated with increased risks of breast and prostate cancer [8]. Ruhe et al. [9] discovered a Tyr367Cys mutation in FGFR4 gene by a comprehensive analysis of tyrosine kinase gene family in cancer cell lines. Later, FGFR4-Y367C was established as a biochemical link of mutation to an oncogenic phenotype [10]. Generally, by ligand binding or receptor mutation, FGFR4 becomes trans-phosphorylated, which results in the recruitment of the central adaptor protein FGFR substrate 2 (FRS2) and the subsequent downstream signaling. FGFR signaling leads to the activation of mitogen-activated protein kinase (MAPK) in breast cancer cells and thereby stimulates proliferation [6,11]. These findings indicated an important role of FGFR4 in cancer cell that it can be self-activated without ligand binding [10]. Here, we report the preparation of cells over-expressing Y367C-FGFR4 to explore the potential target for screening natural active anti-tumor ingredients.

Site-directed mutagenesis was carried out according to manufacturer’s instructions. Briefly, polymerase chain reaction (PCR) was carried out with mutagenic primers. The end of PCR product was blunted and phosphated by using Blunting Kination Enzyme Mix (Takara, Dalian, China). Then, self-ligation was performed by using Ligation Solution I (Takara). Finally, the recombinant plasmid was amplified in Escherichia coli DH5α cells and extracted by using plasmid DNA purification kit (Omega, Norcross, USA). The mutagenic primers were as follows: forward 5′-GGTGATCCGACACCATCCTCTAGCGCAGTCGTA-3′ and reverse 5′-TGCGCTCCGGGCGGGCTG-3′. The sequence of mutated FGFR4 was verified by DNA sequencing. The mutagenesis plasmid sequencing result was shown in Fig. 1.

The full length of Y367C-FGFR4 CDS region was 2409 bp (NCBI reference sequence: NM_002011). The cDNA fragment of Y367C-FGFR4 was amplified from pOB7T-Y367C-FGFR4 plasmid. The Y367C-FGFR4 specific primers were as follows: forward 5′-GACTCAGAATTCCGCTTGCTGCTGCTGAAGCACCTCCCTTTCAGTGGCC-3′ and reverse 5′-GGACTCAGAATTCCGCTTGCTGCTGCTGAAGCACCTCCCTTTCAGTGGCC-3′. PCR products were purified by using gel extraction kit (Omega). The full-length Y367C-FGFR4 cDNA and pEGFP-N1 vector were digested by HindIII and Xhol restriction enzymes (Takara) and then target fragments were purified and ligated. The recombinant plasmid was amplified in E. coli DH5α and then extracted by the DNA purification kit (Omega) and further identified by sequencing. Thus, the recombinant eukaryotic expression vector pEGFP-N1-Y367C-FGFR4 was finally obtained.

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; HyClone, Beijing, China) at 37°C in 5% CO2. Transfection was performed with pEGFP-N1 and pEGFP-N1-Y367C-FGFR4, respectively, by using lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to manufacturer’s instructions when cells were 60–80% confluent. The translected HEK293 cells expressing green fluorescent protein (GFP) were selected by G418 and then survival cells were planted into fresh growth...
medium containing 1 g/l G418. After 14-day selection, G418-resistant clones were picked up with expanding culture. Thus, stable Y367C-FGFR4-transfected cells were obtained.

Single-stranded cDNA were synthesized from total RNA by using M-MLV reverse transcriptase (Invitrogen). Real-time PCR was carried out with specific primers for FGFR4 and GAPDH. Primers sequences were as follows: for Y367C-FGFR4, 5′-CCATAGGGACCCCCTCGAA-3′ (sense) and 5′-CTGGACAGCGGAACTTGA-3′ (anti-sense), for GAPDH, 5′-ATGTTCGTCATGGGTGTGAA-3′ (sense) and 5′-TGTGGTCATGAGTCTTCCA-3′ (anti-sense).

Stably transfected cells were collected and lysed in cell lysis buffer. Supernatants were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the gel was transferred onto polyvinylidene difluoride membranes (Bio-Rad, Indianapolis, USA). The membranes were blocked with 5% non-fat dry milk for 2 h, incubated with polyclonal rabbit anti-FGFR4 antibody (1 : 200; Santa Cruz, Santa Cruz, USA), and subsequently with horseradish peroxidase-goat anti-rabbit IgG secondary antibody (1 : 10,000; Jackson ImmunoResearch, Langcaster, USA). The immunoreactive proteins were visualized by using enhanced chemiluminescence kit. To detect the location of FGFR4 protein, immunofluorescence assay was carried out. The cells were incubated with rabbit anti-FGFR4 antibody (1 : 100; Santa Cruz) and cy3-conjugated AffiniPure goat anti-rabbit IgG (1 : 200; Santa Cruz). The red fluorescence was apparently observed on cell membrane and cytoplasm.

Figure 1 Nucleotide information of Y367C-FGFR4 gene  Alignment of the sequence of Y367C-FGFR4 gene compared with FGFR4 gene. The red arrow represented mutant site A → G.

Figure 2 The expressions and location of Y367C-FGFR4 in different HEK293 cell groups  (A) The immunofluorescence assay was carried out to verify the efficiency of FGFR4. The red fluorescence was apparently observed on cell membrane and cytoplasm. (B) The Y367C-FGFR4 mRNA expression in HEK293 cells. GAPDH was used as an internal standard. Lanes 1 and 2, Y367C-FGFR4 stably transfected HEK293 cells; lane 3, pEGFP-N1 stably transfected HEK293 cells. (C) The Y367C-FGFR4 protein expression in HEK293 cells. GAPDH was used as an internal standard. Lanes 1 and 2, Y367C-FGFR4 stably transfected HEK293 cells; lane 3, pEGFP-N1 stably transfected HEK293 cells. **P < 0.005 and ***P < 0.001.
Jackson ImmunoResearch). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole to detect intact cells.

Effect of Y367C-FGFR4 on HEK293 cells proliferation was evaluated by cell counting. The pEGFP-N1 stably transfected HEK293 cells were used as control.

The full length of Y367C-FGFR4 gene transcript variant CDS region was 2409 bp, the mutation point was the 1101th base pair of Y367C-FGFR4, and the mutation was G to A. Sequencing results showed that the artificial introduction of mutations appeared at expected site (Fig. 1) and then the recombinant pOBT-7-Y367C-FGFR4 plasmid was digested and cloned into pEGFP-N1 vector. The exact sequence information of full-length Y367C-FGFR4 gene from pEGFP-N1-Y367C-FGFR4 plasmid was also verified by sequencing (AuGCT, Beijing, China). These results showed that the FGFR4 cDNA was successfully inserted into pEGFP-N1 plasmid.

Reverse transcriptase-PCR and immunoblot assays demonstrated that the FGFR4 gene expressed highly in HEK293 cells, when compared with pEGFP-N1-HEK293 cells [Fig. 2(B,C)]. Compared with pEGFP-N1 stable-transfected HEK293 cells, Y367C-FGFR4 stably transfected HEK293 cells exhibited more FGFR4 expression in cytoplasm and cell membrane [Fig. 2(A)].

Previous studies have reported that mutations in FGFRs occurred frequently in various types of tumor [12–15]. Our results showed that the proliferation rate of Y367C-FGFR4 cells was ~2 fold higher than that of pEGFP-N1-HEK293 cells from 24 to 96 h (Fig. 3), suggesting that Y367C-FGFR4 promoted host cell proliferation.

In summary, we have successfully constructed the recombinant eukaryotic expression plasmid, pEGFP-N1-Y367C-FGFR4, containing human Y367C-FGFR4 gene. The Y367C-FGFR4 gene was effectively expressed in HEK293 cells and over-expressed Y367C-FGFR4 promoted HEK293 cells growth. Dysregulation of FGFR activity can contribute to cancer development by increasing cell proliferation, angiogenesis, and inhibiting apoptosis [6,8,10]. Genetic aberrations in FGFRs have indeed been found to be associated with cancer development [16]. Phosphorylation of FRS2α caused by the activation of FGFR leads to the recruitment and activation of Grb2/Sos1 complex, which then interacts with Ras to activate MAPK pathway [17–19]. In future experiments, the activation of FRS2α and ERK1/2 should be investigated.

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**References**


