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Original Article

Effect of FGFR2IIIc^{S252W} overexpression upon osteosarcoma U2OS cell proliferation via activating ERK1/2 and JNK signaling pathways

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Abstract: Objective: The genetic mutation of FGFR2^{S252W} in Apert syndrome leads to the incidence of pre-differentiation of osteoblasts. Methods: FGFR2^{S252W} as a gain of function mutation was found in some endothelial cell-derived cancerous tissues. We found over-expression of FGFR2IIIc^{S252W} in osteosarcoma U2OS cells could induce the cell apoptosis. Therefore, we explored the mechanism underlying whether FGFR2IIIc^{S252W} could inhibit the proliferation of U2OS cells. Results: Compared with vector controls, FGFR2IIIc^{S252W} and FGFR2IIIc^{WT} groups, FGFR2IIIc^{S252W} not only deformed cellular morphology, but also activated the ERK1/2 and JNK signaling pathways. In spite of the growth inhibition probably associated with caspase signaling pathway, we found Egr-1, a transcription factor, was significantly up-regulated accompanied with FGFR2IIIc^{S252W} over-expression. The promoter activation assay revealed that FGFR2IIIc^{S252W} increased Egr-1 promoter activity in a dose-dependent manner. Subsequent results demonstrated that transient over-expression of Egr-1 in U2OS cells could also induce cell apoptosis. Conclusion: Taken together, our results suggested that FGFR2IIIc^{S252W} could inhibit U2OS cell proliferation with the activated ERK and JNK signaling pathways accompanied with up-regulated expression levels of Egr-1, which plays a pivotal role in the growth inhibition by FGFR2IIIc^{S252W} overexpression in osteosarcoma U2OS cells.

Keywords: FGFR2IIIc^{S252W}, osteosarcoma cell, growth inhibition, Egr-1

Introduction

FGF/FGFRs play an essential role in controlling cell proliferation, differentiation, apoptosis, and cell-to-cell communication. FGFs depend upon high affinity FGF receptors (FGFRs) to activate the intracellular signaling pathways [1]. Up to now, 5 kinds of FGFRs have been identified. Except FGFR5 [2], FGFR1~4 is the trans-membrane protein with three extrinsic Ig-like domains and intrinsic tyrosine residues [3]. The site of FGFs binding to FGFRs located between the 2nd and 3rd Ig-like domains. FGFR1, 2, 3 have IIIb and IIIc subtypes due to different mRNA splicing. IIIb subtype is expressed in epithelial cells whereas IIIc subtype is identified in mesenchymal cells [4]. FGFs binding to FGFRs leads to receptor dimmerization and phosphorylation of intra-membrane tyrosine residues, which elicits the downstream signal pathways including MAPK, PI3K and PLCγ [5].

Mutation of FGFRs results in multiple craniosynostosis syndromes, such as Crouzon, Pfeiffer, Jacksom-Weiss, Apert *and others* [6-8]. It is possibly associated with the mutated receptors' constitutive activation due to changes of receptor dimmerization, ligand binding specificity or affinity [9, 10]. Apert syndrome (AS), one of severe bone defects of craniosynostosis, is a genetic disease caused by FGFR2 mutation with Ser252Trp (S252W) or Pro253Arg (P253R), which FGFR2^{S252W} accounts for 65% and FGFR2P253R for 35% [11, 12]. At present, AS is primarily caused by enhancement of osteoblast differentiation and two missense mutations also serve as the gain-of-function mutations [13]. It was showed that S252W mutation promoted alkaline phosphatase (ALP), type I Collagen (ColA1), osteocalcin (OC), osteopontin (OPN) expression and enhanced cellular mineralization ability eventually lead to early closure of skull sutures, reduced brain capacity, and tip

deformities formation *in vivo* and *in vitro* [14-16]. Except promoting cell differentiation, the gain-of-function mutation of FGFR2IIIc can also aggravate osteoblast apoptosis. *In vitro* osteoblasts from AS patients had a higher apoptosis rate than normal osteoblasts [14]. Overexpression of FGFR2^{S252W} could also significantly arrest mouse osteoblasts' proliferation, whereas cell apoptosis was increased if the cells were treated with dexamethasone simultaneously [17].

Two AS patients suffering from malignant tumors have been reported, probably associated with congenital embryonic rhabdomyosarcoma and an ovarian dysgerminoma, [18, 19]. Despite the vast majority of tumor cell lines have not FGFR2 mutation [20, 21], S252W mutation was found in endometrial and ovarian cancer samples [22-24]. These tumors are originated from epithelial cells with FGFR2-IIIb subtype. But overexpression of FGFR2IIIc^{S252W} could result in osteogenic differentiation of osteosarcoma cells [16]. When over-expressing FGFR2^{S252W} with FGF9 inducing in PC12 cells, the ability of neuronal differentiation was reduced compared with the wild-type group [25]. Therefore, whether FGFR2^{S252W} inhibits the growth of the tumors originated from mesenchymal cells or not remains to be elucidated.

Osteosarcoma is malignant tumor originated from mesenchymal bone tissue. U2OS is ARF-deficient but p53 is a wild-type cell [26, 27]. In our study, the expression level of FGFR2^{S252W} was significantly up-regulated in U2OS by using adenovirus system compared with wild type group, suggesting that cell morphology were changed , cell proliferation was inhibited, and cell death rate was increased. The further data identified ERK1/2 and JNK MAPK pathways were activated. However, the cell death occurrence was not associated with these two pathways but caspase pathway. The transcription factor Egr-1 was up-regulated by FGFR2IIIc^{S252W}. The more assays displayed that the mutation receptor elicited Egr-1 promoter activity and its relative cells death rate could be reduced by Egr-1 siRNA. This is the first study reported that FGFR2IIIc^{S252W} could inhibit cell proliferation accompanied with ERK1/2 and JNK pathways and up-regulation of Egr-1 expression. The data could provide certain clues for understanding the FGFR2IIIc^{S252W} function and for osteosarcoma therapy.

Materials and methods

Cells and agents

The U2OS (osteosarcoma) cells were obtained from the ATCC. Cells were routinely maintained in F12 medium (Gibco) containing 10% fetal bovine serum (FBS) and 100 μM L-glutamine. HEK 293A cells were purchased from Qbiogene Company and cultured in DMEM containing 10% FBS and 100 μM L-glutamine. Both cells lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in air. Antibodies against total ERK, phospho-ERK, total JNK, phospho-JNK total AKT, phospho-AKT were purchased from Cell Signaling Technology Ltd. Antibodies against Beta-actin, FGFR2IIIc (BEK) and PKCα were purchased from Santa Cruz Biotechnology. PD98059 (an inhibitors for ERK1/2 MAPK), SP600125 (an inhibitor for JNK MAPK) and Z-AD-FAM (an inhibitor for Caspase signal pathway) were obtained from Calbiochem. Transfection reagent Lipofectime 2000TM were purchased from Invitrogen.

Establishment of expression vectors and transient recombinant cell lines

Adenoviral expression vectors used included pAd-Vector (as negative control), pAd-FGFR2-IIIc^{WT}, pAd-FGFR2IIIc^{S252W} (Ad-easy system). Recombinant adenovirus was produced in HEK 293A cells following the manufactures' protocols of Qbiogene. Human breast cancer cell lines MCF-7 and MDA-MB-231 were selected for subsequent experiment. For transient expression, U2OS cells were grown in 6-wells plates and infected at 80% confluence with recombinant adenovirus directly. After 48 hours, the infected cells were collected for next analysis. Plasmids included pcDNA3.1 (as negative control), pcDNA-FGFR2IIIc^{WT}, pcDNA-FGFR2IIIc^{S252W}, pcDNA-Egr-1, pGL3, pGL-Egr-1 and pTK. U2OS cells were transfected at 80% confluence with recombinant plasmids using Lipofectime 2000TM guided by the manual of Invitrogen company.

Cell proliferation and cell apoptosis assays

For cell proliferation assay, U2OS cells were cultured in 96-well plates (10^4 cells per well) and were infected with recombinant adenovirus directly or transfected with recombinant vectors by using Lipofectime 2000TM at 80% cell

confluence. The recombinant cell growth rate was evaluated by MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay [28]. For cell death testing, U2OS cells were cultured in 6-well plates (10^5 cells per well) and infected or recombinant adenovirus directly or transfected with recombinant vectors using Lipofectime 2000TM at 80% cell confluence, then the recombinant cells were subject to Trypan blue staining [29] or flow cytometry with propidium iodide(PI) staining [30].

Western blot

Cells were rinsed three times with cold PBS, and lysed with lysing buffer. Protein concentrations in cell lysates were measured using the BCA protein assay reagent (Pierce, Rockford, IL). An equal quantity of proteins was loaded in each lane. Cell lysates were resolved under reducing conditions by 10% SDS-PAGE and then transferred to PVDF membranes. After being blocked with 5% BSA in Tris-buffered saline (TBS) with 0.1% Tween 20, the membranes were incubated with antibodies against activated or total forms of protein overnight at 4°C, washed three times with 0.1% Tween 20-TBS, and then incubated for 60 min with1: 2000 peroxidase-conjugated anti-rabbit IgG of anti-mouse IgG (Pierce, Rockford, IL). The membrane-bound peroxidase activity was detected using ECL Plus Western blotting detection kits (Pierce, Rockford, IL) and autoradiographic film (Kodak, Japan).

Real-time quantitative PCR

Total RNA was extracted using the total RNA isolation system (Promega, USA). cDNA was generated from 1 mg total RNA per sample using anchored oligo dT primers (Shengong Synthesis; China. RT qPCR was performed by using the LightCycler and the FastStart DNA Master SYBR Green 1 kit (Roche Applied Sciences) as described previously. PCR primers were: FGFR2-F: 5'tgtcattacgtcaacgcaa c-3', FGFR2-R: 5'ttcactgaagagaatacagg-3'; Egr-1-F: 5'gcagcagcagcaccttcaac3'; egr-1-r: 5'tctcgttgttcagagagatg3.

siRNA transfection

Gene silencing was performed using human Egr-1 sequence specific duplex siRNA: 5-ccucuuacccgucccccggutt-3 and 5-accggggacgggguaagagg tt-3, a control non-silencing scramble siRNA si-NC: 5-gttctccgaacgtgtcacgt-3,5-acgtgacacgttcggagaatt-3 (antisense); positive control si-GAPDH5-gtatgacaacagcctcaag t-3; 5-a cttgaggctgttgtcatactt-3 all purchased from GenePharma, Ltd. U2OS cells were fed in a 6-well plate the day before transfected at 80% confluence in suspension state. siRNAs with or without plasmids were co-transfected to cells by Lipofectime 2000TM following the manufactures' protocols of Invitrogen. Total proteins and RNA were extracted at 48 h post-transfection. The protein concentrations were determined by Bradford assay (Bio-Rad), and silencing efficiency of the EGR-1 siRNA was examined by qPCR and Western-blot analysis using anti-EGR-1 antibody (Cell Signal Technology). qPCR was performed as described above.

Luciferase reporter system for analysis of Egr-1 promoter activity

Using Luciferase reporter system, Egr-1 promoter activity was evaluated by transfected different concentrations of pcDNA-3.1 vector, pcDNA-FGFR2IIIc-WT, pcDNA-FGFR2IIIc^{S252W} respectively at 48 h. The relative expression of Egr-1 in cells transfected with different Egr-1 siRNAs consisting of si-601, si-1516, si-1611 was quantitatively measured and scramble siRNA (si-NC) was used as negative control and GAPDH siRNA (si-GAPDH) as positive control.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The average values of six samples were calculated from at least three distinct experiments. Statistical data were analyzed by Student's *t*-test. A *P* value of less than 0.05 was considered as statistical significance.

Results

Over-expression FGFR2IIIc^{S252W} inhibited U2OS cell proliferation

To assess whether FGFR2IIIc^{S252W} could enhance cancer cells proliferation, we used recombinant adenovirus particles to infect different cancer cells, including breast cancer cell MCF-7 and MDA-MB-231 and U2OS. Multiplicity of infection (MOI) of recombinant adenovirus was 100 to ensure cells to be infected completely. The findings failed to support that overexpression FGFR2IIIc^{S252W} improved these cancer cell growth but inhibited U2OS cell proliferation specially. Western blot assay and indirect

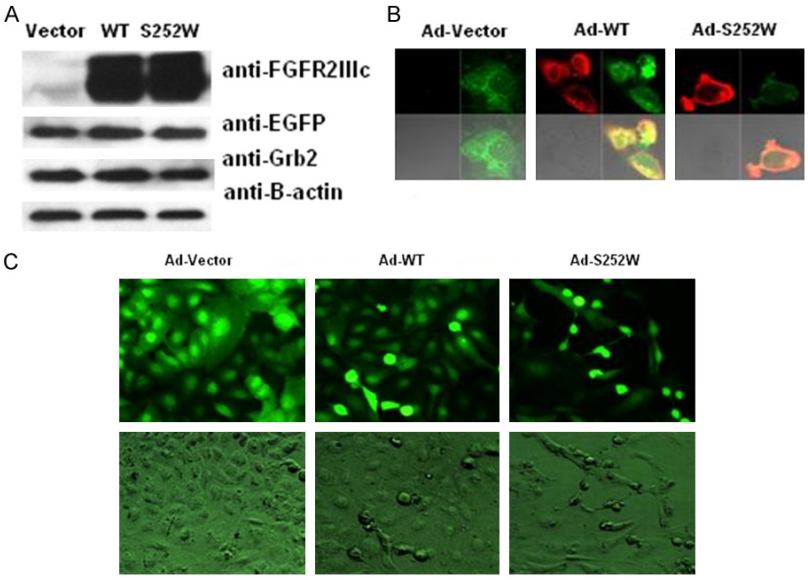

Figure 1. Overexpression FGFR2IIIc^{S252W} modified the U2OS cells phenotype. A. Western Blott evaluating FGFR2IIIc^{WT} and FGFR2IIIc^{S252W} expression levels in transient recombinant cell lines. B. Indirect immunity fluorescence assay identifying FGFR2IIIc under laser scanning confocal fluorescence microscope (LSCF); C. Cell morphologic changes observed under fluorescent microscope.

immunofluorescence test revealed that the expression levels of WT and S252W did not significantly differ in U2OS (**Figure 1A**). Compared with the WT group, the morphology of S252W group cells was deformed, became around and cell adhesion capacity was significantly reduced (**Figure 1B**).

To explore how FGFR2IIIc^{S252W} causes the phenotype changes of U2OS cells, a series of experiments were performed. MTT assay showed that the cell growth rates significantly differed between two groups at different time points. In the S252W group, the cell growth rate was significantly decreased from 24 h to 48 h, whereas slight changes were observed in the WT group compared with negative control vector group. Typan blue staining showed that the cell survival rate was significantly decreased in the S252W group at 48 h after infection of adenovirus. As a marker of cell apoptosis, Annexin V was detected by adding its antibody labeling PE into culture medium in each group at 48 h after infection, but no visible distinction was observed under fluorescent microscope. Flow cytometry demonstrated that the percentage of cells in G0/G1 phase was higher in the WT group and the cell apoptosis rate in the S252W group was increased at 6, 12, 24 and 48 h after infection (**Figure 2**).

Over-expression FGFR2IIIc^{S252W} activated ERK1/2 and JNK signaling pathways

It has been reported that FGFR2IIIc^{S252W} activates ERK MAPK signaling pathway directly. So we detected the relative pathways by Western blot. The results showed that ERK and JNK MAPK pathways had significantly activated, and PKCα, a key protein in PLC-γ pathway, was upregulated. The other MAPK pathway p38 did not significantly differ from PI3K pathway. Specifically, JNK pathway was first to found to be activated by FGFR2IIIc^{S252W} over-expression.

Cell proliferation inhibition is partly associated with Caspase signaling pathway

To analyze if cell proliferation was inhibited by activation of ERK or JNK pathway, PD98059 and SP600125 of different concentrations were used to block the relative signal. However, MTT assay revealed that neither inhibitor returned the tendency of growth inhibition when FGFR2IIIc^{S252W} was over-expressed to promote the inhibitory effect upon cell growth. The growth rate was significantly decreased along with addition of PD98059 in a dose dependent fashion. The significant inhibition effect was observed after blocking JNK pathway with SP600125 at dose of 50 μM. It was possible that the activation of the two pathways is not the main reasons to inhibit cell proliferation. Z-VAD-FMK, an inhibitor of caspase pathway, was used at a dosage of 20 μM. Flow cytometry demonstrated that cell hypodiploid rate was deduced in S252W group. However, MTT test revealed that the cell growth rate was also decreased with addition of Z-VAD-FMK, suggesting that although Z-VAD-FMK could reduce cell death rate, but also interfered cell growth. We supposed that over-expression of FGFR2IIIc^{S252W} increased the cell sensitivity to drugs or inhibitors, which is consistent with previous findings. Therefore, cell death rate was

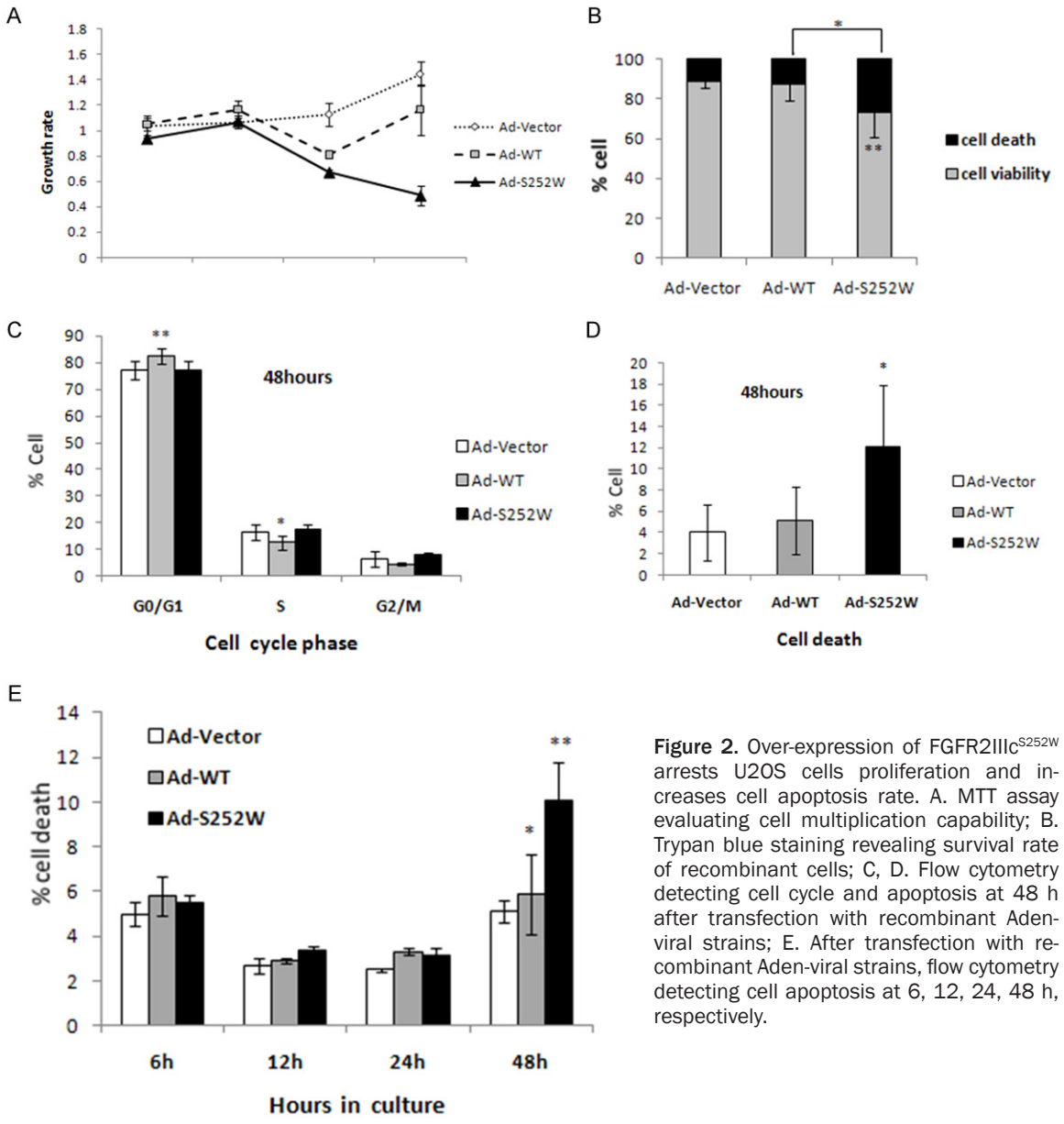

Figure 2. Over-expression of FGFR2IIIc^{S252W} arrests U2OS cells proliferation and increases cell apoptosis rate. A. MTT assay evaluating cell multiplication capability; B. Trypan blue staining revealing survival rate of recombinant cells; C, D. Flow cytometry detecting cell cycle and apoptosis at 48 h after transfection with recombinant Aden-viral strains; E. After transfection with recombinant Aden-viral strains, flow cytometry detecting cell apoptosis at 6, 12, 24, 48 h, respectively.

significantly increased with addition of inhibitors in the S252W group.

Up-regulating expression of Egr-1 by overexpression of FGFR2IIIc^{S252W}

Expression levels of multiple transcription factors related to tumorigenesis were evaluated by RT-PCR (**Figure 3**). Egr-1 was significantly up-regulated in S252W mutation group, subsequently validated by Western blot results. FGFR2IIIc^{S252W} could up-reulate the expression of Egr-1 by up-regulation of Egr-1 promoter activity. Transient over-expression of Egr-1 could inhibit the proliferation of U2OS cells by transfection with recombinant plasmid, and interfere of Egr-1 expression with siRNA could reverse cell growth inhibition. Addition of the Egr-1 siRNA also could reverse the growth inhibition partly by overexpression of FGFR2-IIIc^{S252W}, whereas no significant effect was observed in the WT group.

Discussion

Because FGFR2IIIc plays an important role in bone development and Apert syndrome caused by S252W or P253R mutation in FGFR2 is one

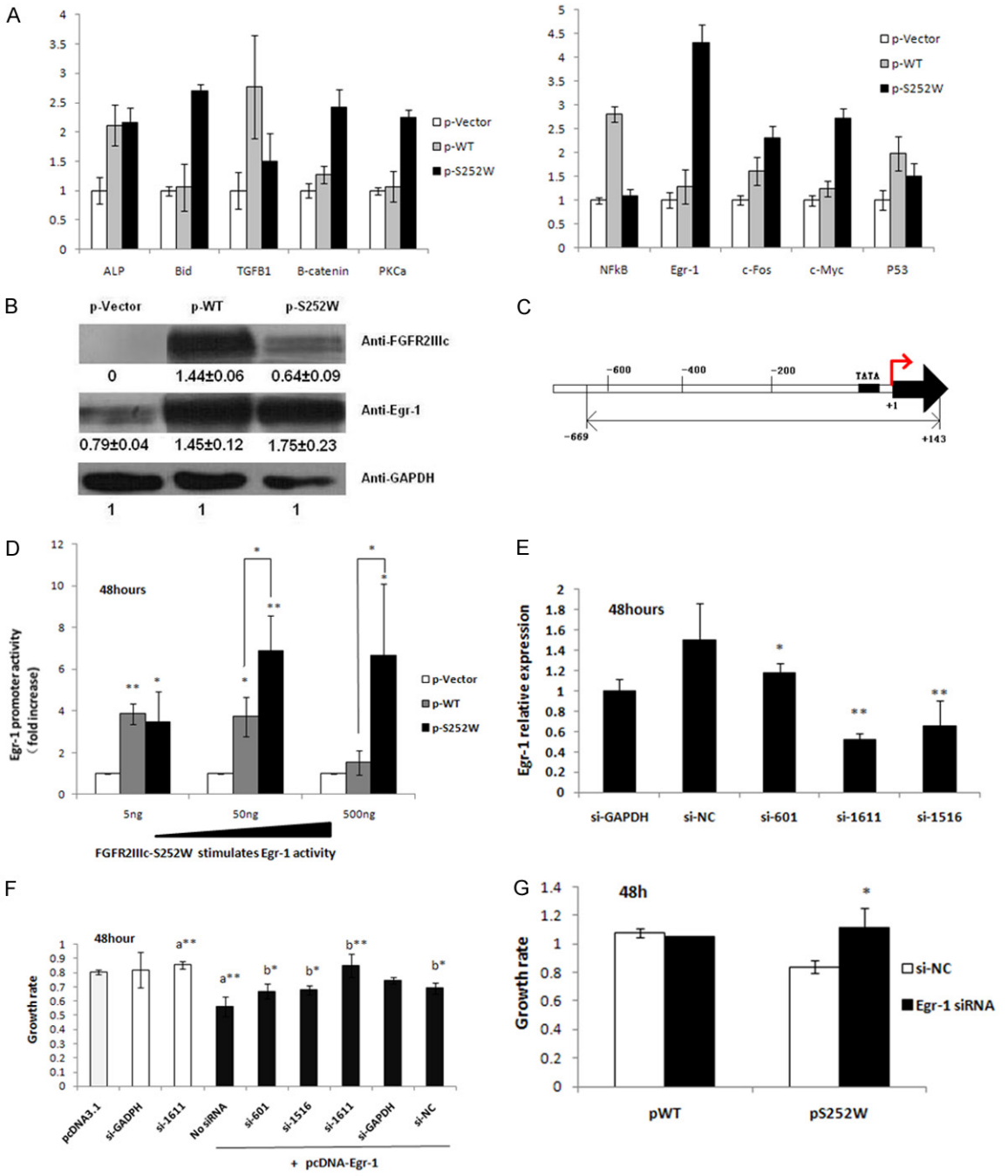

Figure 3. Over-expression FGFR2IIIc^{S252W} in U2OS cell up-regulates Egr-1 expression. A. RT-PCR demonstrated that the expression levels of certain genes were significantly changed at the transcription level in overexpression of FGFR2IIIc^{S252W} group. Specifically, Egr-1 was significantly up-regulated in S252W group. B. Western blot revealing Egr-1 expression at 48 h when cells were transfected with pcDNA- FGFR2IIIc^{S252W} plasmids. C. Schematic representation of Egr-1 promoter. D. Using Luciferase reporter system, Egr-1 promoter activity was evaluated by transfected with different concentrations of pcDNA-3.1 vector, pcDNA-FGFR2IIIc-WT, pcDNA- FGFR2IIIc^{S252W} at 48 h. E. Egr-1 relative expression in cells transfected with different Egr-1 siRNAs including si-601, si-1516 and si-1611. Scramble siRNA (si-NC) was used as negative control and GAPDH siRNA (si-GAPDH) as positive control. F. Overexpression of Egr-1 by pcDNA-Egr-1 in U2OS cells with or without Egr-1 siRNAs, the cell growth rate was evaluated by MTT assay. G. Overexpression of FGFR2IIIc^{WT} and FGFR2IIIc^{S252W} by pcDNA-FGFR2IIIc^{WT} and pcDNA-FGFR2IIIc^{S252W} in U2OS cells with or without Egr-1 siRNAs.

of the most serious craniosynostosis, we focused on exploring whether FGFR2IIIc^{S252W} could arrest osteosarcoma growth. The two cell lines have different characteristics that U2OS is ARF-/- and p53 wild-type originated from 15-year-old female patients [26, 27]. In our study, U2OS was infected by recombinant adenovirus with FGFR2IIIc^{WT} and FGFR2IIIc^{S252W}. Flow cytometry demonstrated that the percentage of MG63 cells at G0/G1 phase in S252W group was higher compared with that in the WT group. These results were consistent with the findings of Tanimoto et al. that the FGFR2IIIc^{S252W} promoted the MG63 osteogenic differentiation [31]. Consequently, U2OS cell line acts as a potential cell model to further confirm the mutation receptor ability of growth inhibition.

In the present study, transient overexpression of U2OS and FGFR2IIIc^{S252W} significantly activates the ERK1/2 signaling pathway. The activation of JNK pathway was also observed, which was not found in previous reports. Compared with the WT group, the cell death rate in S252W group achieved 15%, whereas no evident tendency of cell apoptosis was observed. Consequently, low cell death rate could be caused by activation of JNK MAPK pathway because the JNK signaling pathway was associated with cell apoptosis. Further studies are required to validate whether the U2OS cell growth inhibition is associated with ERK, JNK or caspase signaling pathway. However, the results demonstrated that cell apoptosis was not related to ERK or JNK signaling pathway but partly correlated with caspase signaling pathway, probably due to the overexpression of FGFR2IIIc^{S252W} by adenovirus enhanced cell sensibility to external environment. We also postulated that other intracellular proteins play a key role in cell death. Subsequently, RT-PCR demonstrated that Egr-1 was found to be significantly up-regulated in the S252W group.

Egr-1, a vital transcription factor, is able to regulate the expression of multiple tumorigenesis or tumor-suppressive genes. A variety of extracellular signals, including radiation, growth factors, cellular factors, could induce Egr-1 expression. Many signaling pathways are considered as up-stream of Egr-1, in which MAPK pathway is most reported [32]. It is believed that ERK1/2 MAPK pathway played a key role in upstream signal activity of Egr-1 [33]. Egr-1 expression was significantly increased accompanied with the ERK1/2 phosphorylation activation. Otherwise, to inhibit ERK1/2 activation by PD98059h could decrease the Egr-1 expression [34]. Egr-1 is also regards as an anti-cancer protein to promote cell apoptosis because it could up-regulate the expression of PTEN and p53. In our study, FGFR2IIIc^{S252W} overexpression significantly up-regulated the level of Egr-1 with increasing cell death rate.

According to our prophase study and Ladbury team report, the expression level of exogenous protein is of clinical significance in protein function study. Consequently, adenovirus system was selected to ensure adequate expression level of FGFR2IIIc^{WT} and FGFR2IIIc^{S252W}. But the cell death rate was high in the S252W group with adenovirus system but plasmid system. It is probably the synergy phenomenon by both exogenous protein and adenovirus because the virus packing proteins could influent cell growth. Otherwise, it is possible that the high expression level of FGFR2IIIc^{S252W} is increased over the threshold value which diverts cell proliferation to cell apoptosis.

Conclusion

In prophase study, the recombinant U2OS cell lines were stably established by lentiviral expression system. The data showed that cellular morphology and proliferation in the S252W group were significantly not changed along with FGF-2 induction compared with the WT group. Consequently, we estimate that the recombinant cells lowly express FGFR2IIIc^{S252W}. High expression levels of FGFR2IIIc^{S252W} exert no positive effect upon cell survival.

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Disclosure of conflict of interest

None.

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