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EWS-WT1 Chimeric Protein in Desmoplastic Small Round Cell Tumor is a Potent Transactivator of FGFR4

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Abstract

Desmoplastic small round cell tumor (DSRCT) is a rare but highly aggressive malignant neoplasm that typically involves the abdominal or pelvic peritoneum in children and young adults. This tumor is characterized by the presence of a specific EWS-WT1 fusion gene, which is the result of recurrent chromosomal translocation, t(11;22)(p13;q12). EWS encodes a putative RNA binding protein of unknown function with an N-terminal domain that mediates potent transcriptional activation when fused to heterologous DNA binding domains. WT1 is a tumor suppressor gene initially identified based on its inactivation in Wilms tumor. The chimeric proteins resulting from these chromosomal translocations usually possess gain-of-function transcriptional activities and define histologically and biologically distinct tumor types. EWS-WT1 has two isoforms of EWS-WT1(-KTS) and EWS-WT1(+KTS). Previous studies have identified several EWS-WT1(-KTS) target genes, most of which are involved in growth factor signaling. In the current study, using an exogenous EWS-WT1(-KTS) induction system along with the selection from candidates for target genes based on the microarray data, we identified fibroblast growth factor receptor 4 (FGFR4) as a potential EWS-WT1(-KTS) target and this induction accompanied with increased phosphorylation form of Akt and MAPK, suggesting a post-transcriptional modulation by EWS-WT1(-KTS). In addition, CTNNB1 was also identified as a potential EWS-WT1(-KTS) target that defines epithelial characteristics of DSRCT. Furthermore, up-regulation of CTNNB1 driven by EWS-WT1(-KTS) was independent of FGFR4 regulation. Expressions of FGFR4 and CTNNB1 in DSRCT clinical samples were confirmed by immunohistochemistry. This study provides regulatory mechanism of FGFR4 in DSRCT and also novel insights into the acquisition of epithelial characteristics in DSRCT.

Keywords: Desmoplastic small round cell tumor; EWS-WT1; Target gene; FGFR4; CTNNB1

Introduction

Desmoplastic small round cell tumor (DSRCT) is a rare but highly aggressive malignant tumor that typically involves the abdominal or pelvic peritoneum in children and young adults [1]. This tumor is characterized histologically by solid nests of small round-cell tumor cells expressing epithelial, muscular, and neural markers, surrounded by a dense reactive stroma. This tumor is also characterized by the presence of specific EWS-WT1 fusion genes related to the recurrent chromosomal translocation, t(11;22)(p13;q12) [2,3]. EWS encodes a putative RNA binding protein of unknown function with an N-terminal domain that mediates potent transcriptional activation when fused to heterologous DNA binding domains [4]. The Wilms tumor suppressor (WT1) was initially identified based on its inactivation in Wilms tumor [5]. The chimeric proteins resulting from these chromosomal translocations usually possess gain-of-function transcriptional activities, and define histologically and biologically distinct tumor types. Alternative isoforms of either EWS-WT1(-KTS) or EWS-WT1(+KTS) have also been shown to differ in DNA binding affinity and specificity [6,7]. The molecular targets and associated mechanisms of these fusion proteins have not yet been fully characterized. The molecular targets of EWS-WT1(-KTS) chimeric proteins that have been reported include insulin-like growth factor-I receptor (IGF-IR) gene [8-10]; the beta-chain of the interleukin-2/15 receptor (IL-2/15Rβ) [11]; platelet-derived growth factor-A (PDGFA) [12]; BAI1-associated protein 3 (BAIAP3), which encodes a protein implicated in regulated exocytosis; a potential regulator of growth-factor release [13]; and T-cell acute lymphoblastic leukemia-associated antigen 1 (TALLA-1) [14]. In addition, expression of the transmembrane protein, leucine-rich repeat containing 15 (LRRC15), was found to be induced by EWS-WT1(+KTS) [15]. Recently, equilibrative nucleoside transporter 4 (ENT4), which encodes a pH-dependent adenosine transporter has been identified to be transcriptionally activated by both isoforms of EWS-WT1 [16]. In addition to genes involved in growth signaling that define biological behavior, it is apparent that the tumor-specific chimeric gene products also define histological characteristics [17]. However, genes that modulate histological features of DSRCT have not yet been identified as EWS-WT1 targets.

The fibroblast growth factor (FGF) family of signaling molecules plays important roles in development, angiogenesis, and cancer [18]. The FGF family is composed of 22 structurally related polypeptides that bind to 4 receptor tyrosine kinases (FGFR1-FGFR4) and 1kinase-deficient receptor (FGFR5) [18]. Interactions between FGF proteins and FGFR1-4, specifically FGF19 and FGFR4, result in receptor

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homodimerization and autophosphorylation, and subsequential initiation of multiple signaling pathways [18]. Overexpression of FGFR has also been implicated in neoplastic transformation. For example, FGFR4 is overexpressed in several types of human tumors, including colon, liver, breast, neuroendocrine, pancreatic carcinomas, hepatocellular carcinoma, and pediatric rhabdomyosarcomas [19-26]. Furthermore, comparison of gene expression profiles of apoptosis-sensitive breast cancer cells and corresponding apoptosis-resistant clones led to the identification of FGFR4 as a gene with increased gene expression in response to treatment with doxorubicin or cyclophosphamide [27]. By cDNA microarray analysis, FGFR4 has been identified as one of the most highly expressed tyrosine kinase receptors in DSRCT [28], and this gene has also been described previously as a candidate target of EWS-WT1(-KTS), although this has not been validated [11]. In the present study, we confirmed that FGFR4 was highly expressed in DSRCT clinical samples and in a JN-DSRCT cell line, and that FGFR4 overexpression was primarily restricted to EWS-WT1(-KTS) transfected cells. Furthermore, the use of an exogenous EWS-WT1 induction system revealed that CTNNB1, a Wnt signaling component, which is also involved in FGF/FGFR signaling, might be a potential downstream target of EWS-WT1(-KTS).

Materials and Methods

Cell culture and preparation of cell blocks of JN-DSRCT

The JN-DSRCT cell line was kindly provided by Dr. Iwasaki, Fukuoka University, Japan [29], and grown in a 1:1 mixture of DMEM and Ham's F-12 (Kyokuto Pharmacology, Tokyo, Japan), supplemented with 10% FCS and kanamycin sulfate (100 μg/ml). At 80% confluency, cells were treated with trypsin and harvested by centrifuge to make a cellblock of JN-DSRCT for immunocytochemical analysis. In addition, breast cancer cell lines and synovial sarcoma cell lines were incubated in appropriate culture media and used as control samples for the following RT-PCR and western blotting.

RT-PCR

RNA was extracted from various cell lines and the same amount of RNA from each cell line was converted to cDNA. The relative expression level of FGF19 was semi-quantitatively examined by amplifying the same amount of template cDNA with various cycles (25, 30, 35 and 40 cycles). TATA-binding protein (TBP) was used as an internal control. Primer pairs are available upon request.

Western blotting

Cells were lyzed with RIPA buffer (10 mM Tris (pH 7.4), 5 mM EDTA, 300 mM NaCl, 10% Glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF) and centrifuged at 14,000rpm for 30 min. Supernatants were mixed with 4× SDS-PAGE sample buffer (Invitrogen) and heated at 100°C for 5 min prior to resolution by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were preincubated with 5% non-fat dry milk in TBS-T (TBS with 0.1% of Tween20) before incubation with specific primary antibodies for 2 h. Specific molecules were visualized with horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, UK). The following antibodies were used: rabbit polyclonal anti-FGFR-4 IgG (sc-124, carboxyl terminal human FGFR-4; Santa Cruz Biotechnology), mouse monoclonal antibody β-catenin (BD transduction lab, clone 14), anti-active β-catenin (anti-ABC), (Upstate, clone 8E7), anti-cytokeratin (Becton Dickinson, clone CAM5.2), rabbit monoclonal antibody to human cyclin D1 (DBS, clone SP4), MAPK (Cell Signaling Technology, clone L34F12), pMAPK (Cell Signaling Technology, clone 20G11), Akt (Cell Signaling Technology, #9272), pAKT (Cell Signaling Technology, clone 587F11). Furthermore, mouse monoclonal anti-α-tubulin (Sigma, B-5-1-1) antibody and mouse monoclonal anti-GAPDH (SantaCruz, 6C5) antibody were used as alternative internal controls.

Immunohistochemistry and immunocytochemistry

Two DSRCT clinical samples were prepared for immunohistochemistry. Immunohistochemical analysis was performed using the following antibodies: rabbit polyclonal anti-FGFR-4 IgG (Santa Cruz Biotechnology, clone sc-124) diluted at 1:200, mouse monoclonal antibody β-catenin (BD transduction lab, clone 14) diluted at 1:200, and mouse monoclonal antibody E-cadherin (BD transduction lab, clone 36/E-cadherin) diluted at 1:1000. In addition, immnocytochemical analysis was performed using the JN-DSRCT cell block. For immunocytochemistry, SKBR3-a breast cancer cell line, was used as a positive control. The antibodies used for immunocytochemistry were as follows: rabbit polyclonal anti-c-erbB-2 antibody (DakoCytomation Envision, #A0485), rabbit polyclonal anti-ErbB-3antibody (Santa Cruz, C-17), and mouse monoclonal anti-epidermal growth factor Receptor (EGFR) antibody (Zymed Laboratories, Clone 31G7).

Establishment of tetracycline-regulated EWS-WT1(-KTS) inducible cell line

T-Rex HeLa cells (Invitrogen, life technologies Japan) were cultured in 10 cm dishes with DMEM supplemented with 10% FBS (Tet system approved FBS; BD Bioscience, NJ). At 70% confluency, cells were transfected with 2 μg of DNA of pcDNA4-TO-EWS-WT1(-KTS), which is tagged with myc, by using Fugene6 (Roche) transfection reagent, as previously described [17]. At 48 h after transfection, drug selection was started in a fresh medium supplemented with 400 μg/ml Zeocin (Invitrogen) for 6 weeks, and drug-resistant colonies were isolated. Cells from isolated colonies were treated with 1 μg/ml tetracycline (Invitrogen) to induce EWS-WT1(-KTS)expression.

Immunofluorescence

HeLa cell lines were grown on several glass cover slips in DMEM with 10% FCS and transfected with pcDNA4-EWS-WT1(-KTS) for 48 h. Attached cells on the cover slips were fixed in 4% paraformaldehyde for 10 min and then treated with 0.1% Triton X-100 in PBS for 5 min. The fixed cells were incubated overnight at 4°C for double staining with rabbit polyclonal anti-FGFR-4 IgG (sc-124, carboxyl terminal human FGFR-4; Santa Cruz Biotechnology) diluted at 1:200 and with mouse monoclonal myc-antibody (Invitrogen) for myc-tagged-EWS-WT1(-KTS) diluted at 1:1000. After rinsing 3 times, a secondary antibody (Alexa Fluor 488-conjugated anti-mouse immunoglobulin; Molecular Probes, Eugene, OR) was allowed to react with the preparation, and after a brief rinse, Alexa Fluor 568-conjugated anti-rabbit immunoglobulin (Molecular Probes) was allowed to react for 60 min. The results were visualized by indirect immunofluorescence.

Knockdown of FGFR4 in the JN-DSRCT cell line

To see whether FGFR4 affects β-catenin expression level in a JN-DSRCT cell line, knockdown of FGFR4 was performed using siRNA according to the manufacturer's protocol. Both FGFR4 and non-targeting siRNAs were purchased from Dharmacon (Thermo Fisher Scientific K.K.). JN-DSRCT cells were harvested to extract protein for

western blotting 48 h after transfection. Furthermore, cell proliferation rates were counted at every 24 hrs after each siRNA transfection.

Results

FGFR4 is overexpressed in JN-DSRCT cell line and human DSRCT

Tyrosine kinase receptors were previously found to be highly expressed in DSRCT, based oncDNA microarray data from 139 sarcoma samples and 17 cell lines or xenografts representing 5 different sarcoma histologic subtypes (Supplementary Table 1) [28]. These expression microarray data are freely available at http://cbio.mskcc.org/Public/sarcoma_array_data/. In addition to ERBB2 (HER2) and FGFR4 listed in Supplementary Table 1, the expressions of ERBB3 (Her3) and EGFR were examined by immunocytochemistry in the JN-DSRCT cell line. Among them, FGFR4 was confirmed by immunocytochemistry to be the most highly expressed tyrosine-kinase receptor gene product in the JN-DSRCT cell line (Figure 1A-G). In addition, overexpression of FGFR4 was shown by immunohistochemistry in one of the 2DSRCT clinical samples (Figure 1H); weak FGFR4 expression was observed in the second clinical sample (data not shown).Using western blot, we revealed that FGFR4 was highly expressed at the protein level in the JN-DSRCT cell line compared to other cell lines (Figure 2A).

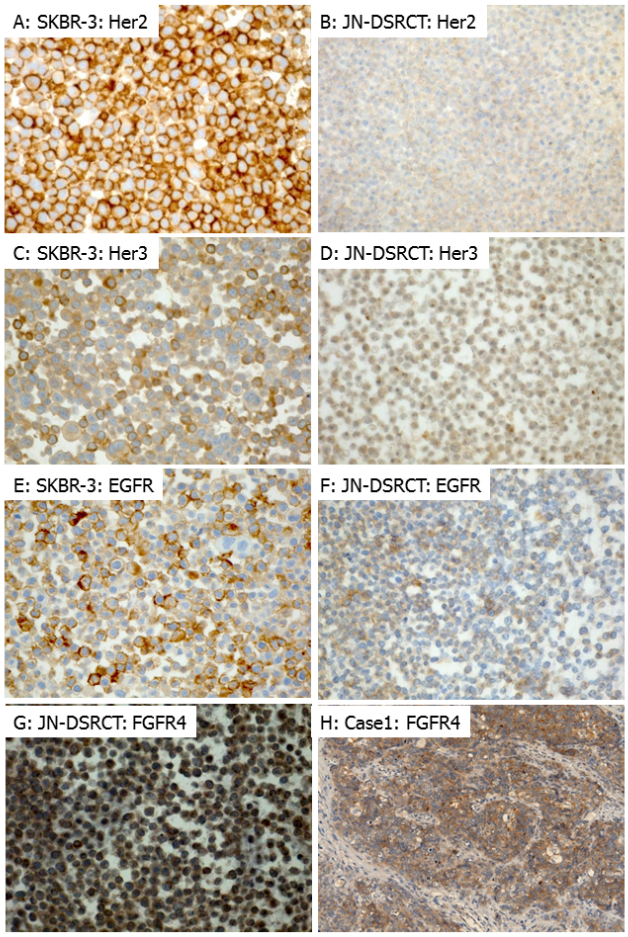

Figure 1: Immunocytochemical analysiswas performed using cell blocks of JN-DSRCT and SKBR-3 cell lines. In this assay, SKBR-3, a breast cancer cell line, was used as a positive control for HER2, HER3, and EGFR expressions. HER2, HER3, and EGFR expressions were not observed in JN-DSRCT cell line (B, D, and F), though expressions of these proteins were seen in SKBR-3 cells (A, C, and E). This analysis confirmed FGFR4 overexpression in a JN-DSRCT cell line (G). FGFR4 was overexpressed in one of the 2DSRCT clinical samples (H).

FGF19, the ligand for FGFR4, is expressed at lower levels in the JN-DSRCT cell line

We next examined the mRNA expression level of FGF19, the ligand for FGFR4, by semi-quantitative RT-PCR. Compared to the FGF19 expression level in other cell lines except SKBR-3 and AU565, the expression level of FGF19 in the JN-DSRCT cell line was relatively lower (Figure 2B).

Exogenous expression of EWS-WT1 in HeLa cells induced FGFR4 and β-catenin expression

First, to see whether FGFR4 is a potential target of EWS-WT1(-KTS), immunofluorescence was performed after transfection of EWS-WT1(-KTS) in HeLa cells. FGFR4 expression was restricted to the EWS-WT1(-KTS) transfected cells (Figure 3A), suggesting that FGFR4 is a potential target of EWS-WT1(-KTS). This was also confirmed by the induction of EWS-WT1(-KTS) in HeLaTrex cells (Figure 4A). Next, because it has been shown that Wnt and FGF signaling cross-talk during a variety of cellular process [30] and that DSRCT has epithelial characteristics, we evaluated the expression levels of β-catenin in EWS-WT1(-KTS)-induced cells after confirmation of β-catenin expression in DSRCT clinical samples. β-catenin expression in DSRCT was observed at membrane and cytoplasm, and E-cadherin membranous expression was also noted (Figure 3B). The total expression level of β-catenin was increased in EWS-WT1(-KTS)-induced HeLa cells (Figure 4A). Interestingly, β-catenin has also been shown to be one of the over expressed genes in DSRCT compared to other translocation-sarcomas (data not shown).

Induction of exogenous EWS-WT1 expression accompanied by an increase in pMAPK and pAkt

We further evaluated a possible change in the expression level of keratin as a marker for epithelial differentiation; however, no changes were observed (Figure 4A). This exogenous EWS-WT1 expression was accompanied by increased expression of the phosphorylated forms of MAPK and Akt, though total expression level of MAPK and Akt kept almost the same level (Figure 4A).

Knockdown of FGFR4 does not influence active β-catenin expression level in the JN-DSRCT cell line

Because, it has been demonstrated that the loss of FGFR4 leads to reduced β-catenin pathway signaling and decreased tumor growth *in vivo* and clonal growth *in vitro* [31], we next knocked down FGFR4 transiently by using siRNA in the JN-DSRCT cell line. However, we did not observe alterations in the expression level of cyclinD1 or β-catenin isoforms, including the active form of β-catenin (Figure 4B), suggesting that β-catenin overexpression driven by EWS-WT1(-KTS) was independent of FGFR4 overexpression. Furthermore, this transient knockdown of FGFR4 did not cause an obvious difference in the cell proliferation rate (Figure 4C).

Discussion

The immunohistochemical profile of DSRCT shows divergent differentiation characterized by the co expression of epithelial and mesenchymal markers with occasional expression of myogenic and

neurogenic markers. In immunohistochemical analyses, DSRCTs show keratin expression in 86% and epithelial membrane antigen in 93% [32] higher than that observed in synovial sarcoma, which is well-known to exhibit epithelial characteristics. In this study, we observed membranous expression of β-catenin in 2 DSRCT clinical samples. In addition, β-catenin expression was up regulated by the exogenous expression of EWS-WT1(-KTS) in HeLa cells, suggesting that CTNNB1 could be a potential target of EWS-WT1(-KTS). β-catenin is a multi-functional protein that plays an important role in maintaining cell-cell adhesion and acts as a downstream effector of the Wnt-signaling cascade. Thus, it is possible that EWS-WT1(-KTS) is involved in the acquisition of epithelial characteristics in DSRCT; however, we could not confirm an increase in the keratin expression in our exogenous EWS-WT1(-KTS) expression model by using the HeLa cell line in which transcription of the E-cadherin gene was constitutively and strongly repressed. Regarding this point, the authors have experienced that E-cadherin protein expression was not observed using the same tetracycline-regulated exogenous SYT-SSX expression system regardless of the increased E-cadherin mRNA expression [17].

A recent study has shown that co activation of FGF and Wnt signaling pathways in tumors leads to more malignant phenotypes [30]. Furthermore, it has also been demonstrated that FGFR4

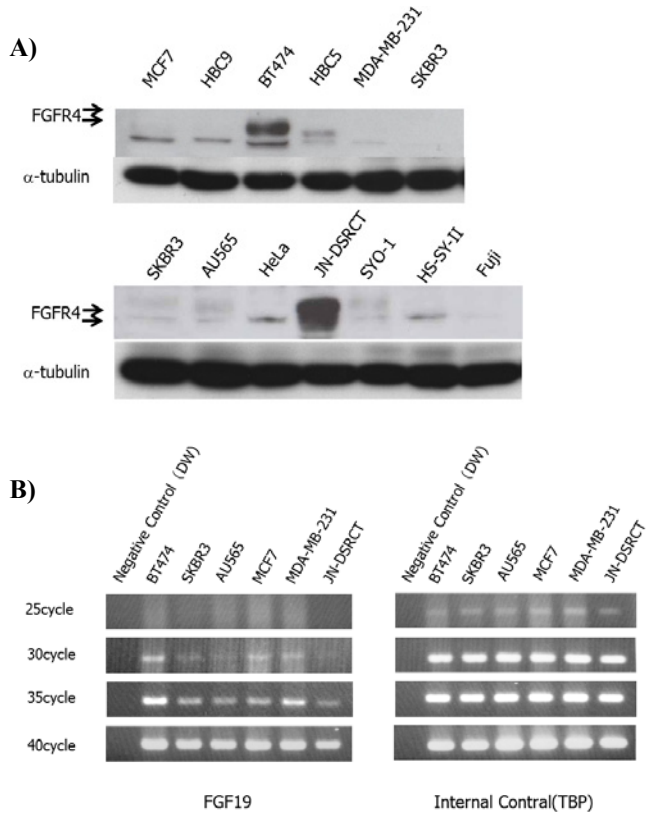

Figure 2: FGFR4 and FGF19 expression in various cell lines. A) FGFR4 expression in various cell lines. FGFR4 expression at the protein level was shown to be higher in a JN-DSRCT cell line than in other cell lines. MCF7, HBC5, HBC9, BT474, SKBR3, AU565 and MDA-MB-231 are breast cancer cell lines. SYO-1, HS-SY-II and Fuji are synovial sarcoma cell lines. B) FGF19 expression level by RT-PCR in various cell lines. Expression level of FGF19mRNA in JN-DSRCT is lower than that in other cell lines except SKBR-3 and AU565.

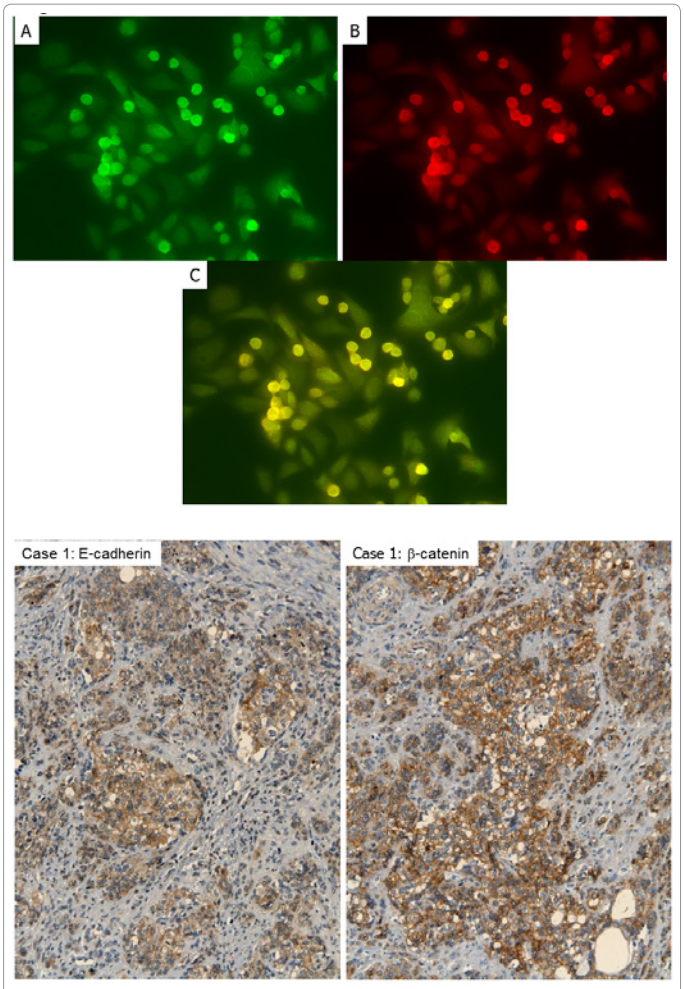

Figure 3: using HeLa cells. A) Mergedphotograph (C) shows that FGFR4 expression (A: red) is restricted to EWS-WT1(-KTS) transfected cells (B: green). B) Immunohistochemical analysis of DSRCT clinical sample. E-cadherin and β-catenin membranous expression was seen in DSRCT case (case 1). Note that some cytoplasmic staining of β-catenin is also seen.

knockdown resulted in reduced β-catenin pathway signaling and decreased tumor growth *in vivo* and clonal growth *in vitro* and that FGF19 increased GSK-3β phosphorylation and active β-catenin [31]. Therefore, we assessed the possible cross-talk between FGF19/FGFR4 signaling and Wnt signaling in a JN-DSRCT cell line. Regarding this point, we confirmed the presence of β-catenin cytoplasmic staining and membranous expression in both DSRCT clinical samples, as well as FGFR4 overexpression and lower mRNA expression of FGF19 in a JN-DSRCT cell line. However, a transient knockdown of FGFR4 in JN-DSRCT cell line did not alter the expression of β-catenin isoforms, including the active form. Furthermore, the expression of cyclinD1, one of the downstream targets of the Wnt signaling pathway [32,33], was not either altered by this treatment. By transient knockdown of FGFR4 using siRNA transfection in JN-DSRCT cell line, we confirmed that β-catenin overexpression and subsequent possible Wnt signaling activation in DSRCT is independent of FGFR4 expression. Thereby, in this experiment cyclin D1 expression which we selected as a marker of Wnt signaling activation, although this is not always the case in soft tissue sarcomas [34], was not altered after FGFR4 siRNA transfection. Taking into consideration that FGF19 was expressed at much lower

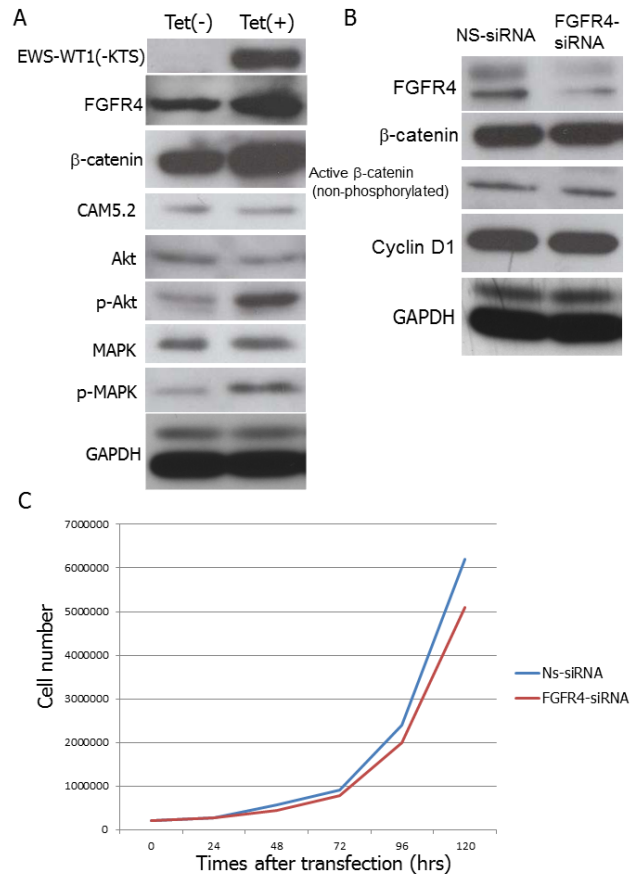

Figure 4: A) Induction of EWS-WT1 by tetracycline in HeLaTrex cells. After induction of EWS-WT1(-KTS), the total expression levels of β-catenin were increased, together with the phosphorylated forms of Akt and MAPK. B) Transient knockdown of FGFR4 in a JN-DSRCT cell line did not alter the protein expression levels of β-catenin, the activated form of β-catenin, or cyclinD1. C) Transient knockdown of FGFR4 in a JN-DSRCT cell line did not affect significant change in cell proliferation rate.

levels in the JN-DSRCT cell line, these findings suggest that cross-talk between FGF19/FGFR4 and Wnt signaling pathways does not have an important role in the development of DSRCT.

In some malignancies, overexpression of tyrosine kinase receptors is associated with oncogenic mutations in the tyrosine-kinase receptor genes themselves, for example, c-kit and PDGFRA in gastrointestinal stromal tumor [35], and EGFR in lung adenocarcinoma [36]. A recent study characterized FGFR4 mutations in only a subset of embryonal rhabdomyosarcoma, and it has also been shown that mutations in genes involved in the growth signaling pathway were absent in a relatively large set of DSRCT [37]. A two-fold increase in FGFR4 has also been observed in an EWS-WT1(-KTS)-inducible system in a U2OS osteosarcoma cell line; however, this finding has not yet been replicated [11]. In the present study, we also showed overexpression of FGFR4 in a JN-DSRCT cell line and in 1 of the 2 DSRCT clinical samples. Although we could not examine the types of EWS-WT1 isoforms in the 2DSRCT clinical samples used in this study, 2 isoforms of EWS-WT1 with differing oncogenic properties were previously characterized in DSRCTs [7]. It has also been shown that the JN-DSRCT cell line expresses a variant form of EWS-WT1 [29]. Considered in conjunction with data reported by Wong et al. [11], our findings suggest that overexpression of FGFR4 in DSRCT is partly derived from the transcriptional up-regulation of directed by EWS-WT1(-KTS).

Although, some DSRCT cases are responsive to multi-drug chemotherapy [38,39], it has been shown that conventional chemotherapy is not generally effective in DSRCT. Therefore, the development of effective therapeutic strategies is the primary goal of DSRCT research. A recent study has shown that antibodies for IGF-IR, previously identified as a target of EWS-WT1(-KTS), showed preliminary evidence of durable antitumor activity combined with anmTOR inhibitor [40]. Furthermore, a recent study has demonstrated that FGFR4 gene expression was up-regulated in doxorubicin-treated, apoptosis-resistant breast cancer cell clones and that ectopic expression of FGFR4 in cancer cells led to reduced apoptosis sensitivity on treatment with doxorubicin or cyclophosphamide [27]. These chemotherapeutic drugs are currently used for the treatment of DSRCT. Thus, FGFR4 expression driven by EWS-WT1(-KTS) in DSRCT might be associated with chemo-resistance in this tumor. Furthermore, it has been reported that targeting FGFR4 inhibits hepatocellular carcinoma in preclinical mouse model [25]. In addition, it has been demonstrated that FGFR4 blockade exerts distinct antitumorgenic effects in human rhabdomyosarcoma, especially in alveolar phenotype [24]. On the other hand, it has been shown that FGF19 expression correlates with tumor progression and poorer prognosis of hepatocellular carcinoma [26], and that therapeutic inactivation of FGF19 could be beneficial for the treatment of colon and liver cancer [41]. Therefore, inhibition of the FGF19/FGFR4 signaling pathway was also expected to have antitumorigenic effects on DSRCT cells. However, lesser expression level ofFGF19 in JN-DSRCT cells suggests that the inactivation of FGF19 would be less effective for blocking the FGFR4 signaling pathway in DSRCT. Furthermore, the blocking of FGFR4 would be predicted to be so far less effective, given that we did not observe differences in the cell proliferation rates after treatment of a JN-DSRCT cell line with FGFR4 siRNA. However, further studies are necessary to evaluate the therapeutic effects of constitutive inhibition of FGFR4 signaling.

Conclusions

EWS-WT1(-KTS) chimeric protein is a potential transactivator of FGFR4. In addition, CTNNB1 could be a potential target of EWS-WT1, and this transactivation of CTNNB1 is likely to play an important role in defining epithelial characteristics in DSRCT.

Co f Interest Statement

All authors declare that we have no conflict of interest.

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