IDENTIFICATION OF P21 GENE AS A DIRECT TARGET OF EWS-FLI1 FUSION PROTEIN IN EWING'S SARCOMA CELLS

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Introduction
The translocation t (11; 22) is a common chromosomal abnormality detected in Ewing's family of tumors (ET) including Ewing's sarcoma and primitive neuroectodermal tumor (PNET). The translocation result in an EWS-FlI1 fusion gene, made up of the 5' half of EWS gene on chromosomal 22 fused to the 3' half of the FlI1 gene on chromosomal 11 which contains ets like DNA binding domain. We have previously reported that EWS-FlI1 antisense oligonucleotide inhibits proliferation of various ET cell lines and alters cell cycle distribution, blocking in the cell cycle at G0/G1 phase. We also elucidated the altered expression of G1 regulatory molecules by the antisense oligonucleotide both at mRNA and protein levels, including downregulation of the G1 cyclins (cyclin D1, cyclinE), and induction of cyclin-dependent kinase inhibitors (p21, p27), Cyclin D1 and p21 genes contain ets like binding sites in their promoters. Thus, these genes might be one of the direct targets of EWS-FlI1 fusion protein. Here, we investigated the regulation of p21 gene expression by EWS-FlI1 fusion protein.

Materials and Methods
Human ET cell line SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37 under 5% CO2 plus 95% air. Murine fibroblast Balb3T3 cells were grown in DMEM containing 10% calf serum (CS). p21 promoter sequence (-2320/+75) was cloned into Hind III site of pG2 basic vector (Promega). Digestion of pG2-p21construct with unique restriction enzymes and religation yield the various sets of deletion reporter plasmid constructs which lack ets binding domains. Prior to the transfection, 1x10^5 SK-N-MC cells were seeded into each well of 6-well plates. Twenty-four hours later, transfections were performed using Fugene6 (Roche) according to the manufacture's protocol. Briefly each well was transfected with 1.0g of each reporter construct and 0.1g of pRL plasmid vector (Promega) to standardize for transfection efficiency. Twenty-four hours after transfection, 10 M of sense oligonucleotides or antisense oligonucleotides were added to the medium and incubated for further 48hrs, then cells were solubilized by the passive lysis buffer (Promega) and subjected to the luciferase assay. Balb3T3 cells were cotransfected with p21 promoter and EWS-FlI1, FlI1 expression plasmid or Mock plasmid vector. Twenty-four hours after the transfection, luciferase assays were performed. pGL3-Control and pRL-SV40 (Promega) were used as a positive control and an internal control for normalization of transfection efficiency, respectively. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and Microplate Plus LB 96V (EG&G Berthold). Electromobility shift assays (EMSAs) were performed to detect the direct interaction of EWS-FlI1 fusion protein and ets binding sites on the p21 promoter. Nuclear extracts of SK-N-MC were incubated with ^32P-labeled wild type probes which contain ets binding sites of p21 promoter with or without wild-type and mutant competitors (1st ets binding site wild type, 5'-GGGTTCCTGGCCATCAGGAACATGTCCCAAC-3', 1st ets binding site mutation, 5'-GGGTTCCTGGCCATCAGGAACATGTCCCAAC-3', 2nd ets binding site wild type, 5'-GGCGAGCTGCTTTAGAGGAAGAAGACTGGGCATGTCTGGGC-3', 2nd ets binding site mutation, 5'-GGCGAGCTGCTTTAGAGGAAGAAGACTGGGCATGTCTGGGC-3') on the gel shift assay kit (Stratagene) according to the manufacturer's instructions.

Results
In SK-N-MC cells, p21 promoter activity was very low level. However, when the cells were treated with the antisense oligonucleotide, expression of EWS-FlI1 was completely abolished and p21 promoter activity of the treated cells was enhanced to 3-fold of that of control cells. (Fig1) Moreover, deletion of ets binding sites form p21 promoter resulted in enhancement of the luciferase activities of the SK-N-MC cells. Deletion of the first ets binding site induced relative luciferase activity approximately 2.5-fold over the control. Deletion of 2nd ets binding site did not influenced the p21 promoter activity. However, when both 1st and 2nd ets binding sites were deleted, synergistic enhancement of luciferase activity was observed. In cotransfection assays, FlI1 induced luciferase expression of the longest p21 reporter construct approximately 2-fold over the control in balb3T3 cells, whereas EWS-FlI1 suppressed the promoter activity by 65%.(Fig.2) EMSAs demonstrated that EWS-FlI1 fusion protein bind to the p21 promoter sequence on both of the ets binding sites. This binding was greatly attenuated by a molar excess of unlabeled wild type sequence of the ets binding sites, not by the oligonucleotide containing mutation at the ets-binding site.

Discussion
The EWS-FlI1 fusion gene is a potent single-step-transforming gene in NIH3T3 cells. However, little is known about the basis for this transformation and the biological significance of EWS-FlI1. We have shown that treatment with antisense oligonucleotides reduced the expression of EWS-FlI1 and enhanced the activity of p21 promoter. The present study indicated that p21 might be a direct target of EWS-FlI1 transcription factor. Recent study showed that TGF (transforming growth factor)-beta receptor was downregulated directly by the EWS-FlI1 fusion protein. Although TGF-beta might influence the cell cycle regulation, our data suggested the direct association of EWS-FlI1 and cell cycle regulatory molecule p21. EWS-FlI1 fusion protein did not downregulate the expression of p21. The cell cycle inhibitor protein p21 modulates cyclin-dependent kinase activity resulting in cell growth arrest. In addition, p21 plays important roles in the control of cell senescence, apoptosis, and carcinogenesis. In conclusion EWS-FlI1 fusion protein directly inhibited the p21, which may partly explain the tumorgenetic activity of EWS-FlI1 fusion protein.