The Prognostic and Therapeutic Relevance of p27kip1 in Ewing’s Family Tumors

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ABSTRACT

**Purpose:** Ewing’s family tumors (EFTs) display the characteristic fusion gene EWS-Fli1. We have reported EWS-Fli1 may promote the cell cycle progression accompanied by the suppression of the expression of cyclin-dependent kinase inhibitor p27kip1 in EFT cells. Here, we describe the prognostic and therapeutic relevance of p27 in EFTs.

**Experimental Design:** We examined tumor samples taken from 21 patients with primary EFTs for the expression of p27 protein immunohistochemically and evaluated its correlation with clinical outcome. We also investigated the usefulness of p27 as a therapeutic strategy in *vivo* and *in vitro* using p27 expression adenovirus. Finally, we examined the process of EWS-Fli1-mediated reduction of p27 expression.

**Results:** Immunohistochemical analysis showed that a low expression level of p27 protein was related to poor event-free survival in an univariate analysis and that the expression level of p27 correlated more significantly with patient survival than several clinical factors in a multivariate survival analysis. Overexpression of p27 with the adenoviral vector remarkably inhibited the cell growth in all EFT cells tested and further induced apoptosis in the wild-type p53 EFT cells. In *vivo* studies demonstrated a reduction in tumor growth of EFT xenograft in nude mice treated with the intratumoral injection of p27-expressing adenovirus. EWS-Fli1 did not significantly affect the p27 promoter activity and p27 mRNA levels. However, the challenge of the proteasome inhibitor caused accumulation of p27 protein in EFT cells. These data strongly suggest EWS-Fli1 might attenuate p27 protein level via activation of the proteasome-mediated degradation pathway.

**Conclusions:** Our findings provide the first evidence of the prognostic relevance of p27 expression in EFTs. We propose p27 as a novel and powerful therapeutic factor for the molecular target therapy of EFTs.

INTRODUCTION

Ewing’s sarcoma and primitive neuroectodermal tumors are small, round cell tumors that arise in the bone and soft tissues and are classified as Ewing’s family tumors (EFTs). EFTs are rare tumors and account for ~2% of childhood cancers (1). EFTs are aggressive malignant tumors with a high propensity for recurrence and distant metastasis. Despite some improvements in treatment and outcome, the 5-year survival rate for EFTs is ~50% (2), indicating the needs for new treatment strategies.

A specific translocation between chromosomes 11 and 22 is found in >85% of EFTs (3). As a result of this translocation, the EWS gene on chromosome 11 fuses to the Fli1 gene, a member of the ETS family of transcription factors, on chromosome 22, which generates the EWS-Fli1 chimeric gene (4). This chimeric gene product, EWS-Fli1 protein, has functions as an aberrant transcription factor. A number of studies have suggested that EWS-Fli1 can act as an oncogene. The transduction of EWS-Fli1 transformed NIH3T3 cells (5). Forced expression of the antisense RNA against EWS-Fli1 resulted in the loss of tumorigenicity in EFT cells (6).

Previously, we have reported that the use of antisense oligonucleotides to suppress EWS-Fli1 expression caused the arrest of EFT cell proliferation at the G1-G0 phase in the cell cycle (7) and that EWS-Fli1 reduced the expression of p21WAF1 and p27kip1 (p27) in EFT cells (8, 9).

p27 was first identified as a cell cycle inhibitor in the cells arrested by transforming growth factor β, and the expression of p27 is regulated by growth inhibitory cytokines and contact inhibition (10–14). p27 is one of cyclin-dependent kinase inhibitors, which inhibit cyclin-dependent kinase activities and cause cell cycle arrest. Clinically, a low level of p27 expression has been reported to correlate inversely with the tumor progression or the poor prognosis in a variety of human malignancies, including breast, prostate, colon, and lung cancers (15–18). A recent study using p27 knockout mice indicated that p27 plays an important role in the inhibition of tumor development and shows the characteristics of a tumor suppressor gene (19).
Overexpression of p27 by replication-deficient recombinant adenovirus resulted in apoptosis and diminished malignancy potential in human breast, lung, and prostate cancer cell lines (20–22). Because EWS-Fli1 caused the down-regulation of p27 in vitro (8), p27 expression may have clinical correlation with disease phenotype, therapeutic responsiveness, and patient outcome. Furthermore, if the loss of p27 plays a causative role in EFTs, it might provide a new target for the therapeutic intervention of EFTs.

In the present study, we examined whether the expression of p27 could reliably predict the prognosis of patients with EFTs. We also investigated the effects of the ectopic overexpression of p27 using replication-deficient recombinant adenovirus in EFT cells in vitro and in vivo.

**MATERIALS AND METHODS**

Oligonucleotides and Reagents. The sequences of the antisense and sense phosphorothioate oligodeoxynucleotides against EWS-Fli1 (AS-ODNs and S-ODNs, respectively) were described previously (7). A proteasome inhibitor, Z-Leu-Leu-Leu-H (aldehide; ZLLLHal) was purchased from Peptide Institute (Osaka, Japan). The antibodies used are as follows: mouse monoclonal antihuman p27 (Kip1; Transduction Laboratories, Lexington, KY); mouse monoclonal anti-Ki-67 (clone MIB1; Immunotech, Marseille, France); mouse monoclonal antihuman retinoblastoma protein (G3-245; PharMingen, San Diego, CA); rabbit polyclonal antihuman phosphorylated retinoblastoma protein (Rb) (Ser̴780; Cell Signaling, Beverly, MA); mouse monoclonal anti-actin (Chemicon, Temecula, CA); rabbit polyclonal antihuman Fli1 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-PARP p85 fragment pAb (Promega, Madison, WI); and horseradish peroxidase-conjugated antimouse or antirabbit secondary antibodies (Santa Cruz Biotechnology).

**Patient Population.** A series of 21 patients preoperatively diagnosed with EFTs at Kyushu University Hospital, National Kyushu Cancer Center and National Kyushu Medical Center, between 1982 and 2002 were used in this study. The diagnosis of EFTs was made based on the histological features. There were 13 male and 8 female patients, ranging from 8 to 75 years old, with median age of 18 years. All cases presented with primary EFTs and were treated with systemic chemotherapy based on VACA or VAIA protocol [VACA: 12 courses of vincristine, cyclophosphamide, and doxorubicin (Adriamycin) alternating with actinomycin D; VAIA: using ifosfamide as a substitute for cyclophosphamide)] (23). The average follow-up period was 41.4 months from the time of diagnosis (range, 7–100 months). Five patients had metastatic disease at the time of diagnosis. Primary tumors were located in the lower extremities in 3 patients, in the upper extremities in 2 patients, and in the trunk in 16 patients. One patient developed a local recurrence and 14 patients developed distant metastases, and 12 patients died of disease (Table 1). By using reverse transcription-PCR (RT-PCR) and sequencing, EWS-Fli1 chimeric RNA was detected in 15 of 21 EFTs, with fusion of EWS exon 7 to Fli1 exon 6 (10 of 15; type I), five (2 of 15; type II), or seven (1 of 15; type IV). In two cases, EWS exon 10 to Fli1 exon 5 and EWS exon 9 to Fli1 exon 7 were observed. Event-free survival was defined as the time from study entry until progression of disease, detection of a second neoplasm, or death, whichever occurred first. The volume of the tumor was estimated by computed tomography scan or magnetic resonance imaging measure of the three diameters of the lesion, including soft tissues, following the method reported by Gobel et al. (24). The formulas varied according to the shape of the mass. When the mass was elliptical or spherical, the formula was as following: volume = A × B × C × 0.78 or volume = A × B × C × 0.52, respectively.

Immunohistochemistry. Five-micrometer thick sections of formalin-fixed and paraffin-embedded biopsy specimens taken from the tumors before treatments were used. The experiments were carried out with a combination of the standard streptavidin-biotin-peroxidase method (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) as described previously (25). Anti-p27 (×200; Transduction Laboratories) and anti-Ki-67 (×100; Immunotech) antibodies were applied. Sections of normal gallbladders, which were previously shown to have positive immunoreactivity for p27 (26), were also stained as controls.

The p27 labeling index (p27 LI) was defined as the percentage of tumor cells displaying nuclear immunoreactivity for p27, and it was calculated by counting p27-nuclear stained cells. The p27 LI was defined in the same manner as p27. Univariate and multivariate survival analyses were performed by using the log-rank test or the Cox proportional hazards regression model. The survival curve was estimated using the Kaplan-Meier method. For demonstration of associations between p27 LI and Ki-67 labeling index, Pearson’s correlation coefficient test was used.

**Cell Culture Experiments.** Three EFT cell lines, SK-N-MC and RD-ES obtained from the American Type Culture Collection (Manassas, VA), PNKT-1 established in our laboratory (27), and a murine fibroblast Swiss 3T3 cells and a human fibroblast hERT-BJ1 cells purchased from Clontech (Palo Alto, CA), were cultured at 37°C, 5% CO₂ in DMEM (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD). Other two EFT cells, VH-64 and WE-68, kindly provided by Dr. Frans van Valen, (Westfälische-Wilhelms-University, Münster, Germany), were cultured at 37°C, 5% CO₂ in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.).
Generation of replication-deficient adenoviral vectors that express human p27 (Ax-p27) or LacZ gene (Ax-LacZ) and the adenoviral infection were carried out as described previously (21). For cell growth and viability experiments, the cells were plated at a density of 2 × 10⁴ cells/well on 12-well plates in triplicate. The number of cells was counted every 24 h for 7 days using a Coulter Hematology Analyzer (Beckman Coulter, CA). The number of viable cells was also evaluated by trypan blue exclusion assay using hemocytometer on days 1–6.

ZLLLHal was freshly prepared in DMSO immediately before use. Vehicle control of DMSO was included in the studies. Cells were cultured for 24 h and treated with various concentrations of ZLLLHal for the required time. After the treatment, adherent cells were collected for additional analysis in different studies. In another experiment, SWISS3T3 cells were transfected with the EWS-Fli1 expression vector or empty vector, cultured for 24 h, and subsequently treated with 5 μM ZLLLHal for 12 h.

**In Vivo Tumor Experiments.** The SK-N-MC (5 × 10⁶) cells were injected s.c. in the flank of 6–8-week-old female athymic nu/nu mice. Treatment was initiated 2 weeks after the injection. The mice were randomized and placed into three groups and were transduced by three separate injections spaced 8 h apart with 100 μl of 10¹⁰ plaque-forming units of Ax-p27, Ax-LacZ, or 100 μl of saline, respectively. Every 48 h, the perpendicular diameters of s.c. SK-N-MC tumors were measured with calipers, and tumor volume was calculated as length × width² × 0.52. Statistical analysis was performed using one-factor ANOVA. For demonstration of significance, Fisher’s protected least significant difference test was used. P of <0.05 was considered to be statistically significant.

**Western Blot Analysis.** Cells lysates from infected cells were prepared and subjected to Western blot analysis as described previously (8). Twenty μg of each protein sample were electrophoresed in 4–12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA) and transferred onto a nitrocellulose membrane (Amersham, Arlington Heights, IL). The membranes were immunoblotted with the appropriate primary antibodies.

**DNA Fragmentation Assay.** For DNA fragmentation assay, the cells infected with Ax-p27 or Ax-LacZ at multiplicity of infection (m.o.i.) 10, including floating cells, were scraped and collected on day 3. The cells were dissolved in 100 μl of cell lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), and 0.5% Triton X-100] and then incubated on ice for 10 min. The lysates were then centrifuged at 16,000 × g for 5 min at 4°C. The supernatant was recovered and treated with RNase A (Roche, Indianapolis, IN) for 1 h at 37°C and then with protease K (Roche) for 1 h at 50°C. DNA was precipitated by adding isopropanol and 5 mM NaCl to the mixture, and the precipitates were resuspended in TE [10 mM Tris (pH 8.0)/1 mM EDTA]. The samples were electrophoresed on 1.2% agarose gels, which were stained with 0.5 mg/ml ethidium bromide (Sigma, St. Louis, MO) for 10 min to visualize the DNA ladder.

**Luciferase Assays.** A luciferase reporter plasmid (p27PF) containing a 3.5-kb fragment (~1358 ~ ~12nt) of human p27 gene (28) and a expression vectors for EWS-Fli1 were kindly provided by Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan) and Dr. Christopher T. Denny (Gwynnie Hazen Cherry Memorial Labs, University of California–Los Angeles, CA), respectively. Lysates from SK-N-MC cells cotransfected with p27PF and pRL-SV40 and treated with S- or AS-ODNs for 48 h or SWISS3T3 cells cotransfected with the reporter construct and the expression vector were prepared and subjected to luciferase assays as described previously (9). For each experiment, at least three independent transfections were performed, and the results were expressed as the mean + SD in relative units of reporter gene activity.

**Real-Time Quantitative RT-PCR.** Real-time quantitative RT-PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was performed as described previously (9, 29). SK-N-MC cells (2 × 10⁴) were cultured for 24 h in 35-mm dishes (Falcon-Becton Dickinson) and treated with 10 μM S- or AS-ODNs for 48 h. Total RNA isolations from the cells and reverse transcription reactions were performed as described previously (8). The primers used were forward primer, 5’-CTCTGAGGACACGCATTGGT-3’, and reverse primer, 5’-GAGTAGAAGAATCGTCGG-TTGCA-3’. The sequence of the TaqMan probe used to quantify the RT-PCR products of p27 mRNA was 5’-(Fam)CTTCCCTATTCCTGCGCATTGCTCC-(Tamra)-3’. The primers were custom-synthesized by Hokkaido System Science (Sapporo, Japan), and the probe was custom synthesized by PE Applied Biosystems (Tokyo, Japan). The standard DNA templates containing human p27 or glyceraldehyde-3-phosphate dehydrogenase cDNA sequences were generated by PCR and subcloned into pCR 2.1 TOPO vector (Invitrogen). Mixtures of the primers and probe for glyceraldehyde-3-phosphate dehydrogenase as an internal control were purchased from PE Applied Biosystems. The relative amount of p27 mRNA standardized against the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA was calculated. To test differences in luciferase assays and real-time quantitative RT-PCR between cells regarding treatments with oligonucleotides, the Student’s t test or Fisher’s protected least significant difference test was used. P of <0.05 was considered to be statistically significant.
10% were used as the cutoff point to define negative and positive expression for p27. There were no apparent differences in clinical parameters between the negative and positive expression groups. The survival curves estimated by the Kaplan-Meier method revealed that the length of survival of patients with p27-negative tumors was significantly shorter than that with p27-positive tumors (Fig. 2A and Table 2). The incidence of 5-year event-free survival was 10.7 and 83.3%, respectively ($P < 0.01$). In addition to p27 LI, an univariate survival analysis by the log-rank test using a tumor volume cutoff value of 200 ml demonstrated that event-free survival of patients with EFTs of tumor volume $< 200$ ml was significantly better than that of tumor-volume $\geq 200$ ml ($P < 0.05$; Fig. 2B and Table 2). Age, tumor site, serum lactate dehydrogenase levels, serum C-reactive protein levels, distant metastases at the time of diagnosis, or type of EWS-Fli1 did not significantly correlate with the survival of patients. However, multivariate survival analysis, performed by the Cox proportional hazards regression model, with stepwise selection of variables, revealed that only p27 LI had independent and significant prognostic effects on event-free survival (Table 2). Because p27 negatively affects the progression of the cell cycle through the inhibition of cyclin E-Cdk2, we used Ki-67 immunohistochemistry to estimate the cell proliferation activity in EFTs. The cells at G0 phase in the cell cycle do not express any Ki-67 antigens. In other words, the cells positive for Ki-67 are in cell cycle progression (30). Pearson’s correlation coefficient tests indicated a statistically significant correlation between p27 LI and Ki-67 labeling index ($r = 0.531$; Fig. 2C).

**Inhibition of EFT Cell Growth by the Expression of p27.** Because the expression of p27 might have prognostic significance in treating EFTs, we examined whether p27 introduction could become a new therapeutic strategy for EFTs. We decided to use a p27-expression adenovirus because of its highly effective gene transduction. For evaluation of the gene transduction efficiency of the adenovirus in EFT cells, we performed 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside staining on Ax-LacZ-infected cells. One hundred percent of the cells showed the expression of the reporter gene by the third day after

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**Fig. 1** Histological features of Ewing’s family tumors (EFTs) and immunohistochemical staining for p27. A, a representative p27-positive tumor. A tumor biopsy specimen diagnosed as EFTs (H&E, original magnification, $\times400$). B, immunostaining with the anti-p27 antibody of the same specimen shown in A. Streptavidin-biotin peroxidase method with hematoxylin counterstain was carried out. The figure shows typical immunohistochemistry patterns positive for p27. The p27 labeling index is 37.3%. C, a p27-negative case. A tumor biopsy specimen of a different patient also diagnosed as EFTs. (H&E, $\times400$). D, p27 immunostaining of the same specimen in C. The p27 labeling index is 2.84%, which is defined as negative for p27.

**Fig. 2** A, survival curves based on p27 labeling index (LI) in Ewing’s family tumors (EFTs). Low-level expression of p27 protein is associated with poor prognoses for patients with EFTs. Five-year event-free survival estimated with the Kaplan-Meier method was 10.7% for the patients with low-p27 tumors and ($\leq$10% of total cells; $n = 15$ patients; solid line), whereas 83.3% for those with high-p27 tumors ($>10\%$ of total cells; $n = 6$ patients; dotted line). The difference was significant ($P = 0.0096$). B, survival curves based on tumor volume in EFTs. Huge volume of tumor is associated with poor prognoses for patients with EFTs. Five-year event-free survival estimated with the Kaplan-Meier method was 10.0% for the patients with a tumor volume $< 200$ ml ($n = 10$ patients; solid line) compared with 54.5% for patients with a tumor volume $\geq 200$ ml ($n = 11$ patients; dotted line). The difference was significant ($P = 0.0356$). C, the correlation between p27 LI and Ki-67 LI in EFTs. Low level p27 expression is associated with high cell proliferation. A statistically positive correlation was observed between p27 LI and Ki-67 LI using Pearson’s correlation coefficient test ($r = 0.531$).
the infection in all EFT cell lines tested (data not shown). Preliminarily, we tested EFT cell lines for the susceptibility to growth inhibition induced by Ax-p27 at m.o.i. 1–100. Ax-LacZ at m.o.i. 100 exhibited adenoviral toxicity to the cells, whereas Ax-LacZ at m.o.i. 10 showed no toxicity (data not shown). Therefore, we decided to use m.o.i. 10 in the following experiments. Western blot analysis detected the overexpression of p27 in RD-ES infected with Ax-p27 at day 1, which continued for at least 14 days (data not shown). In contrast, there was no changes in the p27 protein level in the cells infected with Ax-LacZ.

We examined the effects of p27 expression on phosphorylation in Rb in EFT cells (Fig. 3). Fig. 3A shows changes in the expression of p27, Rb, and phosphorylated Rb after the adenoviral treatments. In RD-ES, antiparallel with p27 overexpression, the protein level of phosphorylated Rb expression was reduced from day 1, whereas that of total Rb was not modified (Fig. 3A). We obtained similar results using four other EFT cells, SK-N-MC, PNKT-1, VH-64, and WE-68 (data not shown). To examine the effects of p27 expression on cell proliferation, we counted the number of cells untreated or treated with Ax-p27 or Ax-LacZ for 7 days. All five EFT cells treated with Ax-p27 displayed marked, almost complete, growth inhibition compared with untreated and Ax-LacZ-treated cells (Fig. 3B).

![Figure 3](image)

**Fig. 3** A, time course of expression of p27, total retinoblastoma (Rb) and phosphorylated Rb (phospho-Rb) protein in RD-ES cells after infection with adenoviruses. Cells were infected with multiplicity of infection 10 of Ax-p27 or Ax-LacZ. Whole cell lysates were collected every 24 h and subjected to Western blot analysis. B, effect of Ax-p27 on Ewing’s family tumor (EFT) cells. EFT cells treated with Ax-p27 displayed marked growth inhibition compared with untreated or Ax-LacZ-treated cells. The growth curves of the untreated cells (○), the cells infected with Ax-p27 (■), and the cells infected with Ax-LacZ (●). α ± β represent mean value and SD, respectively. The experiments were triplicated and repeated three times. C, effect of Ax-p27 on the viability of EFT cells. The cells were treated with Ax-p27 (○ or Ax-LacZ (●) at multiplicity of infection 10.

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* Log-rank test.
* Cox proportional hazards model.
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* NS, not significant.

**Table 2** Event-free survival analysis of patients with Ewing’s family tumors

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We next performed a trypan blue exclusion assay to examine cell viability. The percentages of viable cells treated with Ax-p27 or Ax-LacZ at m.o.i. 10 are shown in Fig. 3C. In all EFT cells tested, Ax-LacZ did not affect the cell viability for 6 days. Ax-p27 treatment caused a slight decrease in the number of viable cells of SK-N-MC, PNKT-1, and RD-ES cells. In VH-64 and WE-68, however, the percentage of the viable cells started to decline more sharply at day 3 and decreased to <50% by day 6.

**Antitumor Effect of Ax-p27 in Tumor-Bearing Athymic Mice.** To investigate whether Ax-p27 has an antitumoral activity *in vivo*, we used the athymic mice xenograft model. The tumor-bearing mice were randomized and placed into three groups. Each mouse was treated either by intratumoral injection of 100 μl with 10¹⁰ plaque-forming units of Ax-p27, Ax-LacZ, or 100 μl of saline. The tumors treated with saline or Ax-LacZ grew continuously during the course of therapy. In contrast, the volume of tumors treated with Ax-p27 was significantly small than that of controls (*P* < 0.01; Fig. 4). Nude mice exhibited no weight loss or no skin change surrounding the xenografts such as necrosis, skin pigmentation, or inflammation (data not shown).

The Correlation between p53 Status and Susceptibility to p27-Induced Apoptosis in EFT Cells. Because recent studies reported that the overexpression of p27 resulted in cell apoptosis (20–22), we speculated that the growth inhibition of EFT cells by Ax-p27 would be due to p27-induced apoptosis. As shown in Fig. 3C, although Ax-p27 inhibited the cell growth and decreased cell viability in all EFT cells tested, the sharp decline in cell viability was found only in the p53-wild-type EFT cells, VH-64, and WE-68. Thus, we examined whether susceptibility to p27 would vary in EFT cells according to the p53 status. We evaluated the apoptosis induced by Ax-p27 in EFT cells using two different methods, detection of cleaved poly (ADP-ribose) polymerase (PARP) and DNA ladder formation (DNA fragmentation assay). As shown in Fig. 5A, DNA fragmentation was observed in the p53-wild-type cell lines VH-64 and WE-68 cells treated with Ax-p27 at day 3 but not with Ax-LacZ. However, there was no detectable DNA fragmentation in the p53-mutated EFT cell lines RD-ES, PNKT-1, or SK-N-MC cells infected with Ax-p27. Treatments with adenoviruses did not result in apoptosis in a murine fibroblast cell Swiss 3T3 and a human fibroblast cell hTERT-BJ1 used as normal cells in this study. The process of apoptosis is mediated by the activation of a cascade of proteolytic enzymes belonging to the caspase family (31). PARP is one of the main cleavage targets of caspase-3, and the cleavage of PARP serves as a marker of the cells undergoing apoptosis (32, 33). As shown in Fig. 5B, the cleaved PARP was clearly observed in VH-64 and WE-68 infected with Ax-p27 at day 3; however, it did not appear in RD-ES, PNKT-1, SK-N-MC, or in two fibroblast cells, infected with Ax-p27.

**Regulation of the p27 Expression via Posttranslational Mechanism in EFT Cells.** To elucidate the mechanism through which p27 expression was attenuated by EWS-Fli1, the effects of EWS-Fli1 on p27 promoter were examined. AS-ODNs caused no significant changes in the activity of human p27 promoter compared with S-ODNs (*P* > 0.05; Fig. 6A, right panel). Real-time quantitative RT-PCR (TaqMan PCR) was used to determine the quantity of p27 transcripts in SK-N-MC cells. Consistent with the data of the p27 promoter assay, the expression of p27 mRNA in SK-N-MC cells treated with AS-ODNs for 48 h increased slightly compared with those with S-ODNs or nontreatment; however, the difference was not significant (*P* > 0.05; Fig. 6B).

Several lines of evidence suggest that the level of p27 expression is regulated in the posttranslational phase. The ubiquitin-proteasome pathway is the principal mechanism regulating p27 protein degradation (34–37). Thus, we examined the effects of the proteasome inhibitor, ZLLLHal, on p27 expression using Western blotting. The treatment with ZLLLHal increased p27 protein expression in SK-N-MC cells in dose- and time-dependent manners. The level of p27 protein was increased as early as 10 6 cells/mouse. We next performed a trypan blue exclusion assay to examine cell viability. The percentages of viable cells treated with Ax-p27 or Ax-LacZ at m.o.i. 10 are shown in Fig. 3C. In all EFT cells tested, Ax-LacZ did not affect the cell viability for 6 days. Ax-p27 treatment caused a slight decrease in the number of viable cells of SK-N-MC, PNKT-1, and RD-ES cells. In VH-64 and WE-68, however, the percentage of the viable cells started to decline more sharply at day 3 and decreased to <50% by day 6.

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Several lines of evidence suggest that the level of p27 expression is regulated in the posttranslational phase. The ubiquitin-proteasome pathway is the principal mechanism regulating p27 protein degradation (34–37). Thus, we examined the effects of the proteasome inhibitor, ZLLLHal, on p27 expression using Western blotting. The treatment with ZLLLHal increased p27 protein expression in SK-N-MC cells in dose- and time-dependent manners. The level of p27 protein was increased as early as 10 6 cells/mouse.
DISCUSSION

In this study, we presented the first evidence of the prognostic value of p27 expression in EFTs. A number of studies demonstrated that low protein level of p27 is the negative prognostic indicator in various human tumors (15–18). We have reported that EWS-Fli1 inhibited the expression of two major cyclin-dependent kinase inhibitors, p21WAF1 (p21) and p27, in EFT cells (8, 9). However, immunohistochemical analysis revealed that the expression level of p21 in EFTs did not significantly correlate with the clinical survival rate of the patients (data not shown). The patients with EFTs exhibiting low expression levels of p27, however, had significantly worse prognoses than those with tumors exhibiting high expression levels of p27. In addition to p27 LI, we examined whether other factors could influence event-free survival of the EFT patients. A univariate analysis, not a multivariate analysis, revealed that tumor volume was also significantly associated with prognosis. Age (<15/≥15-year-old), tumor site (trunk/extremity), serum lactic dehydrogenase levels, serum C-reactive protein, distant metastases at the time of diagnosis, and levels or type of EWS-Fli1 (type1/notype1) did not correlate with patient survival in this study (data not shown). In this study, type I and type II EWS-Fli1 chimeric transcripts were observed in 66.7 and 13.3% of cases, respectively, which is roughly concordant with other studies (38). Although type of EWS-Fli1 chimeric transcripts also has been reported as one of already known prognostic factors, there were no correlations in our series between type of EWS-Fli1 chimeric transcripts and prognosis or p27 expression levels (data not shown). Because the number of patients in this study is very small, additional studies are necessary to clarify whether type of EWS-Fli1 fusion is associated with in prognosis or expression levels of p27 in EFTs. However, it is noteworthy that our results indicate that p27 LI correlated more significantly with patient survival than tumor volume, which is thought to be a major prognostic factor in EFTs (39, 40). The distant metastases at the admission were observed in 1 of 6 patients with high p27LI of EFTs and in 4 of 15 patients with low p27LI of EFTs. During follow up, 10 of 16 patients without distant metastases at admission developed distant metastases. Although 1 of the 10 patients displayed high p27LI EFTs, 9 of 10 displayed low p27LI EFTs. The data are quite interesting because no significant correlation could be observed between the level of p27 expression and occurrence of metastases (data not shown). However, a recent study has implicated p27 as a lymph node status of breast carcinoma (41). Additional studies are warranted to clarify the impact of p27 on development of distant metastases.

Our results also showed that p27 LI inversely correlated with Ki-67 labeling index. Because proliferating cells express Ki-67 antigen (30), the low expression level of p27 is directly connected with the higher proliferation of the tumor cells. The results suggest that the reduced p27 expression in EFTs may be related to rapid growth and progression of tumors, which could result in poor prognosis. Thus, the patients with p27-negative EFTs might need to receive more intensive therapy.

Given the observations that EWS-Fli1 may have the potential to reduce the expression of p27 in EFT cells (8) and that the patients with p27-positive tumors survived significantly longer than those with p27-negative tumors, we next determined whether forced expression of p27 could inhibit EFT cell growth. The adenoviral vector system, which we used in this study, is an effective strategy for complete transduction of the target genes in sarcoma cells (42–44). Similar to a previous study on lung cancer cells (21), the overexpression of p27 in EFT cells also reduced phosphorylated Rb, which may be increased indirectly by EWS-Fli1. The induction of p27 arrested the cell cycle in all EFT cells we tested, indicating that p27 could be a promising target for the treatment of EFTs.

In this study, when the susceptibility to p27 expression was tested in EFT cells, we noticed that apoptosis after p27 transduction occurred only in p53-wild-type cells, VH-64 and WE-68. Kovar et al. (45) reported the status of the p53 gene in the EFT cells used in this study; SK-N-MC and RD-ES contain a
mutated p53 gene, whereas VH-64 and WE-68 have a wild-type p53 gene. Because we could not detect p53 protein expression in the PNKT-1 established in our laboratory by Western blot (data not shown), we used PNKT-1 as the cells with a mutated p53 gene. The viability of cells with wild-type p53 declined sharply after Ax-p27 treatment, accompanied with the detection of p27-mediated cell death by DNA ladder formation and cleaved PARP expression. Thus, we concluded that overexpression of p27 could cause apoptosis in EFT cells with wild-type p53.

Because clinical EFT samples rarely have mutations in Rb or p53 genes (45, 46), the introduction of p27 protein in patients with p53- and Rb-wild-type EFTs might induce apoptosis of the tumor as suggested from our results. We also demonstrated that growth of the cells with mutated p53 was significantly inhibited by Ax-p27 and that intratumoral injection of Ax-p27 exhibited antitumor effects in SK-N-MC xenograft model in vivo. Even if the patients have EFTs with mutated p53, the forced expression of p27 protein could inhibit progression of EFTs. Theoretically, it would be best to inhibit EWS-Fli1 function directly to treat EFTs because STI-571 directly inhibits kinase activity of Bcr-Abl in chronic myelogenous leukemia. Because there is, thus far, no specific strategy to inhibit the function of the oncogenic fusion gene, we have to choose an alternative to the direct EWS-Fli1-targeted therapy. Because fibroblasts infected with Ax-p27 showed growth inhibition (data not shown) but did not indicate apoptosis. Other researchers also carried out direct intratumoral injection of adenovirus expressing p27 without major adverse effects (22). Therefore, we suspect that p27-targeted anticancer therapy would be safe and feasible. The present study demonstrates the possibility that the overexpression of p27 could cause cell cycle arrest via hypophosphorylated Rb and subsequently induce apoptosis in EFT cells. The study shows that p27 might be a novel and powerful therapeutic molecule in EFTs. Although several studies reported the apoptotic effects of p27 in tumor cells (20–22), the mechanisms involved in the p27-mediated apoptosis are still unknown. Additional investigation is needed to elucidate its mechanisms.

We also examined the process for the down-regulation of p27 expression by EWS-Fli1. In the luciferase reporter gene assays, we found that EWS-Fli1 did not modulate p27 promoter

Fig. 6 Analysis of the process for the reduction of p27 expression by EWS-Fli1. A, p27 promoter luciferase assays. The treatment with antisense phosphorothioate oligodeoxynucleotide (AS-ODN; 10 μm) did not significantly up-regulate p27 promoter activity in SK-N-MC cells (left panel). Forced expression of EWS-Fli1 in murine fibroblast; Swiss 3T3 cells also exhibited little change in p27 promoter activity (right panel). (N.S., not significant [P > 0.05]). Bottom panel under the graph represents the protein level of EWS-Fli1 in the experiments. B, SK-N-MC cells were incubated with 10 μm S-ODN or AS-ODN for 48 h; total RNA was isolated, reverse transcribed, and subjected to real-time quantitative reverse transcription-PCR to determine the number of p27 transcripts. The relative amount of p27 mRNA, standardized against the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA, was calculated. Bottom panel under the graph represents the protein level of EWS-Fli1 in the experiment. C, accumulation of p27 protein with proteasome inhibitor. The treatment of SK-N-MC cells with ZLLLHal (5 μm) caused up-regulation of p27 expression in time- and dose-dependent manners (left panel and right panel, respectively). The results represent three independent experiments. D, restoration of p27 protein by proteasome inhibitor. The forced expression of EWS-Fli1 in Swiss 3T3 cells reduced p27 protein; however, the following challenge of proteasome inhibitor resulted in accumulation of p27 protein.
activity. Because it has been reported that EWS-Fli1 could play a role in RNA splicing (47–49), it is possible that EWS-Fli1 might influence p27 mRNA stability at the posttranscriptional level. To measure the amount of p27 mRNA, we performed real-time quantitative PCR (TaqMan PCR). This minute assay detected no significant alteration of p27 mRNA levels in SK-N-MC cells after treatment with AS-ODNs where EWS-Fli1 protein expression was inhibited. The results suggest that the down-regulation of p27 in EFTs might be mediated by the posttranslational mechanism. Because the ubiquitin-proteasome pathway mainly regulates the p27 protein level in many cells (34–37), we next investigated whether inhibition of the proteasome function in EFT cells would result in the accumulation of p27 protein. The challenge of the proteasome inhibitor, ZLLL-Hal, clearly caused the accumulation of p27 protein in dose- and time-dependent manners in both endogenously and exogenously EWS-Fli1-expressing cells. Although we could not completely rule out the possibility that EWS-Fli1 might influence p27 mRNA stability, we concluded that p27 might be diminished, at least in part, by the proteasome-mediated degradation in EFTs. However, the precise process of the inhibition of p27 expression by EWS-Fli1 warrants additional investigation. Our previous studies have demonstrated that the expression of p21 was also suppressed by EWS-Fli1 and that EWS-Fli1 significantly inhibited the promoter activity of the p21 gene (8, 9). Thus, the suppression of p21 expression might be mediated at the transcriptional level. EWS-Fli1 might suppress p27 and p21 via different mechanisms.

In summary, we herein present the first evidence of the prognostic value of p27 expression in EFTs. Furthermore, our results indicate that p27 might be proposed as a novel and powerful therapeutic approach for molecular target therapy in EFTs.

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