Treatment of Ewing’s Sarcoma Using an Antisense Oligodeoxynucleotide to Regulate the Cell Cycle

Satoru ASAMI, Motoaki CHIN, Hiroyuki SHICHINO, Yukihiro YOSHIDA, Norimichi NEMOTO,
Hideo MUGISHIMA, and Takashi SUZUKI

*Research Unit of Clinical Medicine, College of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba
274–8555, Japan; b Department of Pediatrics and Child Health, Nihon University; c Department of Orthopedic Surgery, Nihon University; and d Department of Pathology; School of Medicine, Nihon University; 30–1 Oyaguchikami-cho, Itabashi-ku, Tokyo 173–0032, Japan.

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Ewing’s sarcoma (ES) is one of the most malignant tumors of bone and soft tissue in children and young adults. ES belongs to a group of small round cell tumors (SRCTs) that also includes neuroblastoma, rhabdomyosarcoma, and malignant lymphoma. However, ES exhibits several specific chimeric genes (EWS–FLI1, EWS–ERG, EWS–ETV1, EWS–E1AF, and EWS–FEV) caused by chromosomal translocations that are not shared by other SRCTs. These chimeric genes regulate the expression of various other genes; that is, they activate inhibitors of DNA binding 2 (Id2) gene expression or they suppress transforming growth factor β II (TβRII) receptor gene expression. The regulation of these chimeric genes may affect critical cell signal transductions, such as signals involved in cell cycle and apoptosis in ES tumor cells. Using an antisense oligodeoxynucleotide against a sequence containing the ATG initiation codon of the EWS–FLI1 chimeric gene that specifically reacts with the EWS–FLI1 and EWS–ERG chimeric genes, we were able to regulate the cell cycle through the down-regulation of Id2. Here, we report that treatment with an antisense oligodeoxynucleotide against this chimeric gene was very useful for inducing the regression of ES tumor growth; thus, this chimeric gene may be an important target for the treatment of ES patients.

Key words Ewing’s sarcoma family tumor; EWS–ets chimeric gene; antisense oligodeoxynucleotide; cell cycle

Ewing’s sarcoma (ES), which originates in bone and soft tissue, is one of the most malignant tumors in children and young adults. ES belongs to a group of small round cell tumors (SRCTs).1–3) As primitive neuroectodermal tumor (PNET), Askin’s tumor, and ES have a common chromosomal translocation (t(11; 22)(q24; q12)).2–7) These tumors are classified as Ewing’s sarcoma family tumors (ESFTs). However, many solid childhood tumors containing ESFTs, such as neuroblastoma, malignant lymphoma, and rhabdomyosarcoma, are referred to as SRCTs. Therefore, ESFTs must be differentially diagnosed from other tumors using histopathological and molecular biological methods. The t(11; 22)(q24; q12) translocation is a common characteristic of ESFTs and is found in 88–95% of these tumors.8–10) We previously identified a chimeric gene resulting from this chromosomal translocation. The gene is coded by exons 1—7 of the Ewing’s sarcoma (EWS) gene and exons 6—9 of the Friend leukemia virus integration 1 (FLI1) gene, forming a new chimeric gene by joining the exon at the 3′ end of the EWS gene with the exon at the 5′ end of the FLI1 gene.11,12) EWS forms some chimeric genes with other avian erythroblastosis virus E26 homologue (ets) family genes, such as ets-related gene (ERG), ets translocation variant 1 (ETV1), fifth Ewing variant (FEV), and E1A enhancer-binding protein (E1AF). The chimeric protein produced by the EWS–FLI1 gene reportedly functions as a new transcriptional factor because the transcripational activation domain within the N-terminal domain of the EWS protein is joined with the ETS DNA-binding domain of the FLI1 protein. Moreover, this chimeric protein appears to regulate the transcription of other genes.12–19)

For instance, the cell cycle is reportedly inhibited by the binding of retinoblastoma (Rb) protein to E2 promoter binding factor (E2F).20) However, once the Rb protein becomes phosphorylated, E2F separates from it; this leads to the formation of a complex of cyclin E and cyclin-dependent kinase 2 (CDK2). This complex induces the cell cycle to proceed from G1 phase to S phase.21) Moreover, an inhibitor of DNA binding 2 (Id2) gene is expressed by the EWS–ets chimeric genes; this protein binds to Rb protein and frees E2F and also induces the cell cycle to proceed from G1 phase to S phase through the activities of cyclin E and CDK2.22,23)

Here, we report that the use of an antisense oligodeoxynucleotide to down-regulate the EWS–FLI1 and EWS–ERG chimeric genes, thereby reducing the progression of the cell cycle to S phase.

MATERIALS AND METHODS

Cell Lines and Cultures As shown in Table 1, we used 3 ESFT cell lines (SK-N-LO, KP-EW-MS, and SCMC-ES1) containing the EWS–ets chimeric genes and 3 cell lines (MCF-7 [breast cancer] (ATTC, Rockville, MD, U.S.A.), Jurkat [leukemia] (Dainippon Pharmaceutical Corporation, Osaka, Japan), HL-60 [leukemia] (ATTC, Rockville, MD, U.S.A.).

Table 1. Characteristics of Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>EWS–ETS chimeric gene</th>
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<tbody>
<tr>
<td>SK-N-LO</td>
<td>PNET</td>
<td>EWS (exon 7)–FLI1 (exon 6)</td>
</tr>
<tr>
<td>KP-EW-MS</td>
<td>ES</td>
<td>EWS (exon 10)–FLI1 (exon 5)</td>
</tr>
<tr>
<td>SCMC-ES1</td>
<td>ES</td>
<td>EWS (exon 7)–ERG (exon 6)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast cancer</td>
<td>—</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Leukemia</td>
<td>—</td>
</tr>
<tr>
<td>HL-60</td>
<td>Leukemia</td>
<td>—</td>
</tr>
</tbody>
</table>

PNET: peripheral primitive neuroectodermal tumor, ES: Ewing’s sarcoma.

* To whom correspondence should be addressed. e-mail: suzuki@pha.nihon-u.ac.jp © 2008 Pharmaceutical Society of Japan
U.S.A.) that did not contain these chimeric genes. All the cell lines were cultured in RPMI 1640 medium (Gibco Life Technologies, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, NY, U.S.A.), 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 7.5% (w/v) sodium bicarbonate solution (Gibco Life Technologies, NY, U.S.A.); the cells were then incubated in 5% CO2 at 37 °C.

Antisense Oligodeoxynucleotide Treatment An antisense oligodeoxynucleotide (AS-ODN) directed against a sequence including the ATG initiation codon of the EWS–FLI1 chimeric gene (final concentration: 1 mM, sequence: 5'-ATC CGT GGA CGC CAT TTT CTC TCC T-3' [target nucleotides: 34–58]) was added to each cell culture (1 x 10⁶ cells/dish) in FBS-free medium, and oligomers were supplied every 24 h. These cells were incubated for between 0 and 96 h. All the cultures were compared with controls cultured under the same conditions but without the AS-ODN. The sense oligodeoxynucleotide (S-ODN) sequence corresponding to AS-ODN was also used as a control.

Total RNA Extraction Total RNA was extracted from the cultured cells using an EASY Prep RNA extraction kit (TaKaRa, Shiga, Japan) and the acid guanidinium thiocyanate–phenol–chloroform method. The concentration of total RNA was determined using Gene Spec III (HITACHI, NY, U.S.A.).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) An RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, Shiga, Japan) was used with the primer supplied by the manufacturer. RT was performed using oligo-dT. The mixture was annealed at 42 °C for 30 min followed by incubation at 99 °C for 5 min and then held at 4 °C. The PCR conditions were one cycle of template denaturing at 94 °C for 120 s followed by 24 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s, and then holding at 4 °C. The primer sequences and sizes are listed in Table 2. The concentration of cDNA was determined using a bioanalyzer (Agilent, CA, U.S.A.).

Quantitative Real-Time PCR Total RNA (0.8 μg) was subjected to a reverse transcription reaction using an RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Shiga, Japan) and the PCR products were detected using SYBR Premix EX Taq subjected to a reverse transcription reaction using an RNA marker.

RESULTS AND DISCUSSION We examined some AS-ODNs which we originally designed and have been reported (data not shown). We used one of the most useful AS-ODNs to down-regulate EWS–ets chimeric genes and it was also one of the reported AS-ODNs. Figure 1 shows the expression levels of the EWS–FLI1 and EWS–ERG chimeric genes several days after the addition of AS-ODN (Figs. 1A, B) and gel-like images (Fig. 1C). The relative gene expression level was each gene expression divided by the expression of GAPDH as an internal marker on the gel-like image. Figure 1A shows that EWS–FLI1 chimeric genes were expressed in two cell lines (the EWS (exon 10)–FLI1 (exon 5) chimeric gene was expressed in KP-EW-MS, and the EWS–FLI1 (exon 6) chimeric gene was expressed in SK-N-LO); these expression levels gradually decreased since 2 d after the addition of AS-ODN (Figs. 1A, B) and gel-like images (Fig. 1C). The relative gene expression level was each gene expression divided by the expression of GAPDH as an internal marker on the gel-like image. Figure 1B shows that the expression of the EWS–ERG chimeric gene was only found in SCMC-ES1, containing the EWS–ERG chimeric genes, although each effective time is different. Thus, this AS-ODN may be useful for the treatment of ES family tumors containing these chimeric genes, which are found in 88–95% of ES tumors.

Flow Cytometry Cells were collected by trypsinization and washed in phosphate-buffered saline (PBS). The cells were then washed once again in PBS and incubated with 1 ml of propidium iodide (PI, 50 μg/ml final concentration; Sigma, MO, U.S.A.) for 30 min in the dark at 4 °C. The cell suspension was incubated with 0.25 mg/ml of RNase (Sigma, MO, U.S.A.) for 30 min at 37 °C. The fluorescence of the cells treated with PI was measured using a Cytomics FC500 Flow Cytometer (Beckman Coulter, CA, U.S.A.).

**Table 2. PCR Primers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EWS–FLI1</td>
<td>Sense 5'-CTG CTC TCC GAG GAC ACT GA-3'</td>
<td>328, 646</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GAG GCC CAA GAA CAA CAT CCA-3'</td>
<td>433</td>
</tr>
<tr>
<td>EWS–ERG</td>
<td>Sense 5'-TGC TCC GTG TTC ATC AGC TC-3'</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-TCC TCT GAC TAC AGC GAC ACC-3'</td>
<td>328, 646</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5'-TCC TCT CTC TTC TTG TGC TCT TG-3'</td>
<td>328, 646</td>
</tr>
</tbody>
</table>
dition of AS-ODN, only the rates at 96 h after treatment were significantly different \((p<0.01)\) from the rate at 0 h (Figs. 2A-(a), 2A-(c)). While the rates for SK-N-LO, which contains an EWS–FLI1 chimeric gene, the rate had decreased at 48 h after the addition of AS-ODN and the rates at both 48 h and 96 h after AS-ODN treatment were significantly different from the rate at 0 h \((p<0.01\) and \(p<0.001\), respectively) (Figs. 2A-(b), 2B).

However, the transition rates from the G1/G0 phases to the S phase of the cell cycle did not change in the MCF-7 (breast...
The expression levels for each gene are shown (A): Id2, (B): E2F1) after treatment with antisense oligodeoxynucleotide (0, 2, 4, 8, 16, 26 h). The X-axis shows the number of hours after the addition of antisense oligonucleotide and the Y-axis shows the ratio of each gene’s expression to that of an internal control (GAPDH). The level of expression for each gene after AS-ODN or S-ODN is shown (* p<0.05, compared with value at 0h using an ANOVA). The solid line shows the expression level for each gene after AS-ODN or S-ODN treatment, and the dotted line shows the level of expression for each cell in genes with no treatment.

**REFERENCES**


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cancer), Jurkat (leukemia), or HL-60 (leukemia) cell lines that did not contain EWS–ets chimeric genes (Figs. 2A-(d)—2A(f)). Therefore, the AS-ODN suppressed the transition of S phase in only ESFT cell lines, effectively suppressing cell growth.

The level of Id2 gene expression concerning with the regulation of Rb and E2F proteins by AS-ODN or S-ODN treatment is shown in Fig. 3A. Both the expression of Id2 and E2F1 was observed in SK-N-LO cells, but only the expression of Id2 gene was suppressed by the addition of AS-ODN, with a significant difference noted at 4 h and 24 h after treatment (Fig. 3). However, no significant increases in the expression of the E2F1 gene were noted at any time (Fig. 3B).

The addition of S-ODN had no effect on the expression of these genes in SK-N-LO cells. Moreover, these two genes were minimally expressed in Jurkat cells without EWS–ets chimeric genes and their expressions were not suppressed or up-regulated by either AS-ODN or S-ODN. Therefore, AS-ODN may regulate proteins (e.g., Rb, E2F) that suppress cell growth in ESFTs by down-regulating Id2 genes without the regulation of E2F1 gene located its down-stream.

These results suggest that this AS-ODN against these chimeric genes may be useful for suppressing ESFT tumor growth and may be an important target for the treatment of ES patients.