**Abstract.** Background: p73 is a tumor-suppressor gene with significant homology to p53. Abnormal promoter methylation of p73 is present in different types of cancer. However, the promoter methylation status of p73 in chondrosarcoma (CS) is unknown. Materials and Methods: p73 promoter methylation status was evaluated by quantitative polymerase chain reaction (PCR), p73 protein expression by western blot, and the relationship between p73 methylation and clinical data was analyzed. Results: In 42 tumor tissues with CS, we found that three cases (7%) maintained methylation levels between 51% and 75%, and 39 cases (93%) had levels between 76% and 100%. p73 methylation level was significantly (p<0.05) positively associated with histological grade. Loss of p73 protein expression was correlated with high methylation of the p73 promoter; p73 expression was restored after exposure to a demethylating drug. Conclusion: p73 is epigenetically silenced in CS due to promoter methylation, which suggests the utility of p73 methylation as a biomarker.

Chondrosarcoma (CS) is a malignant tumor of cartilage of bone and the second most common primary bone cancer after osteosarcoma (1). CS accounts for more than 20% of primary bone malignancies and is annually diagnosed in approximately 600 patients in the United States (2, 3). In the clinic, the primary diagnostic methods for CS rely on imaging and histology. However, even with adequate cytology and radiographic imaging, predicting the biological behavior as well as the distant disease potential of this malignancy is often difficult (1). Not only is diagnosis and prognosis a challenge, but CS is notoriously resistant to both chemotherapy and radiation treatments (4). Currently, surgical resection is the major treatment approach for localized lesions. Radiotherapy is useful for the definitive treatment of inoperable micro-lesions and the palliation of local symptoms (5). The outcome of patients with CS is relatively poor due to the potential for local invasion and distant metastasis (6). Thus, there is an urgent need to explore novel strategies to improve the outcomes of patients with CS.

p73 is a member of the p53 suppressor gene family. Because of the structural similarity between p73 and p53, p73 is also considered a tumor-suppressor gene. p73 can act as a transcription factor and regulate the expression of several p53-responsive genes, including: p21WAF1, murine double minute 2 (MDM2), BCL2-associated X (BAX), p53 up-regulated modulator of apoptosis (PUMA), and 14-3-3s (7). Transcriptional activation of these genes can subsequently induce cell-cycle arrest and apoptosis (8, 9). In vivo, reports show that mice lacking p73 develop spontaneous tumors, particularly lung adenocarcinomas, and were more sensitive to chemical carcinogenesis (10). Thus, p73 is thought to be key in suppressing cancer development due to its overlapping function with p53. However, little is known about the relationship between p73 status and CS.

Epigenetics modification, especially DNA methylation, may be critical for p73 expression. Methylation usually exists at a CpG island in the promoters of tumor-suppressor genes and subsequently inactivates gene expression (11). Aberrant DNA methylation of p73 has been found in hematological malignancies, lung cancer, gastric carcinoma and cervical cancer (12-15). However, no studies have shown a relationship between p73 methylation and CS, as far as we are aware.

In this study, we aimed to assess the status of p73 DNA methylation in CS cell lines and CS tumor tissues.
Materials and Methods

Cell lines and cell culture. The human CS cell line SW1353 was obtained from the American Type Tissue Collection (Rockville, MD, USA). The human CS cell line CS-1 was established in our laboratory as previously described (16, 17). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (all purchased from Invitrogen, Carlsbad, CA, USA). Cells were maintained in a humidified incubator containing an atmosphere of 5% CO₂-95% air at 37°C.

Demethylation treatment. When cells were near-confluent monolayers, demethylation treatment was carried out with trypsin-EDTA. CS cell lines SW1353 and CS-1 were seeded in 6-well plates at a density of 5x10⁵ cells per well, and grown overnight for cell-to-well attachment. Cells were then treated with the demethylating drug 5-aza-2’-deoxycytidine (5-Aza-dC; Sigma-Aldrich, St Louis, MO, USA) dissolved in RPMI-1640 medium at 1, 5, and 10 μmol/l for 72 h. Fresh medium was exchanged every 24 hours with the same concentration of 5-Aza-dC. Cells were washed twice with Phosphate-buffered saline and collected for western blot analysis at the end of a treatment course.

Patient specimens and clinical data. Forty-five fresh frozen tumor tissue samples of histologically confirmed CS were identified using the Massachusetts General Hospital (MGH) Cancer Registry and the Orthopedic Oncology database. Histopathological subtype and tumor grade were evaluated by pathologists at MGH. The patient data was obtained as follows: age, gender, histological subtype, tumor grade, tumor location, metastasis and recurrent status. The study was approved by the Partners Human Research Committee (number: 2007P-002464). All patients gave their informed consent to be entered in the MGH Sarcoma Tumor Bank databases for future research studies. The clinical characteristics of CS patients are detailed in Table I.

DNA isolation. Extraction of DNA from CS tumor tissues and cell lines were performed using QIAmp DNA Micro kit (Qiagen, Valencia, CA, USA). The extraction was carried out following the manufacturer’s instructions. In detail, CS tumor tissue sample or cell pellet from cell lines of approximately 8 mg was transferred to a 1.5 ml microcentrifuge tube and 180 μl of ATL buffer was immediately added. After equilibrating to room temperature (25°C), 20 μl of proteinase K was added and cells were mixed by vortexing for 15 sec. Subsequently, the sample tube was incubated at 56°C overnight until the sample was completely lysed. The next day, 200 μl of AL buffer (Qiagen) was added and the cells were mixed again by vortexing for 15 seconds. Next, 200 μl of ethanol (96-100%) was added. The subsequent mixture was loaded onto a QIAampMiniElute spin column provided by the kit and washed with AW1 buffer followed by AW2 buffer. DNA was finally eluted with 60 μl of AE buffer and preserved at −20°C until use.

Quantitative real-time polymerase chain reaction (PCR) of p73 promoter methylation. Located upstream of exon 1 (Figure 1A), CpG island methylation of the extrinsic promoter of p73 was analyzed in CS cell lines and tumor tissues using MethylScreen technology and the EpiTect Methyl II Array (Qiagen). Using the EpiTect Methyl II PCR System, methylated sequences are separated from unmethylated ones via methylation-sensitive (Ms) and/or methylation-dependent (Md) restriction enzymes that are responsive to CpG islands. After digestion, the remaining DNA sequence in each reaction was quantified by real-time PCR. The relative fractions of methylated and unmethylated DNA were then calculated after comparing each reaction of digested DNA fragments with a mock reaction (Mo, no enzymes added). Briefly, 125 ng of genomic DNA was equally divided between four reaction mixtures, which were subsequently added to the mock digestion reaction or the digestion of Ms or Md enzymes added. The amount of target DNA sequence of each reaction was quantified by real-time PCR using primers with SYBR Green ROX Mastermix (Qiagen) and carried out by StepOnePlus Real-time PCR System (Applied Biosystems). The p73 promoter primer used in the procedure was purchased from Qiagen. PCR cycling conditions were as follows: 95°C for 10 min, 3 cycles of 99°C for 30 seconds, 72°C for 1 min, 40 cycles of 97°C for 15 seconds and, 72°C for 1 minute with real-time signal acquisition. The melting curve was performed by the default program on the instrument after the cycling program.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Total</th>
<th>p73 methylation level (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>42</td>
<td>95.20±1.84</td>
<td>0.751</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤45</td>
<td>20</td>
<td>94.46±2.94</td>
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<tr>
<td>&gt;45</td>
<td>22</td>
<td>95.87±2.32</td>
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</tr>
<tr>
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<tr>
<td>Male</td>
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<td>95.12±2.09</td>
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<tr>
<td>Female</td>
<td>10</td>
<td>95.46±2.03</td>
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<tr>
<td>Histological subtype</td>
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<td>0.278</td>
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<tr>
<td>Hyaline and/or myxoid</td>
<td>21</td>
<td>96.60±2.04</td>
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<tr>
<td>Dedifferentiated</td>
<td>11</td>
<td>93.95±4.45</td>
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</tr>
<tr>
<td>Mesenchymal</td>
<td>3</td>
<td>99.49±0.41</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>5</td>
<td>87.65±8.50</td>
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<td>Grade</td>
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<tr>
<td>Present</td>
<td>9</td>
<td>96.08±2.18</td>
<td>0.829</td>
</tr>
<tr>
<td>Absent</td>
<td>33</td>
<td>94.96±2.27</td>
<td>0.3</td>
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<td>Metastatic</td>
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<td>0.409</td>
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<tr>
<td>Present</td>
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<td>93.74±4.11</td>
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<tr>
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<tr>
<td>Limbs</td>
<td>25</td>
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<tr>
<td>Trunk</td>
<td>17</td>
<td>92.85±3.47</td>
<td></td>
</tr>
</tbody>
</table>

SE: Standard error; NOS: not otherwise specified; *represents ρ<0.05.
to 27 cycles and the difference between the C_T value of Msd and mock to be greater than 3. The methylation percentage was calculated by the ΔC_T method according to the manufacturer’s protocol. Due to the inversely proportional correlation between threshold cycle and the amount of target DNA, and because of the doubling of PCR product with every cycle in the exponential phase of the reaction, the initial DNA amount in each sample before PCR was expressed as:

\[ C_{Mo} = 2^{-\Delta C_T(Mo)}; C_{Ms} = 2^{-\Delta C_T(Ms)}; C_{Md} = 2^{-\Delta C_T(Md)}; C_{Msd} = 2^{-\Delta C_T(Msd)} \]

The fraction of DNA in each digestion was obtained by normalizing the DNA amount to the amount of digestible DNA. The amount of digestible DNA is equal to the total amount of DNA determined from the mock digest minus the amount of DNA resistant to DNA digestion (determined from the double digest).

\[ F_{HM} = \frac{C_{Ms}}{C_{Mo} - C_{Msd}} = \frac{2^{-\Delta C_T(Ms)}}{2^{-\Delta C_T(Mo)} - 2^{-\Delta C_T(Msd)}} \]

Figure 1. Gene structure and methylation assessment of p73 in human chondrosarcoma (CS). A: The location of the CpG island in the p73 promoter. The target CpG island is located in Chr1: 3607096-3607553. B: Methylation profile of p73 in CS cell lines. The methylation of the promoter of p73 was found to be highly frequent in CS cell lines CS-1 (methylation (M): 95.98%; unmethylated (UM): 4.02%) and SW1353 (M: 98.80%; UM: 1.20%). C: Representative amplification plots from real-time polymerase chain reaction (PCR) of p73 in CS cell lines. Yellow curve: DNA subjected to no enzyme (Mo), represents the total amount of input DNA. Green curve: DNA subjected to methylation-sensitive enzyme (Ms), represents DNA in which all CpG sites are methylated. Dark blue curve: DNA subjected to methylation-dependent enzyme (Md), represents unmethylated DNA. Pink curve: DNA subjected to both methylation-sensitive and methylation-dependent enzyme (Msd), represents DNA refractory to enzyme digestion (background signal). The X-axis shows the cycle number of real-time PCR, and Y-axis shows fluorescence intensity. TSS: Transcription start site.
Unmethylated (UM) DNA fraction:
\[ F_{UM} = \frac{C_m}{C_m - C_{Msd}} = \frac{2^{C_{M0}}}{2^{C_{M0}} - 2^{C_{Msd}}} \]

Intermediately methylated (IM) DNA fraction:
\[ F_{IM} = 1 - F_{HM} - F_{UM} \]

Methylated (M) DNA fraction:
\[ F_m = F_{HM} + F_{IM} \]

Final data for gene methylation are described as the percentage of unmethylated and methylated fraction of DNA. Due to the experimental design, methylated sites pertain to the target sequence of the p73 promoter with two or more methylated CpG sites.

**Western blot.** Total protein was obtained with 1×RIPA lysis buffer (Upstate Biotecology, Charlottesville, VA, USA), which was added to complete protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were accessed with the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of denatured proteins (20 μg) were resolved on NuPAGE® 4-12% Bis-Tris Gel (Life Technologies) and transferred onto a nitrocellulose membrane (Bio-Rad). After blocking in 5% non-fat milk in Tris-buffered saline with Tween (TBST) for 2 h, the membranes were probed with primary antibodies to p73 (1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), which recognizes endogenous total p73 protein, and to β-actin (dilution 1:2,000; Sigma-Aldrich) and kept at 4˚C overnight. The membranes were then washed with TBST and incubated with their respective secondary antibodies (LI-COR Biosciences) and quantified with Odyssey software 3.0 (LI-COR Biosciences).

**Statistical analysis.** All statistical analyses were performed with the SPSS 17.0 software (Chicago, IL, USA). All data are expressed as the mean±SEM. A detailed analysis of continuous variables was performed with those not within the normal distribution by the Shapiro–Wilks test. Then the Wilcoxon analysis and Kruskal–Wallis test were used to evaluate the differences in methylation level between groups sorted by clinical characteristics such as age, gender, metastasis, recurrence, grade, histological subtype, and location. p-Values for differences were two-sided and a value of p<0.05 was considered as statistically significant for all statistical tests.

**Results**

**p73 promoter methylation level in CS cell lines and in patient tissues.** We found 95.98% and 98.80% promoter methylation of p73 in CS cell lines CS-1 and SW1353, respectively (Figure 1B). Representative amplification curves of p73 in the two cell lines are shown in Figure 1C. We further examined the methylation level of p73 in 45 tumor specimens from patients with CS. Three out of the 45 cases were excluded since their C_{T} values did not pass quality control after three attempts. The p73 methylation level in 42 CS tissue samples was distributed with CS. Western blot revealed silencing of p73 in CS cell lines

**DNA methylation silences p73 expression.** Since p73 promoter methylation was found in CS cell lines, western blot analysis was performed to further determine the protein expression of p73. As expected, p73 exhibited loss of expression in both CS-1 and SW1353 cell lines (Figure 3). After a demethylation process with 5-Aza-dC, p73 expression was restored (Figure 3). Quantitative analysis of western blot data showed that 5-Aza-dC restored expression in a dose-dependent manner (Figure 3).

**Association between p73 promoter methylation and clinical parameters.** We analyzed the potential relationship between p73 methylation level and different clinical variables. Among the cases classified by histological subtype, one case of diffuse large cell lymphoma and one juxtacortical case were excluded since the number of cases was too small for statistical analysis. Across the cases classified by grade, one case was excluded because the grade of the tumor was unavailable. The results are summarized in Table I. No significant correlation of p73 promoter methylation was found with gender, age, histological subtype, metastatic status, recurrent status, and location. However, there was a significant positive correlation between p73 methylation level and tumor grade (p=0.037) (Figure 4). The methylation level was significantly higher in grade 2 (p=0.034) and grade 3 (p=0.031) tumor than in grade 1, whereas there was no significant difference between grade 2 and grade 3 (p=0.283).

**Discussion**

Our analysis of p73 extrinsic promoter methylation in CS cell lines and patient samples alongside the results of previous studies collectively suggest a link between aberrant methylation and expression of p73 in CS. Previous studies reported aberrant DNA methylation of several tumor-related genes in CS. For example, in a panel of 63 patients with CS, samples with down-regulated runt-related transcription factor 3 (RUNX3) expression were found to have a substantial promoter hypermethylation (18). Historically, p73 silencing due to corresponding promoter methylation has been reported in different cancer types, including acute lymphoblastic leukemia and solid tumors, such as gastric carcinoma (12, 14, 19).

Our data suggest that p73 promoter methylation is an important epigenetic mechanism that regulates p73 expression in CS. Western blot revealed silencing of p73 in CS cell lines.

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DNA methyltransferase inhibition by 5-Aza-dC resulted in promoter demethylation. We observed a gradual up-regulation of p73 protein expression upon exposure to a series of increasing 5-Aza-dC concentration. Considering our results, we hypothesize that the frequent methylation of p73 in CS tissue samples may play an important role in tumor progression.

Figure 2. Methylation level of p73 in tissues from patients with chondrosarcoma (CS). A: Methylation level of p73 in each specimen is presented. B: Distributions are shown for the level of methylation in the 42 CS tumor samples.
Low expression of p73 is partly due to alterations in DNA methylation, which has been reported in different cancer types in comparison with their normal tissue counterparts (20-24). Recent work provides preclinical evidence that 5-Aza-dC treatment in myeloid leukemia can induce p73 downstream of p21 and facilitate tumor sensitivity to apoptosis by p73 demethylation (25). Although the role and expression profile of p73 in patients with CS needs to be further studied, our work provides exploratory data on p73 as a potential therapeutic target against CS.

Previous work showed a dynamic expression level of p73 in a multistep model for the malignant transformation of human fibroblasts, and an elevated level of p73 was found in fully transformed fibroblasts (26). Similarly, p73 expression was found to be an early event in human oral carcinogenesis, and patients with epithelial dysplasia positive for p73 had malignant transformation and developed squamous cell carcinoma by their 2-year follow-up (27). In agreement with these reports, our data showed a correlation between p73 methylation and CS histological grade, which is classified based on cellularity, atypia, and pleomorphism (28, 29). In particular, there was significantly lower p73 methylation in grade 1 CS compared to that of grade 2 and grade 3, thereby indicating an association between CS transformation/differentiation and p73 methylation-regulated protein expression.

We also explored the association of p73 methylation with other clinical parameters by performing statistical analysis, yet these yielded no significant associations. A larger study may be necessary to discover associations between p73 methylation and clinical parameters.

In summary, there was an absence of protein expression of p73 due to its high frequency of methylation in CS cell lines. Low methylation levels of p73 were associated with low grade CS, suggesting p73 methylation to be a novel predictor for diagnosis of grade. Further studies on the mechanism of p73 methylation may yield innovative insight into the role of this tumor-suppressor gene in CS.

**Conflicts of Interest**

The Authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the article apart from those disclosed.

**Acknowledgements**

This work was supported, in part, by the Gattegno and Wechsler funds. Z Duan is supported, in part, through a grant from Sarcoma Foundation...
of America (SFA), a grant from National Cancer Institute (NCI)/NIH, UO1, CA 151452-01, a pilot grant from Sarcoma SPORE/NIH, and a grant from an Academic Enrichment Fund of MGH Orthopaedics.

References