Abstract. Background: Malignant fibrous histiocytoma (MFH) of bone is a rare primary malignant neoplasm. Recent studies indicate a positive correlation between the telomere maintenance mechanism and tumour aggressiveness in sarcomas. This study was undertaken to analyse the clinical significance of telomere factors in primary tumour samples from patients with MFHs of bone. Materials and Methods: Telomerase activity was measured in ten bone MFH specimens using a PCR-based TRAP assay. Telomere length was assessed using gel hybridisation. Quantitative detection of human telomerase reverse transcriptase (hTERT) was performed by real-time PCR. Results: Telomerase activity and hTERT expression were detectable in 100% of tumour samples and 50% of tumour samples had evidence of engagement of the alternative lengthening of telomere (ALT) mechanisms. ALT was a significant prognostic risk factor (p=0.0316). Conclusion: This study suggests that the presence of ALT telomere maintenance mechanisms indicates a poor prognosis for bone MFH patients.

Human telomeres are the ends of chromosomes containing thousands of TTAGGG DNA repetitive sequences that are important in the protection and replication of chromosomes (1, 2). Telomerase is a reverse transcriptase that adds telomeric repeats to chromosomal ends and permits the synthesis of telomeric DNA (3, 4). Human telomerase reverse transcriptase (hTERT) is the catalytic protein of telomerase and the close relationship between hTERT mRNA expression and telomerase activity has been reported in tumour cells (5-7). Telomerase activity has been detected in approximately 85-90% of human carcinoma samples tested and correlates to patient prognosis (8, 9). However, sarcomas are distinct from carcinomas in that many of the former use alternative lengthening of telomere (ALT) mechanisms. The telomere maintenance mechanism has recently been reported as a prognostic factor for patients with several types of sarcomas (5, 10-17). Malignant fibrous histiocytoma (MFH) of bone is an uncommon malignant sarcoma which mainly affects the long bones of the extremities and is a tumour about which much still remains unknown (18-21). Although recent studies indicate a positive correlation between the telomere maintenance mechanism and tumour aggressiveness in several sarcoma types (22), no studies have been carried out to estimate the prevalence of ALT and little is known concerning telomerase activity and hTERT expression in MFH of bone. This study performed an analysis of the significant clinical factors of telomere length, telomerase activity and hTERT in primary tumour samples from patients with MFH of bone.

Patients and Methods

Patients and tissue procurement. Between 1989 and 2000, ten patients underwent tumour resection for MFH of bone. Tumour samples were obtained from surgical specimens and all samples were immediately frozen and stored until use. There were six male and four female patients with an average age at presentation of 48 years (range, 23-69 years). All patients presented with storiform-pleomorphic histology. Five tumours were located in the femur and one in the clavicle, sacrum, humerus, ilium and tibia. The average duration of postoperative observation was 65 months (range, 6-141 months). Five patients died of the disease (Table I). Induction chemotherapy was not used in all patients but postoperative chemotherapy was used in patients who had metastases. All patients received wide resection.

Telomerase assay. Evaluation of telomerase activity was performed by the telomeric repeat amplification protocol (TRAP) assay. Telomerase extracts were taken as described earlier (4). The concentration of tumour protein was measured using BCA Protein
Assay Kit (Pierce Chemical Co., Rockford, IL, USA), and then 1 μg of tumour protein was used for each TRAP assay. The levels of telomerase activity were measured using TRAPEze XL Telomerase Detection Kit (Intergen Co., Purchase, NY, USA) and were expressed as total product generated (TPG) units/μg protein.

Real-time quantitative RT-PCR. Total cellular RNA was extracted using the Rneasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesised using 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Quantitative detection of hTERT mRNA was performed with the LightCycler TaqMan Master (Roche Applied Science) using the LightCycler instrument (Roche Molecular System, Alameda, CA, USA). The primer pairs 5'-CGGAAGAGTGTCTGGAGCAA-3' and 5'-GGATGAAGCGGAGTCTGGA-3' for hTERT were used for amplification. Expression of the gene GAPDH was also analysed for each tumour sample as an indicator of RNA quality. A 3×10^6 of telomerase-positive HeLa cell was used as a positive control.

Telomere length analysis. Genomic DNA was isolated from the frozen samples by proteinase K digestion followed by phenol-chloroform extraction (23). After 1 μg of DNA was digested to completion with AluⅠ, CfoⅠ, HaeⅢ, HinfⅠ, MspⅠ and RsaⅠ and electrophoresed on 0.7% agarose gels, in-gel hybridisation was performed by using the labelled oligonucleotide containing a TTAGGG probe. The gels were scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and Telometric (download from http://bioinformatics.fccc.edu/software/OpenSource/telometric/telometric.shtml) software was used to estimate the telomere lengths as the mean and semi-interquartile range (SIR) of the hybridisation signal (24). Tumours were determined as ALT-positive when the mean and SIR of the terminal restriction fragment (TRF) length distribution was greater than 16 kb and 4 kb, respectively (5).

Statistical analysis. The overall survival curve for each group was calculated using the Kaplan-Meier method and the resulting survival curves were evaluated using the log-rank test. Each prognostic factor was divided into two groups based on an average value. Data are presented as mean±standard deviation. In all analyses, a p-value <0.05 was considered to indicate significance. All statistical analyses were performed on a personal computer using the statistical package Statview, Version 5.0 (Abacus Concepts, Berkley, CA, USA).

Results

Telomerase activity. Telomerase activity was detected in all tumour samples. The mean level of telomerase activity was 10.1±7.19 TPG (range, 0.50-21.0 TPG) (Table I).

hTERT mRNA expression. hTERT mRNA expression was evident in all tumour samples. The mean level of hTERT was 616±432 (range, 242-1405) (Table I).

Telomere length. The mean telomere length was 21.9±10.5 kb (range, 12.3-38.5 kb) and the mean SIR was 3.9±1.00 kb (range, 2.3-5.5 kb). According to the characteristic high mean and range of the telomere length distribution, 50% of the tumour samples were ALT-positive (Table I).

Prognostic factors. ALT-positive patients had a worse prognosis than other patients (5-year-survival rate, 20% vs. 80%, respectively, p=0.0316; Figure 1) There was no significant correlation between the survival rate and the level of telomerase activity (p=0.923). Similarly, there was no significant correlation between the survival rate and the level of hTERT expression (p=0.722).

Discussion

Telomere maintenance is regarded as an important mechanism in evading senescence in tumour cells and telomeres of human tumour cells have two mechanisms for telomere maintenance, namely by telomerase activation and ALT. Although over 80% of all carcinomas rely on telomerase activity to maintain stable telomere length (8, 9), many types of sarcoma elongate telomeres consistent with ALT in the absence of telomerase activity (5, 11, 12, 15). Recently, telomere- and telomerase-related studies of several sarcomas have investigated their prognostic utility. These

### Table I. Data of ten patients with bone MFH.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Site</th>
<th>Histological sub-type</th>
<th>Prognosis</th>
<th>Survival period (months)</th>
<th>TA (TPG)</th>
<th>ALT</th>
<th>hTERT</th>
<th>Mean TRF (kb)</th>
<th>SIR (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Female</td>
<td>Femur</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>130</td>
<td>13.5</td>
<td>–</td>
<td>–</td>
<td>304</td>
<td>13.7</td>
</tr>
<tr>
<td>65</td>
<td>Female</td>
<td>Femur</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>37</td>
<td>21</td>
<td>–</td>
<td>–</td>
<td>1405.4</td>
<td>14.4</td>
</tr>
<tr>
<td>46</td>
<td>Male</td>
<td>Femur</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>141</td>
<td>15.4</td>
<td>+</td>
<td>+</td>
<td>921.8</td>
<td>30.6</td>
</tr>
<tr>
<td>27</td>
<td>Female</td>
<td>Clavicle</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>92</td>
<td>10.6</td>
<td>+</td>
<td>+</td>
<td>323.1</td>
<td>14.7</td>
</tr>
<tr>
<td>57</td>
<td>Male</td>
<td>Femur</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>93</td>
<td>9.2</td>
<td>–</td>
<td>–</td>
<td>241.7</td>
<td>12.3</td>
</tr>
<tr>
<td>69</td>
<td>Male</td>
<td>Femur</td>
<td>Stori-pleo</td>
<td>DOD</td>
<td>8</td>
<td>0.9</td>
<td>+</td>
<td>+</td>
<td>1278.2</td>
<td>38.1</td>
</tr>
<tr>
<td>67</td>
<td>Male</td>
<td>Sacrum</td>
<td>Stori-pleo</td>
<td>DOD</td>
<td>7</td>
<td>10.7</td>
<td>+</td>
<td>+</td>
<td>324.5</td>
<td>18.2</td>
</tr>
<tr>
<td>38</td>
<td>Male</td>
<td>Humerus</td>
<td>Stori-pleo</td>
<td>DOD</td>
<td>18</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>603.6</td>
<td>38.5</td>
</tr>
<tr>
<td>57</td>
<td>Female</td>
<td>Ilium</td>
<td>Stori-pleo</td>
<td>DOD</td>
<td>6</td>
<td>17.4</td>
<td>+</td>
<td>+</td>
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<td>25.9</td>
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<tr>
<td>27</td>
<td>Male</td>
<td>Tibia</td>
<td>Stori-pleo</td>
<td>CDF</td>
<td>118</td>
<td>1.4</td>
<td>–</td>
<td>–</td>
<td>426.6</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Stori-pleo: Storiform-pleomorphic; TA: telomerase activity; CDF: continuous disease-free; DOD: died of disease.
reports indicate a positive correlation between the telomere maintenance mechanism and tumor aggressiveness in several sarcoma types (5, 10-17, 22). While MFH arising from soft tissue is the most common sub-type of soft tissue sarcoma, MFH of bone is a rare primary bone tumor, accounting for approximately 1% of all primary bone tumors (25). To the best of the Authors’ knowledge, only one report has demonstrated the prevalence of telomerase activity in three cases of bone MFH (16).

ALT cells usually show a heterogeneous telomere length, ranging from very short to abnormally long and several types of sarcomas have been reported to have ALT telomeres without telomerase activation (5, 26). Approximately 50% of sarcomas are telomerase-negative so that ALT appears to be much more common in mesenchymal-originating sarcomas compared to other tumour types (27). However, several studies have shown evidence of the presence of both ALT and telomerase activity (5, 11-14). In the present study, telomerase activity and hTERT were detected in all bone MFH samples examined and 50% of the samples were ALT-positive. Telomere maintenance mechanisms by both telomerase and ALT may be important in evading senescence and necessary for tumourigenesis in bone MFH cells.

Although soft tissue MFH and bone MFH tumours are histologically indistinguishable, these tumours have different oncological characteristics. Based on previous reports, bone MFH patients have a worse prognosis than soft tissue MFH patients, and chemotherapy is more effective for bone MFH than for soft tissue MFH (28). It was previously reported that telomerase activity is detected in 79% of soft tissue MFH samples with the mean level of telomerase activity being 7.57 TPG (12). In the present study, telomerase activity was detected in all bone MFH samples and the mean level of telomerase activity was 10.1 TPG. Therefore, telomerase activity was higher in bone MFH tumours than in soft tissue MFH tumours. This difference in telomerase activity levels may be related to the different oncological characteristics of the two tumour types.

The prognosis for patients with bone MFH depends on histological characteristics, surgical margins, local recurrence and efficacy of chemotherapy (28-39). However, in this study, ALT was a significant prognostic risk factor in patients with bone MFH. There were no significant differences in survival rates according to telomerase activity and hTERT expression. The present study suggested that the presence of ALT telomere maintenance mechanisms indicates a poor prognosis for patients with bone MFH, independent of telomerase and telomere maintenance.

A better understanding of the roles of telomere maintenance mechanisms, especially ALT mechanisms, may lead to novel adjuvant telomerase and telomere-targeting therapy to improve the treatment of patients with bone MFH.

References

Figure 1. Kaplan-Meier survival curves for ALT-positive and ALT-negative patients. ALT-positive patients had a worse prognosis compared to ALT-negative patients (p=0.0316).