Expression Pattern of the *HMGB1* Gene in Sarcomas of the Dog

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**Abstract.** Background: The human high mobility group protein B1 (HMGB1) has attracted considerable interest among oncologists because it sensitises cancer cells to the anticancer drug cisplatin by shielding cisplatin-DNA adducts from nucleotide excision repair. Materials and Methods: Since cisplatin is the cornerstone of adjuvant systemic therapy for osteosarcomas, in both humans and dogs, the expression pattern of the HMGB1 gene in seven canine sarcomas was investigated by Northern blot analysis and semi-quantitative RT-PCR. Results: A strong intertumoural variation of HMGB1 expression was detected by Northern blot analysis and confirmed by the semi-quantitative RT-PCR established herein. Conclusion: The observed variations of HMGB1 expression in canine sarcomas emphasises the role of HMGB1 as a potential marker of clinical interest as its expression level may predict the clinical outcome of therapies based on cisplatin. The semi-quantitative RT-PCR established allows a quick and convenient determination of the HMGB1 expression level as necessary for clinical applications.

The related platinum compounds cisplatin and carboplatin are widely used antitumour drugs for the treatment of a number of malignancies. The main cytotoxic effect of cisplatin/carboplatin is the formation of cisplatin/carboplatin-DNA adducts characterised by intrastrand cross-links and significantly bended and distorted DNA.

Gel mobility shift assays revealed a selective affinity of high mobility group (HMG) proteins for cisplatin-DNA adducts (1). The recognition of cisplatin damage by HMGB1 is assumed to mediate cisplatin cytotoxicity. HMG proteins are chromatin-associated non-histone proteins characterised by low molecular weight, acid-solubility and a high content of charged amino acids. According to their molecular size, sequence and DNA binding capacity, three families have been distinguished: HMGB (formerly HMG1/2), HMGN (formerly HMG14/17) and HMGA (formerly HMG(Y)) (2,3). The HMGB family, comprising HMGB1, HMGB2 and HMGB3, is characterised by its two DNA-binding domains called the "HMG-Box" (4,5).

Interestingly, *HMGB1* gene expression can be induced by oestrogens in breast cancer cells probably due to an up-regulation of the gene, so that *HMGB1* itself can be considered an oestrogen-responsive gene (11). Recently, we were able to explain this observation by the identification of two oestrogen responsive elements within the first intron of *HMGB1* (12). He et al. (10) have shown that, in oestrogen receptor-positive human breast cancer cells, oestrogen can significantly increase the effect of cisplatin by causing an overexpression of *HMGB1*. This finding has led to the conclusion that oestrogen treatment prior to cisplatin therapy may sensitis the cancer cells against that drug. Accordingly, a clinical trial for the treatment of gynaecological tumours with cisplatin has already been approved by the Food and Drug Administration (FDA) (10). On the other hand, the former experiment clearly shows that the quantitation of the intratumoural *HMGB1* expression level may be of high impact for a cisplatin/carboplatin therapy for two reasons. Firstly, it may predict the clinical outcome of the therapy; secondly, it may influence the therapy protocol as, for example, tumours

**Key Words:** Osteosarcoma, cisplatin, *HMGB1* expression, semi-quantitative RT-PCR.
Thus, in this study we analysed the GGGCAAGGAGATCCTAAGAAG 3') (13) and Ex5lo (5’- GAGGAGGATTGGCTGACAAT 3’). A 445 bp cDNA probe detecting the 1.3 kb transcript of the canine GAPDH gene was amplified by PCR with the primer set GAPDH2up (5’- GTGAAGGTCGGAAGTAC 3’) and GAPDHdog5do (5’- GAGGAGGATTGGCTGACAAT 3’). Probes were labelled with 50 µCi(-32P)dCTP (Amersham Biosciences) using the Megaprime Labelling Kit (Amersham Biosciences) for random-primed labelling (14). Hybridisation was performed for 3 h at 68°C in 10 ml of PerfectHyb Plus Hybridisation Buffer (Sigma-Aldrich, Saint Louis, USA). The membranes were washed for 5 min with low stringency at RT in 2x SSC, 0.1% SDS and twice for 20 min with high stringency at 68°C in 0.5x SSC, 0.1% SDS. Signals were visualised using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA). Quantitation of the transcripts of HMGB1 and GAPDH was performed using the software program ImageQuant (Molecular Dynamics).

**Table I. Sarcoma samples analysed in this study.**

<table>
<thead>
<tr>
<th>Sarcoma sample</th>
<th>Tumour</th>
<th>Breed</th>
<th>Sex</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS1</td>
<td>Osteosarcoma</td>
<td>Rottweiler</td>
<td>f</td>
<td>1 yr</td>
</tr>
<tr>
<td>OS2</td>
<td>Osteosarcoma</td>
<td>Crossbreed</td>
<td>f</td>
<td>4 yrs</td>
</tr>
<tr>
<td>OS3</td>
<td>Osteosarcoma</td>
<td>German Shepherd</td>
<td>m</td>
<td>6 yrs</td>
</tr>
<tr>
<td>OS4</td>
<td>Osteosarcoma</td>
<td>Crossbreed</td>
<td>m</td>
<td>9 yrs</td>
</tr>
<tr>
<td>OS5</td>
<td>Osteosarcoma</td>
<td>German Shepherd</td>
<td>m</td>
<td>n.r.¹</td>
</tr>
<tr>
<td>FS</td>
<td>Fibrosarcoma</td>
<td>Bobtail</td>
<td>m</td>
<td>5 yrs</td>
</tr>
<tr>
<td>LMS</td>
<td>Leiomyosarcoma</td>
<td>Crossbreed</td>
<td>f</td>
<td>10 yrs</td>
</tr>
</tbody>
</table>

¹n.r. = not reported

showing a high HMGB1 expression level may be treated with a lower amount of this antitumour drug.

Due to the close similarities of numerous canine diseases to their human counterparts, the role of the dog as a model organism for therapeutic approaches is justified. Furthermore, genes and proteins known to be of high diagnostic and therapeutic impact in man can also be considered to play an important role in the dog.

Osteosarcomas and several types of carcinomas belong to the group of canine malignancies often treated with cisplatin or carboplatin. So far no data are available analysing the expression pattern of the HMGB1 gene in canine sarcomas. Thus, in this study we analysed the HMGB1 expression level in five canine osteosarcomas, one fibrosarcoma and one leiomyosarcoma by Northern blot experiments. Based on the observed strong intertumoural variation of HMGB1 expression, we further established a quick RT-PCR-based diagnostic system for future studies.

**Materials and Methods**

*Tissue samples.* All canine tumour samples used in this study (Table I) were provided by the Clinic for Small Animals, Hanover, Germany. Samples were taken during surgery, immediately frozen in liquid nitrogen and stored at -80°C.

*RNA isolation.* Total RNA extraction of the canine sarcoma samples was performed according to the RNeasy midi protocol for isolation of total RNA from heart, muscle and skin tissue (Qiagen, Hilden, Germany) including a Proteinase K digest. Enrichment of poly A+ mRNA was carried out using the Oligotex mRNA kit (Qiagen).

*Northern blot hybridisation.* For Northern blot analysis, 5 µg of mRNA from each sample were separated on a 1.2% denaturing agarose gel containing 0.65% formaldehyde. RNAs were transferred onto a Hybond-XL charged nylon membrane (Amersham Biosciences, Buckinghamshire, England) by capillary blot overnight. As a probe for hybridisation, a 603 bp cDNA fragment derived from the ORF (exon 2-5) of the canine HMGB1 gene was generated by PCR using the primer pair ToastUP (5’- GGGCAAGGAGGATCCTAAGAAG 3’) (13) and Ex5lo (5’- TCTTCCCTTCCTTCCTCATCC 3’). Semi-quantitative RT-PCR. cDNA synthesis was performed using primer AP2 (5’- AAGGATCTCGTGACATCT 3’) with 500 ng of mRNA with SuperScript Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. In order to determine the expression of HMGB1 in relation to that of the housekeeping gene GAPDH, a duplex PCR was established using the primer sets ToastUP/Ex5lo and GAPDH2up/GAPDHdog5do (see above). PCR reactions were set up according to the "basic PCR protocol" of Taq DNA Polymerase (Invitrogen) using the following PCR program: initial denaturation for 5 min at 94°C, 28 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 55°C and extension for 45 sec at 72°C, followed by a final extension for 10 min at 72°C. The appropriate number of cycles was previously determined so that for both PCR-products amplification was in the exponential range (data not shown). PCR-products were separated on a 1.2% agarose gel stained with VistraGreen (Amersham) and visualised using a Storm PhosphorImager (Molecular Dynamics). Quantitation of the PCR-fragments of HMGB1 and GAPDH was performed using the software program ImageQuant (Molecular Dynamics) measuring pixel intensities.

**Results**

Northern blot hybridisation on a series of 5 osteosarcomas, one fibrosarcoma and one leiomyosarcoma sample of the dog (Table I), using a cDNA probe derived from the ORF (Exon 2-5) of the canine HMGB1 gene, resulted in the detection of two HMGB1 mRNA transcripts of approximately 1.4 and 2.4 kb (Figure 1), which are similar to that observed in human tissues (15-17) and various canine tissues (18). In order to quantify the expression of HMGB1, the blot was rehybridised with a canine GAPDH-specific cDNA probe (Figure 1). Summing up the intensities of the 1.4 and 2.4 kb HMGB1 signals, the HMGB1-RNA / GAPDH-RNA ratios were calculated. As shown in Figure 1, the analysed canine sarcoma samples revealed a strong intertumoural variation in the relative expression of HMGB1. Values obtained by Northern blot analysis for the osteosarcoma samples varied between 0.52 and 1.31, while the fibrosarcoma and the leiomyosarcoma showed ratios of 0.73 and 0.24, respectively (Table II).
In order to confirm the results and to develop a less time- and material-consuming technique, we established a semi-quantitative duplex RT-PCR suitable for detecting intertumoural variation of HMGB1 expression in relation to expression of the house-keeping gene GAPDH (Figure 2). After quantitation of the signals obtained by RT-PCR, the HMGB1-RNA / GAPDH-RNA ratios were calculated. The values for the osteosarcoma samples varied between 0.72 and 1.28, while the ratios for the fibrosarcoma and the leiomyosarcoma were 0.73 and 0.42, respectively (Table II). In order to determine the comparability of the results obtained by the Northern blot hybridisation and RT-PCR analyses, mean values for each test series were calculated, set to one, and relative expression levels were determined (Table II, Figure 3). Statistical analysis using the Pearson’s Correlation Test revealed a significant correlation between the relative HMGB1 expression level obtained by Northern blot hybridisation and the level obtained by the established RT-PCR (r=0.8919, p=0.0071).

**Discussion**

Cisplatin and carboplatin are widely used anticancer drugs, manifesting their cytotoxicity to tumour cells by damaging DNA, generating a distorted DNA duplex. HMGB1 proteins selectively bind with high affinity to cisplatin or carboplatin-DNA adducts and several investigations revealed that this interaction contributes to tumour death by blocking excision repair of the major cisplatin-DNA adducts (9,10).

No features have been identified yet allowing clinicians to predict the response to cisplatin or carboplatin therapies in dogs with osteosarcomas at the time of diagnosis or during treatment (19). Hence, it was the aim of this study to analyse the expression level of HMGB1 in canine sarcomas.

Based on Northern blot and RT-PCR analyses, we were able to show an intertumoural variation of HMGB1 expression levels among canine sarcomas. Very recently, comparable results were obtained for human breast cancer samples (17,20) and a clinical trial designed to increase HMGB1 expression by oestrogen treatment has been approved by the FDA (10). The observed intertumoural variances of HMGB1 expression in seven sarcomas analysed in this study may be of importance for therapeutic approaches based on cisplatin/carboplatin treatment as, for example, tumours showing a high HMGB1 expression level may be treated with a lower amount of this antitumour drug. However, future clinical studies including a greater number of tumours have to be performed to correlate the

<table>
<thead>
<tr>
<th>Sarcoma sample</th>
<th>Absolute HMGB1 / GAPDH-RNA ratios</th>
<th>Relative HMGB1 / GAPDH-RNA ratios1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Northern blot</td>
</tr>
<tr>
<td>OS1</td>
<td>0.95</td>
<td>0.52</td>
</tr>
<tr>
<td>OS2</td>
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<tr>
<td>OS3</td>
<td>1.02</td>
<td>1.05</td>
</tr>
<tr>
<td>OS4</td>
<td>1.28</td>
<td>1.31</td>
</tr>
<tr>
<td>OS5</td>
<td>0.72</td>
<td>0.60</td>
</tr>
<tr>
<td>FS</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>LMS</td>
<td>0.42</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Mean value 0.87 0.75 1.0 1.0

1 Calculated with the mean values of the absolute HMGB1 / GAPDH-RNA ratios set to one.

Figure 1. Northern blot analysis of five osteosarcomas (OS1-5), one fibrosarcoma (FS) and one leiomyosarcoma (LMS) of the dog hybridised with a HMGB1-specific cDNA probe detecting the two canine HMGB1 transcripts of approximately 1.4 and 2.4 kb (upper part). Co-hybridisation of the same membrane with a GAPDH-specific cDNA probe detecting a 1.3 kb transcript (lower part).

Figure 2. Semi-quantitative duplex RT-PCR products of HMGB1 (603 bp) and GAPDH (445 bp) using canine cDNAs of five osteosarcomas, one fibrosarcoma and one leiomyosarcoma after electrophoresis and VistraGreen staining (Amersham Biosciences). Lane 1: DNA molecular weight standard 1 Kb Plus DNA Ladder (Invitrogen). Lanes 2-6: osteosarcoma samples 1-5 (OS1-5). Lane 7: fibrosarcoma sample (FS). Lane 8: leiomyosarcoma sample (LMS). Lane 9: H2O, negative control.
HMGB1 expression level with clinical outcome of cisplatin/carboplatin chemotherapy. The statistically significant correlation of the relative HMGB1 expression levels obtained by Northern blot analyses as well as duplex RT-PCR makes the established PCR approach a quick and convenient method to determine the intratumoural HMGB1 expression.

References


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