Alternate splicing of the p53 inhibitor HDMX offers a superior prognostic biomarker than p53 mutation in human cancer

Kristiaan Lenos1*, Anna M. Grawenda2*, Kirsten Lodder1, Marieke L. Kuijjer3, Amina F.A.S. Teunisse1, Emmanouela Repapi2, Lukasz F. Grochola3, Frank Bartel4, Pancras C. W. Hogendoorn3, Peter Wuerl5, Helge Taubert6, Anne-Marie Cleton-Jansen3, Gareth L. Bond2§, Aart G. Jochemsen1§

1 Department of Molecular Cell Biology, Leiden University Medical Center, P.O. Box 9600, S1-P, 2300 RC Leiden, The Netherlands
2 Ludwig Institute for Cancer Research, University of Oxford, Nuffield Department of Clinical Medicine, Old Road Campus Research Building, Oxford OX3 7DQ, United Kingdom
3 Department of Pathology, Leiden University Medical Center, PO Box 9600, L1-Q, 2300 RC Leiden, The Netherlands
4 Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Magdeburger Str. 14, 06097 Halle/Saale, Germany
5 Department of General and Visceral Surgery, Diakonie-Hospital Halle, Advokatenweg 1, 06114 Halle, Germany
6 University Clinic of Urology, Div. Molecular Urology, FAU Erlangen-Nürnberg, Germany and Nikolaus-Fiebig-Center for Molecular Medicine, FAU Erlangen-Nürnberg, Glückstr. 6, 91054 Erlangen, Germany

The authors declare that there are no conflicts of interests.

*These authors contributed equally to this manuscript.

§Corresponding authors:
A.G.Jochemsen@lumc.nl. Department of Molecular Cell Biology, Leiden University Medical Center, P.O. Box 9600, S1-P, 2300 RC Leiden, The Netherlands.
Gareth.Bond@ndm.ox.ac.uk. The Ludwig Institute for Cancer Research, The Nuffield Department of Medicine, The University of Oxford, Oxford, The United Kingdom.

Running title: A novel role for HDMX alternate splicing in cancer

Keywords: Sarcoma, p53, HDMX, Splicing, Prognosis
Abstract

Conventional high-grade osteosarcoma is the most common primary bone malignancy. Although altered expression of the p53 inhibitor HDMX (Mdmx/Mdm4) is associated with cancer risk, progression, and outcome in other tumor types, little is known about its role in osteosarcoma. High expression of the Hdmx splice variant HDMX-S relative to the full length transcript (the HDMX-S/HDMX-FL ratio) correlates with reduced HDMX protein expression, faster progression and poorer survival in several cancers. Here, we demonstrate that the HDMX-S/HDMX-FL ratio positively correlates with less HDMX protein expression, faster metastatic progression and a trend to worse overall survival in osteosarcomas. We found that the HDMX-S/HDMX-FL ratio associated with common somatic genetic lesions connected with p53 inhibition, such as p53 mutation and HDM2 overexpression in osteosarcoma cell lines. Interestingly, this finding was not limited to osteosarcomas as we observed similar associations in breast cancer and a variety of other cancer cell lines, as well as in tumors from soft tissue sarcoma patients. The HDMX-S/HDMX-FL ratio better defined sarcoma patients with worse survival rates than p53 mutational status. We propose a novel role for alternative splicing of HDMX, whereby it serves as a mechanism by which HDMX protein levels are reduced in cancer cells that have already inhibited p53 activity. Alternative splicing of HDMX could therefore serve as a more effective biomarker for p53 pathway attenuation in cancers than p53 gene mutation.
Introduction

Osteosarcoma is the most common primary bone malignancy in children and adolescents, occurring mostly in patients between 10 and 25 years of age and found in the rapid growth areas of the long bones (1). The vast majority of osteosarcomas arise without a clear hereditary component, with the exception of a small subset including individuals with the Li-Fraumeni and progeria syndromes. Overall long-term (5-year) survival for patients with non-metastatic osteosarcomas has increased from 10 to 65% over the past 20 years, due to improved surgical techniques and multidrug chemotherapy, but further improvement is needed (2). Indeed, 25-50% of patients will relapse or metastasize and 5-year survival rate for metastatic osteosarcomas is only 10 to 20% (3, 4). It is clear that additional therapies are required in order to increase the survival of osteosarcoma patients.

In approximately 50% of all human cancers mutations are found in the TP53 gene, which encodes the tumor suppressor protein p53 (5-7). Indeed, it has been clearly shown that in many of the remaining 50% of cancers, the activity of the wild type p53 protein is greatly attenuated (8). In high-grade osteosarcoma, mutations in the p53 gene are found in only 20% of the tumors (9-11). However, the genomic region that encodes for a key negative regulator of p53, HDM2 (12, 13), was found to be amplified in 10-35% of osteosarcomas and these amplifications were shown to be mutually exclusive to p53 mutations (9-11, 14).

In many of the studies that were aimed at determining the importance of the inactivation of the p53 pathway during the progression of osteosarcoma, only p53 mutational status was studied and the level of HDM2 protein expression was not included, resulting in the possible underestimation of the influence of p53 inactivation on prognosis. Moreover, the somatic mutation or alteration of other crucial effectors of p53 activity has also been under-studied in osteosarcoma. For example, the oncogene HDMX is a close homolog of HDM2 and encodes a
protein product, which also clearly inhibits p53 activity (15, 16). In several tumor types, overexpression and amplification of HDMX has been shown both in tumors (17, 18) and tumor-derived cell lines and is primarily found in tumors that have retained wild-type p53 (19). Indeed, two tumor types that have a very low frequency of p53 gene mutation (retinoblastomas and Ewing sarcomas) have demonstrated elevated HDMX expression (20, 21).

In sarcomas, amplification of the HDMX gene has been found in both soft tissue sarcomas (STS, 16 to 17%) and osteosarcomas (35%) (14, 22). Bartel et al. demonstrated a significant association between HDMX amplification and poor prognosis after initial STS diagnosis (22). In addition, they showed that the mRNA expression of an alternative splice variant of Hdmx, HDMX-S, is upregulated in 14% of STS, and that an increase in the ratio of the transcript levels of the splice variant over full length (the HDMX-S/HDMX-FL ratio) correlates with a higher tumor grade at diagnosis and decreased overall survival (22). The HDMX-S/HDMX-FL ratio was also found to be increased in high-grade glioblastomas (17) and papillary thyroid carcinomas (23). The HDMX-S transcript results from the exclusion of exon 6 (24), resulting in a truncated protein essentially consisting of the p53-binding domain which, in overexpression studies, was found to be stronger inhibitor of p53 shown to bind to and inhibit p53 more efficiently (24). Although studies such as these suggest an oncogenic role for the HDMX-S protein, no conclusive evidence has been published to date to suggest that the protein is produced and active in cells (25). Indeed, our previous work suggests that an increase in the HDMX-S/HDMX-FL ratio associates primarily with reduced levels of full length HDMX protein (25).

Here, we report further on the alterations in the HDMX-S/HDMX-FL ratio in relation with p53 status and cancer progression. The results indicate that changes in the HDMX-S/HDMX-FL ratio could serve as a more effective biomarker for p53 pathway attenuation in cancers than p53 gene mutation.
Materials and Methods

Patient Material

Osteosarcoma cohort

Clinicopathological data of 51 osteosarcoma patients are shown in Supplementary Table S1. Part of the samples were previously described (26). Biopsies were taken before pre-operative chemotherapeutics were administered. The differences in response to chemotherapy were classified as good or poor, using the Huvos criteria (27). All tissue samples were handled according to the National Ethical Guidelines ('Code for Proper Secondary Use of Human Tissue in The Netherlands', Dutch Federation of Medical Scientific Societies).

Soft Tissue Sarcoma Cohort

The clinicopathological data of the 157 soft tissue sarcoma patients included in this study are shown in Supplementary Table S2. A subset was previously described (22). Tumor samples were taken before adjuvant radio- and/or chemotherapeutics were administered. The study adhered to national regulations on ethical issues and was approved by the local ethical committee. All patients gave written and informed consent (Department of Surgery 1, University of Leipzig, Germany).

Cell Culture and Reagents

Cell lines were maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics. Most of the osteosarcoma cell lines have been described recently, including their genotyping and detailed methods on p53 sequencing (28). Osteosarcoma cell lines L2531, L2635, L2792, L2826, L2857 and L2962 were recently established at the Dept. of Molecular Cell Biology, LUMC in the lab of Dr. Szuhai. Status of p53 gene was determined as described by Da Costa et al. (29). Tumor -, normal - and cell line DNA was typed to confirm cell line identity with use of the Cell ID system of Promega. Normal osteoblasts were obtained as
described previously (30). Breast cancer cell lines were a gift from Mieke Schutte (Erasmus MC, Rotterdam). The origin of all breast cancer cell lines and verification of their individual identity has been described previously, including a full description of the p53 gene sequencing(31, 32). RNA and protein was extracted from the cells within 5 passages after receipt. All cell lines are routinely tested for mycoplasm infection. To determine the p53-response cells were treated with 10 μM Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA) for the indicated times. The RNA from the NCI60 panel of cell lines was a generous contribution from the NCI-Division of Cancer Treatment and Diagnosis Repository Molecular Characterization Program.

**RNA isolation, Reverse Transcription, Semi-Quantitative PCR and Q-RT-PCR**

**Osteosarcomas and breast cancer cell lines**

RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer’s protocol, followed by cDNA synthesis following standard protocols. Semi-Quantitative PCR was performed according to standard protocols; for detection of both HDMX-FL and HDMX-S, the HDMX ex3 forward 5’-TGCATGCAGCAGGTGCG-3’ and the HDMX ex8 reverse 5’-CATTACTTCTAGGTGTAT-3’ primers were used. For detection of GAPDH, the GAPDH forward 5’-AATCCCATCACCATCTTCC-3’ and the GAPDH reverse 5’-ATGAGTCCTTCCACGATACC-3’ primers were used. Band intensities were quantified using Odyssey 2.1 analysis software (LI-COR Biosciences, Lincoln, Nebraska USA).

**NCI60 cell lines**

The cDNAs for the NCI60 cell lines panel were derived from RNAs obtained from the National Cancer Institute (NCI)/NIH Developmental Therapeutics Program (DTP), a recognized cell repository. The NCI60 panel of human tumor cell lines are some of the most extensively characterized cell lines. In regards to cell line identity, the cell lines have been characterized using many approaches, such as SNP arrays, oligonucleotide-base HLA typing, spectral karyotyping, screening
for known cancer mutations, and variations in short tandem repeats. Much of these data can be found on the DTP web-site. The mRNA levels of HDMX-S and HDMX-FL were determined as described above. Measurements of HDM2 transcript levels were performed by qRT-PCR using commercially available RoboGene®MDM2 cDNA quantification module, with ABI PRISM 7000 Sequence Detection System. HDM2 measurements were normalised to GAPDH transcript levels.

Soft tissue sarcomas

HDMX-FL and HDMX-S transcript levels were measured by quantitative real-time RT-PCR as described (22). Briefly, the reactions were carried out on a RotorGene 3000 (Corbett Research, Sydney, Australia), with a common forward primer for both FL-HDMX and HDMX-S; X quant fw 5’-CAGCAGGTGCAGCAAGGTGAA-3’ and reverse primers specifically designed for amplification of either FL-HDMX, FL-AS 6 5’-CTGTGCGAGAGCGAGTCTG-3’ or HDMX-S, XS AS 5’-GCACCTTTGCTGTAGTAGCAGTG-3’. Each reaction included 1/10 of the cDNA reaction, 10 μL of 2X Quantitect SYBR Green Master Mix (Qiagen) and 20 pmol of the respective primers in a total volume of 20 μL. The PCR consisted of 50 cycles with 30 sec of denaturation at 95 °C, 30 sec of primer annealing at 58 °C, and synthesis at 72 °C for 30 sec. The HDMX-FL and HDMX-S levels were normalized against GAPDH expression levels.

The p53 mutational status was assessed by sequencing exons 4 through 10. Details of p53 sequencing are provided in Supplementary Information.

Protein extraction, Western blotting and Antibodies

Protein extraction and immunoblotting were performed as described previously (33). Anti-Hdmx and anti-USP7 (A300-287A,A300-033A; Bethyl Laboratories, Montgomery, TX), anti-p53 PAb1801 and DO-1, anti-HDM2 antibodies 4B2 (34) and SMP14 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies goat-anti-mouse-HRP and goat-anti-rabbit-HRP were obtained from Jackson Laboratories.
Statistical Analysis

The GraphPad Instat version 3.06 software was used to compare means. The Kaplan Meier and Cox multivariate regression survival analyses and the analyses of contingency tables were performed using the SPSS 19.0 software. Statistical significance was regarded as p< 0.05.

Results

The **HDMX-S/HDMX-FL ratio in osteosarcoma**

To further explore the importance of the p53 pathway during the progression of osteosarcoma, we initially analyzed the **HDMX-S/HDMX-FL ratio** and its association with HDMX protein levels in 22 osteosarcoma cell lines. The levels of **HDMX-S** and **HDMX-FL** mRNA were determined in logarithmically growing cells for all 22 osteosarcoma cell lines and 1 osteoblast. Semi-quantitative PCR was performed using primers designed to amplify exons 3 to 8 (Fig. 1A). The intensities of the bands for **HDMX-S** and **HDMX-FL** were quantified and normalized to **GAPDH** expression levels (Fig. 1B) and results are summarized in Supplementary Table S3. The protein levels of HDMX were also determined from logarithmically growing cells (Fig. 1C). The intensities of the band corresponding to HDMX were quantified and normalized to **USP7** levels (Supplementary Table S3). The **HDMX-S/HDMX-FL** ratios varied from 0.39 to 3.18 with a median of 1.5, a standard deviation of 0.7 and a normal distribution (Fig.2A). Interestingly, the **HDMX-S/HDMX-FL** ratio significantly negatively correlated with the measured HDMX protein levels (p=0.004, Fig. 2B). Specifically, those five cells with the highest **HDMX-S/HDMX-FL** ratios had 9.5 fold less HDMX protein than those five cells with the lowest ratios (t-test, p=0.0238, Fig. 2C).
In order to explore the potential association of the $HDMX-S/HDMX-FL$ ratio with osteosarcoma progression and survival we studied 51 high-grade osteosarcoma patients from Germany, Holland and Italy (Supplementary Table S1). The patients consisted of 32 males and 19 females with an average age of diagnosis of 17.5 years, ranging from 3-58 years. The levels of $HDMX-S$ and $HDMX-FL$ were determined in biopsies that were taken before pre-operative chemotherapy. The level of each transcript was determined in 49 of the samples (Fig. 3A, Supplementary Table S4) and the intensities of the bands for $HDMX-S$ and $HDMX-FL$ were quantified and normalized to $GAPDH$ expression levels. The $HDMX-S/HDMX-FL$ ratios varied from 0.38 to 4.86 with a median of 1.3 and a standard deviation of 1. Interestingly, the distribution of the $HDMX-S/HDMX-FL$ ratios was not normal (p=0.0011). In fact, 11 tumors demonstrated very high ratios, as defined by one standard deviation above the median (Fig. 3B).

To determine if the $HDMX-S/HDMX-FL$ ratios associated with progression of the high-grade osteosarcomas, we explored whether patients whose tumors had the highest $HDMX-S/HDMX-FL$ ratios had a shorter time to metastasis than patients with the lowest ratios. The metastasis information was available for all osteosarcoma patients. Of the 49 patients where the $HDMX-S/HDMX-FL$ ratios were successfully determined, 38 patients had not yet presented metastasis upon inclusion in the study. Interestingly, when the patients were divided up into three equal groups based on the measured $HDMX-S/HDMX-FL$ ratios in the tumors (12 low, 13 intermediate and 13 high), those patients whose tumor had the highest ratios had the shortest time to metastasis and those with lowest ratios, the longest time (Fig. 3C, p=0.005, Logrank Test). Specifically, the 13 patients whose tumors had the highest $HDMX-S/HDMX-FL$ ratios (average ratio of 2.55, ranging from 1.8 to 4.07) associated with a significantly shorter time of metastasis-free survival than the 12 whose tumors had the lowest ratios (average ratio of 0.8, ranging from 0.44 to 1.0). In two and a half years, 62% of those with the highest $HDMX-S/HDMX-FL$ ratios had presented with a metastasis, compared to only 8% of those with the lowest ratios. Similar, trends were seen in
over-all survival, whereby the 13 patients with highest 
HDMX-S/HDMX-FL ratio associated with shorter over-all survival than the 12 with the lowest ratios (p=0.072, Fig. 3D).

These results show that the HDMX-S/HDMX-FL ratio positively correlates with less HDMX protein expression, faster metastatic progression and worse overall survival of osteosarcoma. We propose that a reason a cancer cell would not retain the oncogenic activity of full length HDMX could be because it has already inhibited p53 signalling by mutations of other key regulatory genes in the pathway and, therefore, is no longer under selective pressure to sustain high levels of HDMX. If true, cancer cells with higher HDMX-S/HDMX-FL ratios should associate with higher frequencies of other p53 pathway mutations. To test this possibility, we determined the status of two well characterized somatic alterations that result in p53 inhibition, p53 gene mutation and over-expression of HDM2, in all 22 osteosarcoma cell lines. The protein levels of HDM2 were determined in logarithmically growing cells, and normalized to USP7 levels (Fig. 1C; Supplementary Table S3). The p53 status of all 22 osteosarcoma cell lines was determined using a combination of direct sequencing, p53 mRNA measurements and measurements of the response of each cell line to Nutlin-3, a small molecule inhibitor of the p53-HDM2 interaction that activates p53 signalling (35). A cell line was only deemed to have functional wild type p53 if no mutations were found in exons 3 to 11, it expressed detectable levels of p53 mRNA and protein, and responded to Nutlin-3 treatment, as measured by the determination of p53 and HDM2 protein levels (Supplementary Fig. S1A), reduced survival (Supplementary Fig. S1B), and inhibition of cell cycle progression (Supplementary Fig. S1C). Of the 22 cell lines, only 6 lines were deemed to be wild type for p53 (Supplementary Table S3). Interestingly, and in support of the hypothesis above, those 6 cell lines contained significantly lower HDMX-S/HDMX-FL ratios than the remaining 16 cell lines (p=0.0325, Mann-Whitney test, Table 1). Similar trends were observed when those cells retaining wild-type p53 and low levels of HDM2 were compared with those with wild-type p53 and higher levels of HDM2 (p=0.1,
Mann-Whitney test, Table 1), thereby lending further support to the hypothesis that higher HDMX-S/HDMX-FL ratios, and therefore lower HDMX protein levels, can arise in cancer cells that have already inhibited p53 signalling through alterations of other key p53 pathway genes.

The HDMX-S/HDMX-FL ratio in breast cancer and NCI60 cells

In order to test this hypothesis further, we explored the associations of the HDMX-S/HDMX-FL ratio in a panel of 37 cell lines derived from breast cancer, a tumor type that is clearly surveyed by the p53 pathway, and in which p53 mutational status of the tumor has clear prognostic value (36). We first determined the levels of HDMX-S and HDMX-FL (Fig. 4A). The HDMX, HDM2 and p53 protein levels and p53 mutational status in all 37 cells have been published before (32, 33). The results are summarized in Supplementary Table S5. The HDMX-S/HDMX-FL ratios varied from 0.12 to 4.35, with a median of 1.1 and a standard deviation of 1.15. The distribution of the HDMX-S/HDMX-FL ratios is not normal (p=0.0241). Of 37 cell lines 9 showed very high ratios, as defined by one standard deviation above the median (Figure 4B). Interestingly, the HDMX-S/HDMX-FL ratio significantly negatively correlated with measured HDMX protein levels (p=2.28x10^-8, Fig. 4C). Furthermore, when the cells were ranked by the measured HDMX-S/HDMX-FL ratios and subsequently divided into almost equal groups of 9, the groups with increasing ratios had significantly decreasing HDMX protein levels (Kruskal-Wallis Test, p=0.0001, Fig. 4D). Specifically, the 9 cell lines with the highest HDMX-S/HDMX-FL ratios had 9.87 fold less HDMX protein than the 9 cell lines with the lowest ratios (Mann-Whitney Test, p= 0.0002).

The 8 breast cancer cell lines with wild-type p53 contained significantly lower HDMX-S/HDMX-FL ratios than the remaining 29 cell lines (p=0.0028, Mann-Whitney test, Table 1). Similar differences were seen when we compared cells retaining wild-type p53 and low levels of HDM2 to those with wild-type p53 and
high levels of HDM2 (p=0.343, Mann-Whitney Test, Table 1). In order to test this hypothesis further in another cell line panel, we determined the HDMX-S/HDMX-FL ratios and measured HDM2 mRNA levels in the National Cancer Institute 60 (NCI60) panel, which consists of 59 human cancer cell lines, representing 9 cancer types (Supplementary Table S6; (37)). Three cell lines overlap with the above-analysed breast cancer cell panel. Of the 59 cell lines, 14 are known to have wild-type p53, 36 have either mutated or deleted p53, while for the remaining cell lines p53 status is inconclusive (38, 39). We determined the levels of HDMX-FL and HDMX-S mRNA (Supplementary Fig. S2A) and the levels of HDM2 mRNA normalised to GAPDH using qRT-PCR (Supplementary Table S6). The HDMX-S/HDMX-FL ratios varied from 0 to 18.65, with a median of 1.25 and a standard deviation of 3.3. The distribution of the HDMX-S/HDMX-FL ratios, similarly to the breast cancer cell panel and osteosarcoma patients, was not normal (Fig. Supplementary S2B). Interestingly, we found that the 14 cell lines with wild-type p53 had significantly lower HDMX-S/HDMX-FL ratios than the remaining 36 cell lines lacking functional p53 (p=0.0227, Mann-Whitney Test, Table 1). Furthermore, a similar significant trend was observed when we compared HDMX-S/HDMX-FL ratios in the wild-type p53 cell lines with either high or low levels of HDM2 mRNA. Specifically, the 7 cell lines with the lowest HDM2 mRNA levels had significantly lower HDMX-S/HDMX-FL ratios than the 7 cell lines with the highest levels of HDM2 (p=0.0364, Mann-Whitney Test, 1-tailed, Table 1).

Together, these observations made in three cell panels comprising a total of 115 different cell lines, lend support to a model that higher HDMX-S/HDMX-FL ratios, and therefore lower HDMX protein levels, arises in cancer cells that have attenuated p53 signalling through alterations of key p53 pathway genes. Indeed, we observed that the 27 cell lines with wild-type p53 in the three panels had significantly lower HDMX-S/HDMX-FL ratios than the 79 cell lines lacking functional p53 (p=0.0001, Mann-Whitney Test, Table 1). Furthermore, in the 27 wild-type p53 cell lines, the 13 lines with the lowest HDM2 mRNA levels had
significantly lower \(HDMX-S/HDMX-FL\) ratios than the 14 with the highest levels of \(HDM2\) (\(p=0.0116\), Mann-Whitney Test, Table 1). When the 106 cell lines with p53 mutational status were separated into almost equal numbers after ranking based on the \(HDMX-S/HDMX-FL\) ratios, we observed that of the 35 lines with the lowest ratios, 12 (34\%) had wild-type p53 and lower HDM2 levels. In contrast, in the 36 cell lines with the highest ratios, only 1 cell line (3\%) had wild-type p53 and lower HDM2 levels (\(p=0.0006\), Fisher’s Exact Test, Fig. 5), compared to 4 of the 35 cell lines with intermediate ratios (11\%, \(p=0.001\), Fisher’s Exact Test).

The \(HDMX-S/HDMX-FL\) ratio and p53 mutation as prognostic markers

Thus far in cells, we have observed that the cancer lines with higher \(HDMX-S/HDMX-FL\) ratios have significantly higher frequencies of two common p53 pathway mutations, namely p53 gene mutation and HDM2 overexpression (Table 1, Figure 5). Inhibition of p53 signalling through p53 mutation has clearly been shown to have prognostic value in multiple cancers (36). If the proposed model is true, the \(HDMX-S/HDMX-FL\) ratio could potentially serve as a more effective prognostic biomarker than p53 gene mutation, because alterations of other key p53 pathway genes that result in p53 pathway attenuation could be captured simultaneously. To begin to explore this possibility, we compared the prognostic value of either p53 tumor mutation or the \(HDMX-S/HDMX-FL\) in another tumor type that is clearly surveyed by p53 signalling, namely soft tissue sarcoma (STS), where an increase of the \(HDMX-S/HDMX-FL\) ratio has been shown to associate with altered survival (22).

We studied a cohort of 157 soft tissue sarcoma patients from Germany (Supplementary Table S2). We were able to receive ample tumor material from 80 patients for the \(HDMX\) transcript analysis and the patients consisted of 36 males and 44 females. The levels of \(HDMX-S\) and \(HDMX-FL\) were determined, as described in the materials and methods, in tumor samples that were taken before therapy. The levels of each transcript were determined and normalized to
GAPDH expression levels. Sixteen tumors (20%) had no detectable HDMX transcript levels, 24 tumors (30%) had more HDMX-FL than HDMX-S transcripts, 24 tumors (30%) had equal amounts of HDMX-S and full length transcripts, while 16 tumors (20%) had more HDMX-S than HDMX-FL transcripts. The p53 mutational status was successfully determined in 104 tumors of the 157 patients. Of the 104 tumor DNAs, 19 were found to have missense mutations in the p53 gene. Interestingly, similar to the observations made in the three cell panels, in the 46 tumors where both the levels of HDMX-S and HDMX-FL and mutational status were determined, we observed that the tumors with higher HDMX-S transcript levels than HDMX-FL were significantly enriched for p53 mutations (p=0.0337, Fisher’s exact Test, one-sided, Fig. 6A). The p53 mutation status alone could not effectively serve as a prognostic factor. Patients that had retained wild-type p53 in their sarcomas had longer survival times than those with mutant p53, although the difference was not significant (p=0.469, Logrank Test, Fig. 6B; Supplementary Fig. S3A). However, the patients whose tumors had more HDMX-S than HDMX-FL transcripts had significantly shorter overall survival than those with equal or lower HDMX-S transcript levels (p=0.024 Logrank Test, Fig. 6C; Supplementary Fig. S3B,S3C) with a 5.746-fold higher risk of tumor-related death (p=0.01, Fig. 6D).

Discussion

In this study, we began with the aim of further exploring the importance of the p53 pathway during the progression of osteosarcoma, through the analysis of the HDMX-S/HDMX-FL ratio and its association with HDMX protein levels in 22 osteosarcoma cell lines, as well as cancer progression and survival in 51 osteosarcoma patients. Previously, we and others had reported increased mRNA expression of HDMX-S in various cancers (17, 22, 23). Interestingly, many tumors that showed increased HDMX-S mRNA levels had less HDMX full-length RNA and protein (22, 23), and correlated with later tumor stage and poorer
survival. For example, we demonstrated that even in cells expressing predominantly \textit{HDMX-S} mRNA, only full length HDMX protein could be detected (Fig. 4A and (25)). We propose that the HDMX-S protein is very unstable or inefficiently translated and, therefore, is unlikely to play an important, dominant role in cell proliferation. Here, we further showed that as the \textit{HDMX-S/HDMX-FL} ratio increased, HDMX full-length protein decreased in the 22 osteosarcoma cell lines and the 37 breast cancer cell lines studied (Figures 1, 2 and 4). For example, the osteosarcoma cell lines with the highest \textit{HDMX-S/HDMX-FL} ratio expressed up to 9.5 fold less HDMX full length protein.

In this study we went on to explore the potential association of the \textit{HDMX-S/HDMX-FL} ratio with osteosarcoma progression, as we and others had previously demonstrated (17). Here, we studied 51 high-grade osteosarcoma patients and, strikingly, found that high \textit{HDMX-S/HDMX-FL} ratios correlated with faster metastatic progression (Fig. 4). Specifically, over two years, 62\% of those with the highest \textit{HDMX-S/HDMX-FL} ratios had presented with a metastasis, compared to only 8\% of those with the lowest ratios. Interestingly, a subset of the cell lines utilized in this study had been previously analysed for \textit{in vivo} growth characteristics, including the capacity to metastasize (40). One of the tested cell lines was found to produce metastases in mice, HOS-143B, whereas the parental HOS cell line did not. Intriguingly, and in line with our observations in the patient cohort, we found a dramatically increased \textit{HDMX-S/HDMX-FL} ratio and significantly decreased HDMX protein levels in HOS-143B cells compared to HOS cells (Fig. 1B and C).

Our observations in an osteosarcoma cell line panel and patient cohort suggest that the \textit{HDMX-S/HDMX-FL} ratio positively correlates with less HDMX protein expression, as proposed earlier (25), and faster metastatic progression, as well as a trend to worse overall survival. In this study, we proposed that a reason why a cancer cell would not retain the oncogenic activity of HDMX is because it has already inhibited p53 signalling by mutations of other key regulatory genes in the
pathway and, therefore, the cell is no longer under selective pressure to sustain higher levels of HDMX. Furthermore, cells with deficient p53 activity could be under positive selection pressure to reduce HDMX levels as suggested by two recent studies, which report that full length MDMX functions as a tumor suppressor in cells with deficient p53-function (41, 42). Indeed, our observations made in three cell panels, comprising a total of 115 different cell lines, lend support to this model. We observed that the cancer lines with higher $HDMX-S/HDMX-FL$ ratios had significantly higher frequencies of two common p53 pathway mutations, namely p53 gene mutation and HDM2 overexpression (Table 1, Fig. 5). An interesting prediction of this model would be that the $HDMX-S/HDMX-FL$ ratio could potentially serve as a more effective prognostic biomarker than p53 gene mutation, because alterations of other key p53 pathway genes (like HDM2 overexpression) that result in p53 pathway attenuation could be captured simultaneously. In this report, we have presented observations that support this in a cohort of soft tissue sarcoma patients, where $HDMX-S/HDMX-FL$ ratios and p53 mutation status could be determined. We observed that the $HDMX-S/HDMX-FL$ ratio was indeed a strikingly better prognostic indicator than p53 mutation. While these observations remain to be replicated in other patient cohorts and cancers, they indicate that the $HDMX-S/HDMX-FL$ ratio could serve as an effective biomarker for p53 attenuation in cancer cells that could be utilized in a further personalization of therapeutic interventions in sarcomas, as well as potentially other cancers.

**Acknowledgments.**

The authors thank Dr. Karoly Szuhai and Danielle de Jong for the use of previously unpublished osteosarcoma cell lines, Dr. Lam for the protein data of the breast cancer cell lines, Dr. Konstantin Agelopoulos and Dr. Massimo Serra for providing the clinical samples from respectively the Westfälische Wilhemsuniversität Münster and the Instituti Ortopedici Rizzoli, Bologna. This work was supported by grants from the Dutch Cancer Society (UL-2006-3595) and (2008-4060), the Ludwig Institute for Cancer Research, the Development
Fund-Oxford Cancer Research Centre-University of Oxford, the Deutsche
Krebshilfe (grant 108424), the Wilhelm-Roux-Programm of the University of
Halle-Wittenberg and the Fritz-Thyssen-Stiftung (Az 10.09.2.117).
The Department of Pathology, Leiden University Medical Center, is partner of the
EuroBoNeT consortium, a European Commission granted Network of Excellence
for studying the pathology and genetics of bone tumors.

References
1. Raymond AK, Ayala AG, Knuutila S. Conventional Osteosarcoma. In: Fletcher
CDM, Unni KK, Mertens F, editors. World Health Organization Classification of
Tumours Pathology and Genetics of Tumours of Soft Tissue and Bone. Lyon: IARC
Improvement in histologic response but not survival in osteosarcoma patients treated with
intensified chemotherapy: a randomized phase III trial of the European Osteosarcoma
Rijswijk CS, et al. Prognostic factors in pulmonary metastasized high-grade
Survival after recurrent osteosarcoma: data from 3 European Osteosarcoma Intergroup
Clinicopathologic implications of MDM2, p53 and K-ras gene alterations in
osteosarcomas: MDM2 amplification and p53 mutations found in progressive tumors.
analysis of p53, MDM2 and H-ras genes in low-grade central osteosarcoma.
12. Kawai H, Wiederschain D, Yuan ZM. Critical contribution of the MDM2 acidic
Table 1. The associations of the HDMX-S/HDMX-FL ratios with p53 pathway mutations in three cell panels.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Ratio</th>
<th>S/FL</th>
<th>wt (n=6)</th>
<th>1.17</th>
<th>1.84</th>
<th>p value 0.0325*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteosarcoma</td>
<td></td>
<td></td>
<td>mut (n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low p53 (n=3)</td>
<td></td>
<td></td>
<td>low HDM2 (n=3)</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high HDM2 (n=3)</td>
<td></td>
<td></td>
<td>high HDM2 (n=3)</td>
<td>1.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td></td>
<td></td>
<td>wt (n=8)</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>low p53 (n=3)</td>
<td></td>
<td></td>
<td>low HDM2 (n=4)</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high HDM2 (n=4)</td>
<td></td>
<td></td>
<td>high HDM2 (n=4)</td>
<td>1.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.343*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI60</td>
<td></td>
<td></td>
<td>wt (n=14)</td>
<td>1.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>low p53 (n=7)</td>
<td></td>
<td></td>
<td>lowHDM2 (n=7)</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high HDM2 (n=7)</td>
<td></td>
<td></td>
<td>high HDM2 (n=7)</td>
<td>2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.0728*</td>
<td></td>
<td>0.0364**</td>
<td></td>
</tr>
<tr>
<td>All Cell Lines</td>
<td></td>
<td></td>
<td>wt (n=27)</td>
<td>1.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>low p53 (n=13)</td>
<td></td>
<td></td>
<td>low HDM2 (n=13)</td>
<td>0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high HDM2 (n=14)</td>
<td></td>
<td></td>
<td>high HDM2 (n=14)</td>
<td>1.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.0116*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mann-Whitney test, 2-tail
** Mann-Whitney test, 1-tail
Figure Legends

Figure 1. Analysis of HDMX-S in 22 osteosarcoma cell lines.

Fig. 1A Schematic drawing of Hdmx protein structure (upper), containing the p53 Binding Domain (p53-BD), Acidic Domain (AD), Zinc-finger (Zn) and RING Domain. Structure of HDMX-FL and HDMX-S mRNA is depicted below. Arrows indicate start and stop codons. Exon 6 is spliced out in HDMX-S resulting in 26 novel amino acids and a premature stop codon. Fig. 1B 22 osteosarcoma cell lines and 1 osteoblast sample were examined for HDMX-FL and HDMX-S mRNA expression using primers for Hdmx exon 3 (Fw) to exon 8 (Rev). GAPDH mRNA expression was examined as an internal control. Fig. 1C Protein levels were analysed with immunoblotting using the indicated antibodies. USP7 expression was analysed as a loading control.

Figure 2. High HDMX-S/HDMX-FL ratios in 22 osteosarcoma cell lines associate with lower HDMX protein levels.

Fig. 2A Column graph depicting the number of cells with a given HDMX-S/HDMX-FL ratio, whereby a normal distribution is observed. Fig. 2B Graph depicting the negative correlation of the HDMX-S/HDMX-FL ratio on the y-axis with HDMX protein levels on the x axis. Fig. 2C Column graph depicting the average HDMX protein levels for the groups of cell lines with varying ratios of HDMX-S/HDMX-FL, whereby cells with higher ratios contain less HDMX protein. The error bars depict standard errors.

Figure 3. Analysis of HDMX-S mRNA expression in osteosarcoma biopsies, and its association with worse clinical outcome.

Fig. 3A The expression patterns of HDMX-FL and HDMX-S mRNA in 51 osteosarcoma biopsies, normal osteoblasts and breast cancer cell line MCF7, analyzed by semi-quantitative PCR with Hdmx primers in exon 3 (FW) and exon 8 (Rev). Fig. 3B Column graph depicting the number of biopsies with a given
HDMX-S/HDMX-FL ratio. Fig. 3C, 3D Kaplan-Meier plots displaying the metastasis free survival (C) or overall survival (D) for patients whose biopsies contain high (n=13), intermediate (n=13) or low HDMX-S/HDMX-FL ratios (n=12). The p-values are derived from a logrank test comparing patients with high ratios to those with low ratios, as is depicted by the * symbol.

Figure 4. Analysis of the HDMX-S/HDMX-FL ratio and its association with HDMX protein levels in breast cancer cell lines.

Fig. 4A. Cell lines were examined for HDMX-FL and HDMX-S mRNA expression using primers for Hdmx exon 3 (Fw) to exon 8 (Rev). Fig. 4B Column graph depicting the number of cells with a given HDMX-S/HDMX-FL ratio. Fig. 4C Graph depicting the negative correlation of the HDMX-S/HDMX-FL ratio on the y-axis with HDMX protein levels on the x-axis. Fig. 4D Column graph depicting the average HDMX protein levels for the groups of cell lines with varying ratios of HDMX-S/HDMX-FL, whereby cells with higher ratios contain less HDMX protein. The error bars depict standard errors.

Figure 5. Cancer lines with higher HDMX-S/HDMX-FL ratios have significantly higher frequencies of two common p53 pathway mutations, namely p53 gene mutation and HDM2 overexpression.

Column graph depicting the percentage of cell lines with wild-type p53 and lower HDM2 in the 106 cell lines that are divided into three groups based on low, intermediate and high expression of the HDMX-S/HDMX-FL ratio.

Figure 6. High HDMX-S/HDMX-FL ratios, but not p53 gene mutation associate with worse clinical outcome of soft tissue sarcoma patients.

Fig. 6A Column graph depicting the percentage of tumors with missense p53 mutations for tumors with either low (n=18) or higher (n=28) HDMX-S/HDMX-FL ratios. Tumors with higher ratios contain a greater percentage of p53 mutations. Fig. 6B A Kaplan-Meier plot that displays overall survival for patients whose tumors contained either wild-type or mutant p53 (n=104). The p-value depicted
on the plot is derived from a logrank test. **Fig. 6C** A Kaplan-Meier plot that displays the overall survival for those patients whose tumors contained high, intermediate or low $HDMX$-$S/HDMX$-$FL$ ratios ($n=64$). The first p-value is derived from a logrank test comparing the patients with higher, equal and lower ratios. The second p-value is derived from a logrank test comparing the patients with higher ratios to those with low ratios, as depicted by the * symbol. **Fig. 6D** Predicted survival curves for patients with different $HDMX$-$S/HDMX$-$FL$ ratios ($n=64$) derived from a Cox multivariate regression analysis that was adjusted to the known independent prognostic factors of soft tissue sarcomas that included tumor stage, resection type, tumor location and tumor type. The p-value depicted on the plot is derived through the comparison of the patients with higher ratios to those with low ratios, as is depicted by the * symbol.
Figure 1

A

HDMX-FL 

1- p53-BD | AD | Zn | RING | 490

HDMX-FL

1  2  3  4  5  6  7  8  9  10  11

AUG  STOP

HDMX-S

1  2  3  4  5  6  7  8  9  10  11

AUG  STOP

B

FL  S

HDMX exon 3 to 8

GAPDH

C

Hdm2

Hdmx

p53

p21

USP7
Figure 2

A

Number of cells

HDMMX-S/ HDMMX-FL ratio

B

Spearman's p=0.004
Corr. Co. -0.583

HDMMX-S/HDMMX-FL ratio vs HDMMX relative protein level

C

Kruskal-Wallis Test p = 0.0379

HDMMX relative protein level

very high
n=5

high
n=6

low
n=6

very low
n=5

HDMMX-S/HDMMX-FL ratio
Figure 4

**A**

Western blot analysis of HDMX protein expression in various cell lines. The blots show the expression levels of HDMX protein (exons 3 to 8) and GAPDH as a loading control. The lanes are labeled with sample identifiers (1-30) and M (marker).

**B**

Bar graph displaying the number of cells in different HDMX-S/HDMX-FL ratio categories. The x-axis represents the HDMX-S/HDMX-FL ratio, while the y-axis shows the number of cells. The data points are color-coded for different categories.

**C**

Scatter plot showing the correlation between HDMX-S/HDMX-FL ratio and HDMX relative protein level. The Spearman's correlation coefficient is provided. The linear regression line is also depicted.

**D**

Kruskal-Wallis test results for HDMX relative protein level across different ratio categories. The p-value is 0.0001, indicating a significant difference in protein levels among the groups.
Alternate splicing of the p53 inhibitor HDMX offers a superior prognostic biomarker than p53 mutation in human cancer

Kristiaan Lenos, Anna M. Grawenda, Kristen Lodder, et al.

Cancer Res  Published OnlineFirst June 14, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-0215

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/06/14/0008-5472.CAN-12-0215.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.