Alternate Splicing of the p53 Inhibitor HDMX Offers a Superior Prognostic Biomarker than p53 Mutation in Human Cancer

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Alternate Splicing of the p53 Inhibitor HDMX Offers a Superior Prognostic Biomarker than p53 Mutation in Human Cancer

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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 show 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 patients with soft tissue sarcoma. The *HDMX-S/HDMX-FL* ratio better defined patients with sarcoma 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. *Cancer Res; 72(16); 4074–84. ©2012 AACR.*

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 nonmetastatic 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% to 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 to increase the survival of patients with osteosarcoma.

In approximately 50% of all human cancers mutations are found in the p53 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% to 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 understudied in osteosarcoma. For example, the oncogene *HDMX* is a close homolog of *HDM2* and

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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, 2 tumor types that have a very low frequency of p53 gene mutation (retinoblastomas and Ewing sarcomas) have showed elevated HDMX expression (20, 21).

In sarcomas, amplification of the *HDMX* gene has been found in both soft tissue sarcomas (STS, 16%–17%) and osteosarcomas (35%; refs. 14, 22). Bartel and colleagues showed 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. Clinicopathologic data of 51 patients with osteosarcoma are shown in Supplementary Table S1. Part of the samples was previously described (26). Biopsies were taken before preoperative 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).

STS cohort. The clinicopathologic data of the 157 patients with STS 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, Leipzig, Germany).

Cell culture and reagents

Cell lines were maintained in RPMI supplemented with 10% FBS 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 Department of Molecular Cell Biology, LUMC (Leiden, The Netherlands) in the laboratory of Dr. Szuhai. Status of p53 gene was determined as described by da Costa and colleagues (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 Netherlands). 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 μmol/L Nutlin-3 (Cayman Chemical) 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, semiquantitative PCR, and quantitative reverse transcription 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. Semiquantitative PCR was carried out 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 glyceraldehyde-3-phosphate dehydrogenase (*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).

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 single-nucleotide polymorphism 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 website. The mRNA levels of *HDMX-S* and *HDMX-FL* were determined as described earlier. Measurements of *HDM2* transcript levels were carried

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out by quantitative reverse transcription PCR (qRT-PCR) using commercially available RoboGeneMDM2 cDNA quantification module, with ABI PRISM 7000 Sequence Detection System. *HDM2* measurements were normalized to *GAPDH* transcript levels.

Soft tissue sarcomas. *HDMX-FL* and *HDMX-S* transcript levels were measured by quantitative real-time reverse transcriptase PCR as described (22). Briefly, the reactions were carried out on a Rotor-Gene 3000 (Corbett Research), with a common forward primer for both *FL-HDMX* and *HDMX-S*; X quant forward 5′-CAGCAGGTGCGCAAGGTGAA-3′ and reverse primers specifically designed for amplification of either *FL-HDMX*, FL-AS 6 5′-CTGTGCGAGAGCGAGAGTCTG-3′ or *HDMX-S*, XS AS 5′-GCACTTTGCTGTAGTAGCAGTG-3′. Each reaction included one tenth of the cDNA reaction, 10 μL of 2× 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 seconds of denaturation at 95°C, 30 seconds of primer annealing at 58°C, and synthesis at 72°C for 30 seconds. 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 carried out as described previously (33). Anti-HDMX and anti-USP7 (A300-287A, A300-033A) were from Bethyl Laboratories. Anti-p53 PAb1801 and DO-1, anti-HDM2 antibodies 4B2 (34) and SMP14 were from Santa Cruz Biotechnology. 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 conducted 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. Semiquantitative PCR was carried out 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

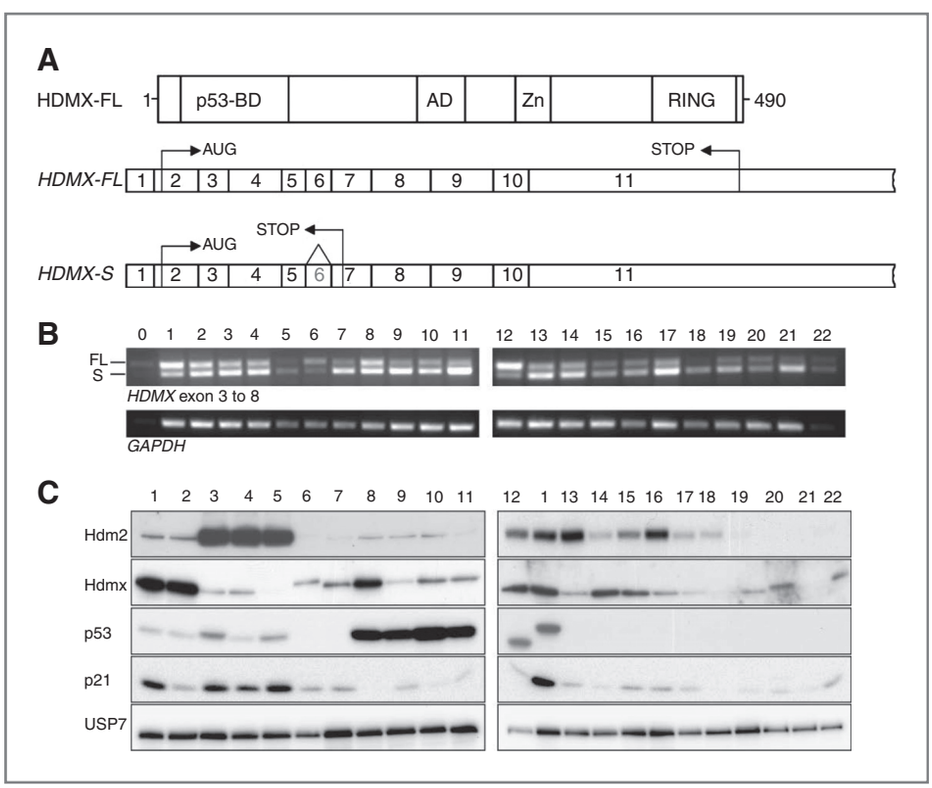

Figure 1. Analysis of *HDMX-S* in 22 osteosarcoma cell lines. A, schematic drawing of Hdmx protein structure (top) containing the p53-binding domain (p53-BD), acidic domain (AD), zinc-finger (Zn), and RING domain. Structure of *HDMX-FL* and *HDMX-S* mRNA is depicted below. Arrows indicate start and stop codons. Exon 6 is spliced out in *HDMX-S*, resulting in 26 novel amino acids and a premature stop codon. B, a total of 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. C, protein levels were analyzed with immunoblotting using the indicated antibodies. USP7 expression was analyzed as a loading control.

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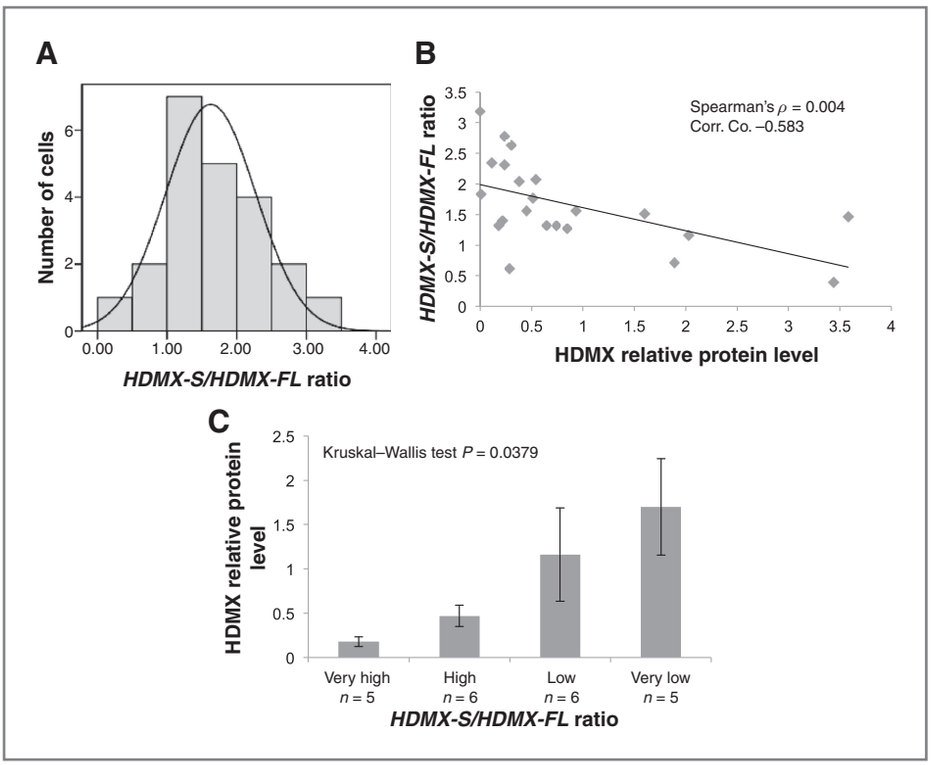

Figure 2. High *HDMX-S/HDMX-FL* ratios in 22 osteosarcoma cell lines associate with lower HDMX protein levels. A, column graph depicting the number of cells with a given *HDMX-S/HDMX-FL* ratio, whereby a normal distribution is observed. B, graph depicting the negative correlation of the *HDMX-S/HDMX-FL* ratio on the *y*-axis with HDMX protein levels on the *x*-axis. C, 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.

Table S3). The *HDMX-S/HDMX-FL* ratios varied from 0.39 to 3.18 with a median of 1.5, an SD 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 5 cells with the highest *HDMX-S/HDMX-FL* ratios had 9.5-fold less HDMX protein than those 5 cells with the lowest ratios (*t* test, $P = 0.0238$, Fig. 2C).

To explore the potential association of the *HDMX-S/HDMX-FL* ratio with osteosarcoma progression and survival, we studied 51 patients with high-grade osteosarcoma 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 to 58 years. The levels of *HDMX-S* and *HDMX-FL* were determined in biopsies that were taken before preoperative 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 an SD of 1. Interestingly, the distribution of the *HDMX-S/HDMX-FL* ratios was not normal ($P = 0.0011$). In fact, 11 tumors showed very high ratios, as defined by one SD above the median (Fig. 3B).

To determine whether 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 patients with osteosarcoma. 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 3 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, $P = 0.005$, log-rank test). Specifically, the 13 patients whose tumors had the highest *HDMX-S/HDMX-FL* ratios (average ratio of 2.55, ranging from 1.8–4.07) 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 0.44–1.0). In 2.5 years, 62% of those with the highest *HDMX-S/HDMX-FL* ratios had presented with a metastasis, compared with only 8% of those with the lowest ratios. Similar, trends were seen in overall survival, whereby the 13 patients with highest *HDMX-S/HDMX-FL* ratio associated with shorter overall 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 signaling 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

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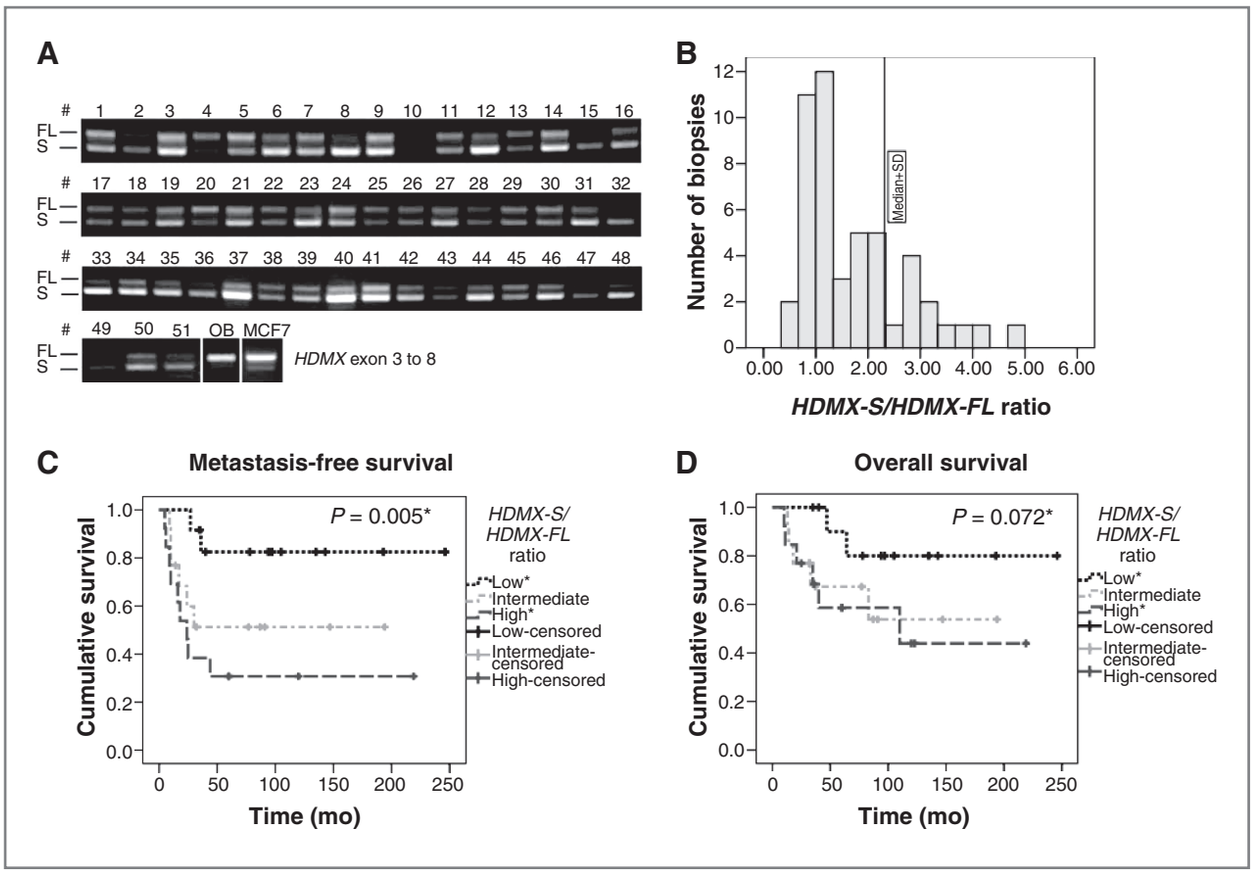

Figure 3. Analysis of *HDMX-S* mRNA expression in osteosarcoma biopsies and its association with worse clinical outcome. A, the expression patterns of *HDMX-FL* and *HDMX-S* mRNA in 51 osteosarcoma biopsies, normal osteoblasts, and breast cancer cell line MCF7, analyzed by semiquantitative PCR with HDMX primers in exon 3 (FW) and exon 8 (Rev). B, column graph depicting the number of biopsies with a given *HDMX-S/HDMX-FL* ratio. C and D, 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 log-rank test comparing patients with high ratios to those with low ratios, as is depicted by the asterisk.

2 well-characterized somatic alterations that result in p53 inhibition, p53 gene mutation, and overexpression 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 signaling (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 signaling through alterations of other key p53 pathway genes.

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

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 an SD of 1.15. The

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Table 1. The associations of the HDMXS/HDMX-FL ratios with p53 pathway mutations in 3 cell panels

			Ratio S/FL
Osteosarcoma			
	p53 ($n = 22$)	wt ($n = 6$)	1.17
		Mut ($n = 16$)	1.84
		P	0.0325[a]
	wt p53 ($n = 6$)	Low HDM2 ($n = 3$)	0.83
		High HDM2 ($n = 3$)	1.51
		P	0.1[a]
Breast cancer			
	p53 ($n = 37$)	wt ($n = 8$)	0.69
		Mut ($n = 29$)	1.76
		P	0.0028[a]
	wt p53 ($n = 8$)	Low HDM2 ($n = 4$)	0.34
		High HDM2 ($n = 4$)	1.03
		P	0.343[a]
NCI60			
	p53 ($n = 50$)	wt ($n = 14$)	1.47
		Mut ($n = 36$)	2.71
		P	0.0227[a]
	wt p53 ($n = 14$)	Low HDM2 ($n = 7$)	0.57
		High HDM2 ($n = 7$)	2.38
		P	0.0728[a]
			0.0364[b]
All cell lines			
	p53 ($n = 106$)	wt ($n = 27$)	1.22
		Mut ($n = 79$)	2.24
		P	0.0001[a]
	wt p53 ($n = 27$)	Low HDM2 ($n = 13$)	0.56
		High HDM2 ($n = 14$)	1.93
		P	0.0116[a]

[a]Mann–Whitney test, 2-tail.
[b]Mann–Whitney test, 1-tail.

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 SD above the median (Fig. 4B). Interestingly, the *HDMX-S/HDMX-FL* ratio significantly negatively correlated with measured HDMX protein levels ($P = 2.28 \times 10^{-8}$, 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). 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; ref. 37). Three cell lines overlap with the above-analyzed breast cancer cell panel. Of the 59 cell lines, 14 are known to have wild-type p53, 36 have either mutated or deleted p53, whereas 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 normalized 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 an SD of 3.3. The distribution of the *HDMX-S/HDMX-FL* ratios, similarly to the patients with breast cancer cell panel and osteosarcoma, was not normal (Supplementary Fig. 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-*

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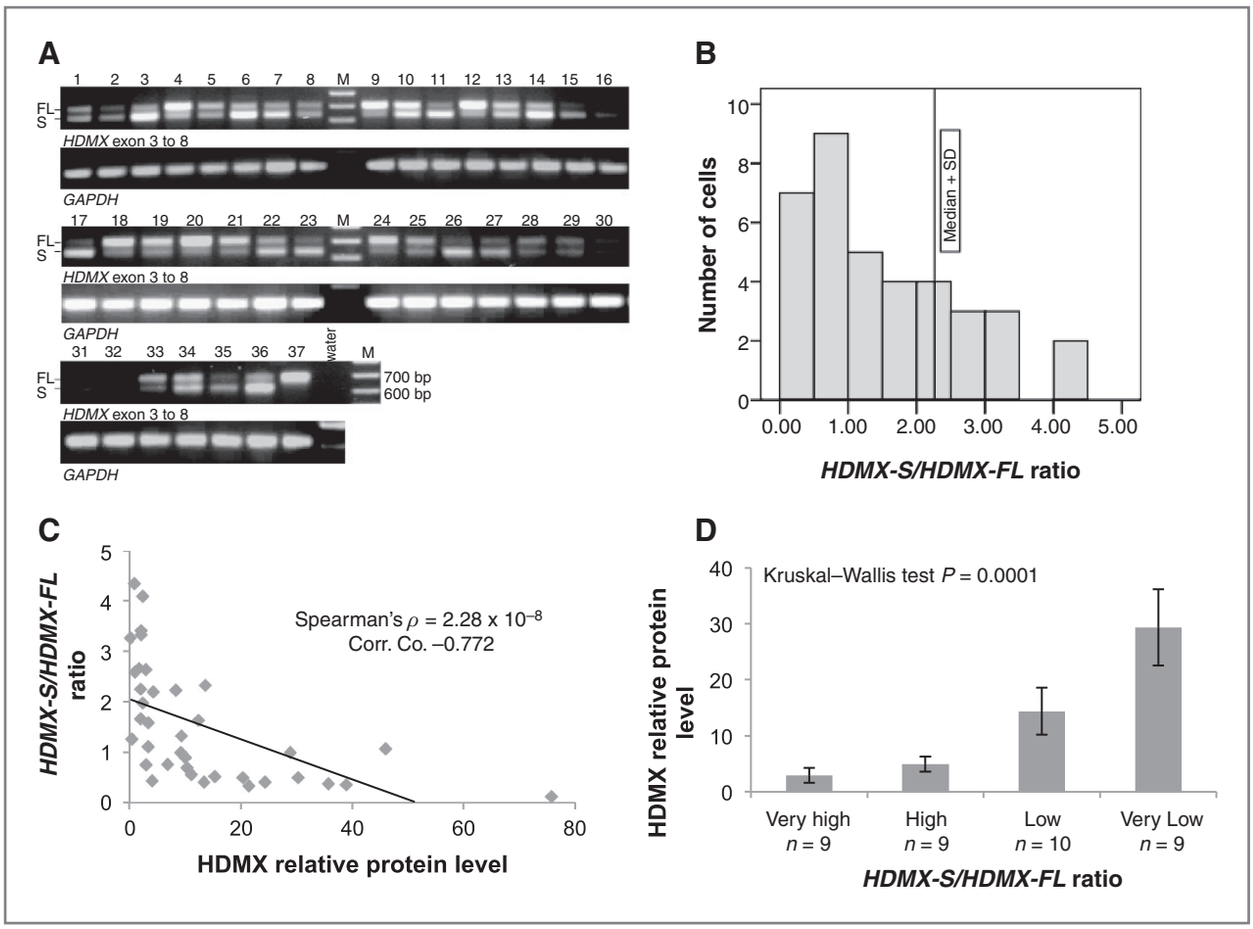

Figure 4. Analysis of the *HDMX-S/HDMX-FL* ratio and its association with HDMX protein levels in breast cancer cell lines. A, cell lines were examined for *HDMX-FL* and *HDMX-S* mRNA expression using primers for Hdmx exon 3 (FW) to exon 8 (Rev). B, column graph depicting the number of cells with a given *HDMX-S/HDMX-FL* ratio. C, graph depicting the negative correlation of the *HDMX-S/HDMX-FL* ratio on the *y*-axis with HDMX protein levels on the *x*-axis. D, 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.

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 3 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 signaling through alterations of key p53 pathway genes. Indeed, we observed that the 27 cell lines with wild-type p53 in the 3 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 exact test, Fig. 5), than 4 of the 35 cell lines with intermediate ratios (11%, $P = 0.001$, Fisher 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 2 common p53 pathway mutations, namely p53 gene mutation and HDM2 overexpression (Table 1, Fig. 5). Inhibition of p53 signaling 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,

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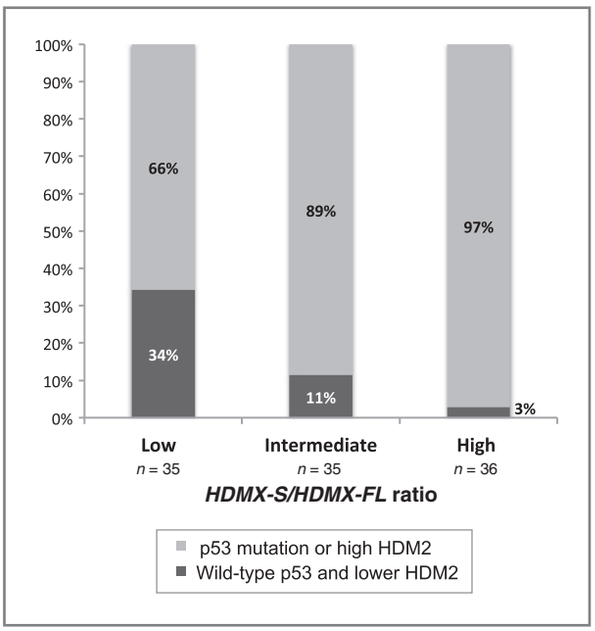

Figure 5. Cancer lines with higher *HDMX-S/HDMX-FL* ratios have significantly higher frequencies of 2 common p53 pathway mutations, namely p53 gene mutation and HDM2 overexpression. Column graph depicting the percentage of cell lines with wild-type p53 and lower HDM2 in the 106 cell lines that are divided into 3 groups based on low, intermediate, and high expression of the *HDMX-S/HDMX-FL* ratio.

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 signaling, namely 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 patients with STS 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, whereas 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 3 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 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 ($P = 0.469$, log-rank 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 ($P = 0.024$ log-rank test, Fig. 6C; Supplementary Fig. S3B and 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 patients with osteosarcoma. 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 showed that even in cells expressing predominantly *HDMX-S* mRNA, only full-length HDMX protein could be detected (Fig. 4A and ref. 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 *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 (Figs. 1, 2, and 4). For example, the osteosarcoma cell lines with the highest *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 *HDMX-S/HDMX-FL* ratio with osteosarcoma progression, as we and others had previously shown (17). Here, we studied 51 patients with high-grade osteosarcoma and, strikingly, found that high *HDMX-S/HDMX-FL* ratios correlated with faster metastatic progression (Fig. 4). Specifically, over 2 years, 62% of those with the highest *HDMX-S/HDMX-FL* ratios had presented with a metastasis, compared with only 8% of those with the lowest ratios. Interestingly, a subset of the cell lines used in this study had been previously analyzed for *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 *HDMX-S/HDMX-FL* ratio and significantly decreased HDMX protein levels in HOS-143B cells compared with HOS cells (Fig. 1B and C).

Our observations in an osteosarcoma cell line panel and patient cohort suggest that the *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 signaling by mutations of other key regulatory genes in the pathway and, therefore, the cell is no longer under selective

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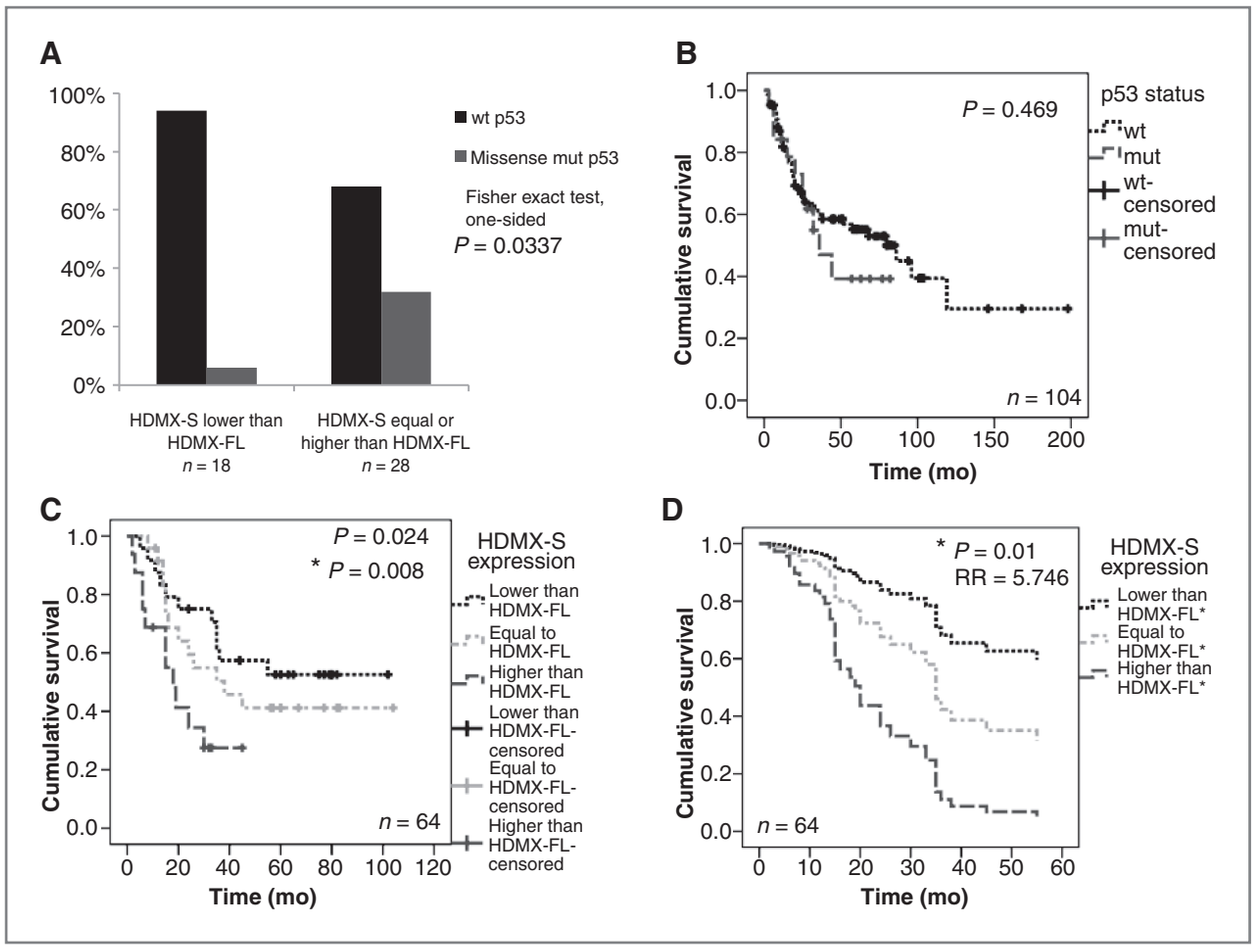

Figure 6. High *HDMX-S/HDMX-FL* ratios but not p53 gene mutation associate with worse clinical outcome of patients with STS. A, column graph depicting the percentage of tumors with missense p53 mutations for tumors with either low ($n = 18$) or higher ($n = 28$) *HDMX-S/HDMX-FL* ratios. Tumors with higher ratios contain a greater percentage of p53 mutations. B, 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 log-rank test. C, 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 log-rank test comparing the patients with higher, equal, and lower ratios. The second *P* value is derived from a log-rank test comparing the patients with higher ratios to those with low ratios, as depicted by the asterisk. D, 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 STSs 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 asterisk.

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 2 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 3 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 2 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 patients with STS, 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 used in a further personalization of therapeutic interventions in sarcomas as well as potentially other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Alternate Splicing of the p53 Inhibitor HDMX Offers a Superior Prognostic Biomarker than p53 Mutation in Human Cancer

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