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Original Article

Inverse association between MDM2 and HUWE1 protein expression levels in human breast cancer and liposarcoma

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Abstract: The ubiquitin E3 ligase MDM2 is best known for its ability to suppress the tumor suppressor p53. However, MDM2 also targets other proteins for proteasomal degradation and accumulating evidence strongly suggests p53-independent roles of MDM2 in cancer. We previously reported that MDM2 promotes degradation of another ubiquitin E3 ligase HUWE1 by ubiquitination, particularly, which confers $HER2^+$ breast cancer cells resistance to the HER2 inhibitor lapatinib. However, it remains unclear whether such a mechanism can operate in other cell types, independently of HER2 inhibitors. Moreover, *in vivo* evidence that supports HUWE1 degradation by MDM2 is missing. In the current study, we performed immunohistochemistry (IHC) to analyze expression levels of MDM2 and HUWE1 in normal organs, two breast cancer cohorts (A, n = 137 and B, n = 27), and a liposarcoma cohort (n = 45). Our results show that HUWE1 is ubiquitously expressed in healthy organs, where the oncoprotein MDM2 is undetectable. Likewise, in the majority of breast cancers regardless of their subtypes, MDM2 is below detectable levels, while HUWE1 is highly expressed. In contrast, in a subset of liposarcoma that is characterized by MDM2 overexpression, only 40% of these showed detectable HUWE1 protein. Importantly, despite the inverse association between MDM2 and HUWE1 protein levels, gene expression analysis in independent datasets revealed no such correlation at the mRNA level. Our results demonstrate the first *in vivo* evidence to support the hypothesis of MDM2-mediated HUWE1 degradation, which may help to understand the regulation of HUWE1 as well as p53-independent roles of MDM2.

Keywords: ARF-BP1, E3 ligase, HECTH9, MULE, TCGA, ubiquitination

Introduction

The ubiquitin E3 ligase MDM2 is known to be a key repressor of the tumor suppressor p53. MDM2 promotes p53 protein degradation by ubiquitination. In addition, MDM2 physically blocks the transcriptional activity of p53. Therefore, MDM2 is a critical factor that determines cellular response to p53-activating agents [1, 2]. Supporting this notion, a number of studies have shown that MDM2 protein levels are frequently upregulated in human cancer [3]. Moreover, MDM2 overexpression is sufficient to cause tumors in mice [4]. Interestingly, MDM2 also ubiquitinates and targets various cellular proteins, other than p53, for proteasomal degradation [5, 6]. Accumulating evidence indeed suggests that MDM2 has oncogenic functions in addition to the suppression of p53 activity [4]. However, which other pathways in addition to p53 can be regulated by MDM2 and how the p53-independent pathways may contribute to tumorigenesis remain elusive.

Previously, we have identified HUWE1 (a.k.a., ARF-BP1, MULE, and HECTH9) as a novel substrate of MDM2 [6]. HUWE1 is an ubiquitin E3 ligase can target its substrate proteins for proteasomal degradation. The substrates of HUWE1 include p53, the anti-apoptotic BCL2 family protein MCL1, MYC, and Protein Phosphatase 5 (PP5) [6-10]. Therefore, enhanced MDM2 activity can promote ubiquitination and degradation of HUWE1, which, in turn, results in the stabilization and accumulation of HUWE1

substrates. In particular, we showed that MDM2-mediated degradation of HUWE1 plays a critical role in conferring breast cancer cell lines resistance to HER2-targeted therapy by stabilizing at least two of the pro-survival HUWE1 substrates, MCL1 and PP5 [6]. It is suggested that HUWE1 may be one of the MDM2 targets that could impact cell fates, independently of p53.

It remains to be elucidated whether MDM2-mediated degradation of HUWE1 is only specific to $HER2^+$ breast cancer cell lines or also occurs in other cancer cell types. In the present study, we extended our initial findings in $HER2^+$ breast cancer cell lines to normal tissues, primary breast cancer, and liposarcoma tissues by analyzing MDM2 and HUWE1 expression levels in tissue microarrays (TMAs) and publically available datasets.

Materials and methods

Tissue microarrays (TMAs)

The creation of the Normal Tissue, the Breast (cohort A), and the Liposarcoma TMAs was approved by the institutional Committee for the Protection of Human Subjects (CPHS) at Dartmouth College and was conducted in the Pathology Translational Research Laboratory (PTRL) at Dartmouth-Hitchcock Medical Center (DHMC). The Normal Tissue and the Liposarcoma TMAs were constructed using the Automated TMA Master-3D Histotech (Caliper Life Solutions, 68 Elm St, Hopkinton, MA 01748-1668). The Breast TMA (cohort A) was compiled using the Manual tissue arrayer; MTA-1 (Beecher Instruments, Inc. 820 Hummingbird Court Sun Prairie, WI 53590). The Breast TMA comprises 0.6 mm diameter cores from 147 consecutive cases (where adequate tissue available) of invasive breast cancers diagnosed at DHMC between 2000 and 2007 with varying histologies (mostly infiltrating ductal carcinomas (IDCa), but also infiltrating lobular carcinomas (ILCa), mucinous/colloid, metaplastic and tubular carcinomas), tumor grades (low, intermediate, high), tumor sizes (ranging from 0.5-10.5 cm), and nodal status (both negative and positive). The distribution of prognostic markers was as follows: 88 cases = $ER^+/PR^+/HER2^-$; 12 cases = $ER^+/PR^-/HER2^-$; 22 cases = $ER^-/PR^-/HER2^-$ (triple negative); and 15 cases = $HER2^+$. In-built controls were normal breast (9 cases). The Liposarcoma TMA comprises 1.0 mm diameter cores of 46 cases of liposarcoma of the following subtypes: myxoid (n = 14), spindle cell (n = 2), round (n = 3), well-differentiated (n = 19), pleomorphic (n = 7). In-built controls included: normal fat (n = 12), skeletal muscle (n = 3), lipoma (n = 3), hibernoma (n = 1), and fat necrosis (n = 1).

The Breast TMA (cohort B) has been previously described [11].

Immunohistochemistry (IHC)

Tissue slices (4 micron thickness), deparaffinized with xylene and hydrated through graded alcohols, were mounted on glass slides and then immunostained using the standardized protocols and reagents of the Bond Max Leica automated stainer (Leica Biosystems Inc. 1700 Leider Lane, Buffalo Grove, Illinois 60089). Using the Leica Bond Polymer Refine Kit, conditions for MDM2 (Calbiochem Cat No OP46) immunostaining were dilution 1:100 with pretreatment Antigen retrieval 2 for 20 mins (Bond Epitope Retrieval solution 2, Ref# AR9640, ph 8.9-9.1 at 25 c) (EMD Chemicals, Inc. San Diego, Ca 92121). Conditions for HUWE1 (ProSci Incorporated Cat # 4213) immunostaining were dilution 1:1000, with antigen retrieval 1 for 20 mins (Bond Epitope Retrieval solution 1, Ref# AR9961, ph 5.9-6.1 at 25 c) (Prosci-Inc, 12170 Flint place, Poway, Ca 92064). Diaminobenzidine (DAB chromagen) (Bond Polymer Refine Detection, Ref# DS98000) was applied for visualization, with a hematoxylin counterstain.

Gene expression analysis

Gene expression data for breast tumor and liposarcoma samples were downloaded from The Cancer Genome Atlas (TCGA) data portal and the NCBI Gene Expression Omnibus (GEO), respectively. The breast cancer data contain expression profiles for 1035 and 527 patients measured by RNA-seq and microarray (Agilent G4502A platform), respectively. The liposarcoma data contain expression profiles for 259 samples measured by RNA-seq (GEO Accession #: GSE30929).

Results

Ubiquitous expression of HUWE1 in normal tissues

It is known that while MDM2 is often overexpressed in cancer, its expression levels are

Table 1. HUWE1 protein expression in normal tissues

Organ	Cell type	% Staining (<1, <50, >50)	Compartment distribution#	Staining intensity*
Tonsil	Crypt epithelium	>50	C	1
	Lymphocytes	>50	C	1
Kidney	Distal tubules	>50	C	2
Cerebellum	Glia	>50	C	2
Adrenal cortex		0		0
Adrenal medulla	Reticularis	>50	C	1
Lymph node	Lymphocytes	>50	C	1
Spleen	Lymphocytes	>50	C	1
Pancreas	Islet cells	>50	C	2
Liver	Hepatocytes	>50	C	1
Ampulla		>50	C	2
Gallbladder	Epithelium	>50	C	3
Omentum		0		0
Gastric fundus	Paneth cells	>50	C	3
Stomach	Epithelium	>50	C	2
Small bowel	Epithelium	>50	C	2
Colon	Epithelium	>50	C	2
Lung		0		0
Heart	Muscle	>50	C	2
Aorta	Fibromuscular	>50	C	1
Bladder	Epithelium	>50	C	1
Breast	Ductal epithelium	<50	C	2
Cervix	Epithelium	>50	C	2
Endometrium	Epithelium	>50	C	2
Ovary		0		0
Fallopian tube	Epithelium	>50	C	2
Placenta	Trophoblast	>50	C	1
Skin		0		0
Synovium	Epithelium	>50	C	1

#Compartment distribution: C = cytoplasmic; N = nuclear. *Staining intensity: 0 = negative, 1 = cytoplasmic blush, 2 = medium, 3 = high.

Table 2. MDM2/HUWE1 protein expression in breast cancer cohort A

MDM2	-	-	+	+	Total
HUWE1	-	+	-	+	
$ER^+/PR^+/HER2^-$ (%)	11 (12.5)	54 (61.4)	1 (1.1)	22 (25)	88
$ER^+/PR^-/HER2^-$ (%)	1 (8.3)	7 (58.3)	0 (0)	4 (33.3)	12
Triple negative (%)	2 (9.1)	19 (86.4)	0 (0)	1 (4.5)	22
$HER2^+$ (%)	2 (13.3)	11 (73.3)	0 (0)	2 (13.3)	15
Total (%)	16 (11.7)	91 (66.4)	1 (0.7)	29 (21.2)	137

very low in normal tissues [2]. Indeed, we did not detect MDM2 protein expression by IHC in any of the normal tissues we examined (**Table 1**). In contrast, HUWE1 is ubiquitously expressed in most of the tissues; gastric fundus and gallbladder epithelium particularly showed high expression of HUWE1 protein (**Table 1**). These results support a previous Northern blot study on HUWE1 levels in various human tissues [8]. Interestingly, our results indicated that HUWE1 was mostly cytoplasmic (**Table 1**), supporting a fluorescent microscopy study that showed the cytoplasmic localization of HUWE1 in MCF10a, a non-transformed human mammary epithelial cell line [12].

High HUWE1 and low MDM2 protein levels in breast cancer

Previously, we have shown that HUWE1 is abundantly expressed in $HER2^+$ breast cancer cell lines [6]. When the cells acquire resistance to HER2-targeted therapy, MDM2 becomes active and directly promotes HUWE1 degradation, although the mechanism of MDM2 activation remains unknown [6]. To examine HUWE1 and MDM2 expression in primary breast cancer tissues, we next analyzed HUWE1 and MDM2 protein levels in two independent breast cancer cohorts. Cohort (A) consisted of 137 breast cancer cases, whereas cohort (B) comprised 27 cases (**Tables 2** and **3**, respectively). In both cohorts, MDM2 was expressed only in a small proportion of the breast cancer cases (**Figure 1** and **Tables 2** and **3**). In contrast, HUWE1 protein was ubiquitously expressed (**Figure 1** and **Tables 2** and **3**). Cohort (A) was divided into the breast can-

Table 3. MDM2/HUWE1 protein expression in breast cancer cohort B

MDM2	-	-	+	+	Total
HUWE1	-	+	-	+	
Total (%)	3 (11.1)	19 (70.4)	1 (3.7)	4 (14.8)	27

cer sub-types, depending on the status of estrogen receptor (ER), progesterone receptor (PR), and HER2 (**Table 2**). There was no correlation between MDM2/HUWE1 expression levels and the breast cancer sub-types (**Table 2**). Interestingly, in the majority of the HUWE1-positive cases, MDM2 protein was below detectable levels (**Tables 2** and **3**; of note, none of the $HER2^+$ breast cancer cases had been treated with lapatinib or other HER2 inhibitors). Whereas MDM2 is a nuclear protein, HUWE1 was mostly cytoplasmic in the breast cancer tissues (**Figure 1**).

A positive correlation between HUWE1 and MDM2 mRNA levels in breast cancer

MDM2 and HUWE1 protein levels in breast cancer may be regulated by gene expression or at the level of protein stability. To assess whether MDM2/HUWE1 protein levels in breast cancer reflect gene expression patterns and to investigate whether there is a correlation between gene expression patterns of *MDM2* and *HUWE1*, we conducted gene expression analysis in TCGA datasets. We analyzed microarray data from the Agilent G4502A platform. Patient samples were grouped based on expression levels of MDM2 or HUWE1 relative to median MDM2 or HUWE1 expression, respectively. That is, samples that exhibited values greater than median MDM2 (HUWE1) expression were scored as high MDM2 (HUWE1), and samples less than median MDM2 (HUWE1) expression were scored as low. Accordingly, patient samples were divided into four groups: $MDM2^{high}$-$HUWE1^{high}$, $MDM2^{low}HUWE1^{high}$, $MDM2^{high}HUWE1^{low}$, and $MDM2^{low}HUWE1^{low}$. *P*-value was calculated using Chi-square test.

Interestingly, patients with $MDM2^{high}$ showed $HUWE1^{high}$ more frequently than $HUWE1^{low}$, while patients with $MDM2^{low}$ exhibited $HUWE1^{low}$ more than $HUWE1^{high}$ (**Table 4**). A similar result was obtained by analysis of an independent RNA-seq dataset (**Table 5**). These results suggest that there is a positive correlation between *MDM2* and *HUWE1* expression levels. Thus, in the two breast cancer cohorts, cases with high levels of both MDM2 and HUWE1 proteins (**Tables 2** and **3**) are likely to be ascribed to high mRNA expression of the two genes. Likewise, breast cancer cases with low levels of both MDM2 and HUWE1 proteins (**Tables 2** and **3**) are likely to be secondary to low expression of the two genes. Importantly, however, this overall pattern of *MDM2/HUWE1* gene expression did not correlate with the MDM2/HUWE1 protein expression pattern, especially considering the presence of a large population (66 and 70%) of MDM2-negative/HUWE1-positive breast cancer cases (**Tables 2** and **3**). Therefore, it is suggested that HUWE1 and MDM2 protein levels, especially in the MDM2-negative/HUWE1-positive population, may be regulated, at least in part, at post-translational levels, likely at the level of protein stability, which supports our previous finding that MDM2 is an E3 ligase for HUWE1 in breast cancer cells [6].

Low HUWE1 and high MDM2 protein levels in liposarcoma

To further characterize the relationship between MDM2 and HUWE1 protein levels, we next sought to analyze a cancer type that is associated with MDM2 overexpression. We chose liposarcoma since a subset of liposarcomas are often characterized by MDM2 gene amplification [13]. We performed immunohistochemical analysis for MDM2 and HUWE1 protein expression in a liposarcoma cohort (n = 45). Our liposarcoma cohort comprised pleomorphic (n = 7), myxoid (n = 14), round cell (n = 3), spindle cell (n = 2), and well-differentiated (n = 19) liposarcomas. In our cohort, a subset of well-differentiated and myxoid liposarcoma cases (13 out of 19 and 6 out of 14 samples, respectively) exhibited high levels of MDM2 protein expression (**Table 6**). Interestingly, in MDM2-positive liposarcoma cases, only 40% of them showed detectable levels of HUWE1 protein by IHC (**Figure 2**, **Table 6**). As was the case with breast cancer, MDM2 was nuclear, while HUWE1 was cytoplasmic in liposarcoma (**Figure 2**). Normal fat tissues did not express MDM2 and HUWE1 proteins at detectable levels by IHC (n = 10).

No correlation between HUWE1 and MDM2 mRNA levels in liposarcoma

The well-differentiated liposarcoma subtype is known to accompany MDM2 gene amplification

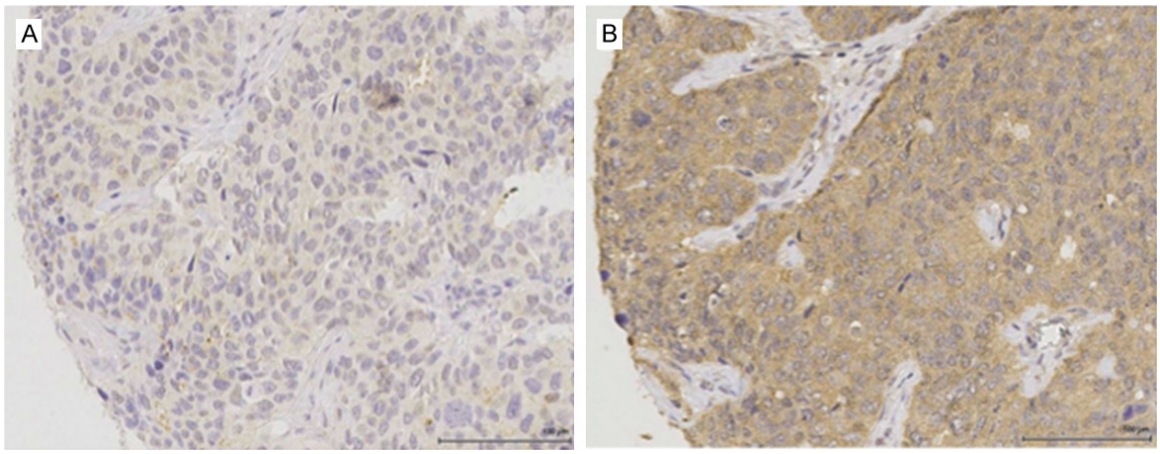

Figure 1. Immunohistochemical analysis of MDM2 and HUWE1 in breast tumors. IHC detection of MDM2 (A) and HUWE1 (B) in invasive ductal carcinoma ($ER^+/PR^+/HER2^-$) is shown. MDM2 is expressed at undetectable levels, while HUWE1 is widely expressed in the cytoplasm. The error bars indicate 100 μm.

Table 4. MDM2/HUWE1 gene expression in breast cancer (TCGA)

Microarray (N = 527)		HUWE1 mRNA	
		Low	High
MDM2 mRNA	Low	147	116
	High	116	148

X-squared = 7.0603, df = 1, *p*-value = 0.007881 (Chi-square test).

Table 5. MDM2/HUWE1 gene expression in breast cancer (TCGA)

RNA-seq (N = 1035)		HUWE1 mRNA	
		Low	High
MDM2 mRNA	Low	305	213
	High	213	306

X-squared = 32.294, df = 1, *p*-value = 1.325e-08 (Chi-square test).

[13]. However, our analysis using a GEO dataset (GEO Accession #: GSE30929) indicated that there was no association between MDM2 and HUWE1 mRNA levels among different liposarcoma subtypes (**Table 7**). In particular, MDM2 expression levels did not affect HUWE1 mRNA levels (**Table 7**). Likewise, in well-differentiated liposarcoma, there was no statistical relationship between MDM2 and HUWE1 mRNA levels (**Table 8**). Taken together, our results suggest that while an increase in MDM2 protein levels in liposarcoma, especially in the well-differentiated subtype, is likely secondary to enhanced *MDM2* transcript levels, diminished HUWE1 protein levels in such liposarcoma cells are not ascribed to *HUWE1* gene expression.

Discussion

A p53-independent role of MDM2 has been described in various settings. Previously, we have demonstrated that MDM2 is an ubiquitin E3 ligase for HUWE1 [6]. In particular, we found that when $HER2^+$ breast cancer cells acquire resistance against the HER2 inhibitor lapatinib, MDM2 promotes degradation of HUWE1, which in turn leads to accumulation of anti-apoptotic substrates of HUWE1, such as MCL1 [6]. Although both HUWE1 and MDM2 bind to p53 and the tumor suppressor protein ARF [8, 14], we showed that MDM2 and HUWE1 interact directly and independently of both p53 and ARF [6]. Although the underlying molecular mechanism still remains elusive, the study demonstrated how MDM2 might impact cellular signaling pathways, independently of p53.

In the present study, we sought to extend our findings in $HER2^+$ breast cancer cell lines to normal tissues, breast cancer and liposarcoma. We analyzed gene and protein expression levels of MDM2 and HUWE1 in breast cancer and liposarcoma. We found that the oncoprotein MDM2 was below detectable levels in normal tissues and most of the breast cancers, while HUWE1 protein was expressed in widespread fashion (**Tables 1-3**). It should be noted that the breast cancers in our cohorts had not been treated with lapatinib or any other HER2 inhibitors prior to sampling. Thus, these results are consistent with our previous finding that HUWE1 is widely expressed in $HER2^+$ breast cancer cell lines before acquiring resistance to HER2 inhibition [6]. It is interesting to find out

Table 6. MDM2/HUWE1 protein expression in a liposarcoma cohort

MDM2	-	-	+	+	Total
HUWE1	-	+	-	+	
Pleomorphic (%)	3 (42.9)	3 (42.9)	0 (0)	1 (14.3)	7
Myxoid (%)	6 (42.9)	2 (14.3)	6 (42.9)	0 (0)	14
Round cell (%)	1 (33.3)	2 (66.7)	0 (0)	0 (0)	3
Spindle cell (%)	0 (0)	1 (50)	0 (0)	1 (50)	2
Well-differentiated (%)	6 (31.6)	0 (0)	7 (36.8)	6 (31.6)	19
Total (%)	16 (35.6)	8 (17.8)	13 (28.9)	8 (17.8)	45

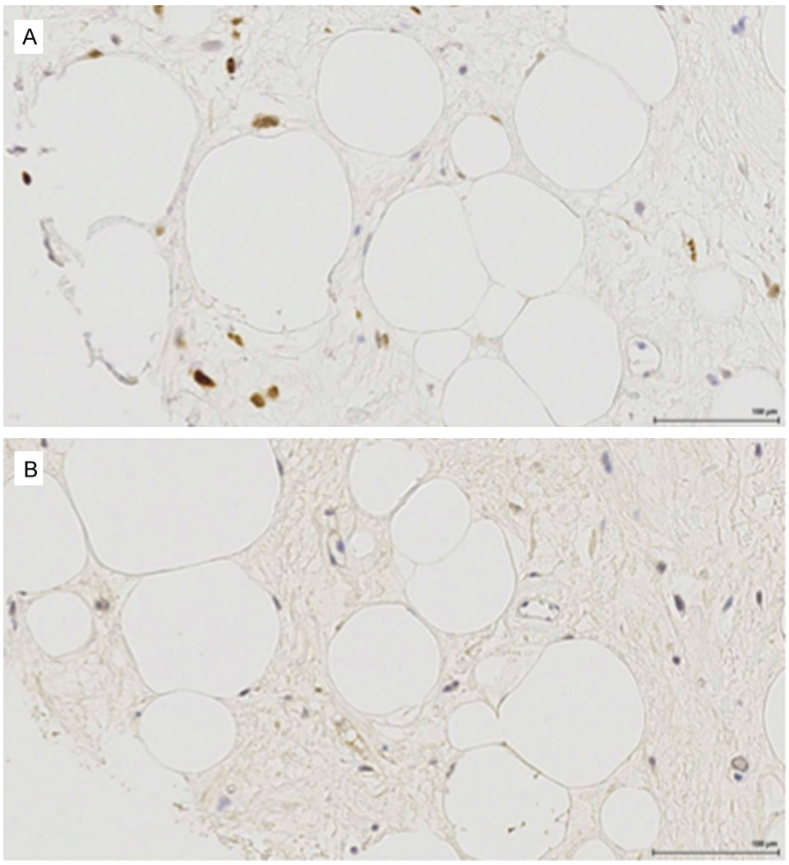

Figure 2. Immunohistochemical analysis of MDM2 and HUWE1 in liposarcoma. IHC detection of MDM2 (A) and HUWE1 (B) in well-differentiated liposarcoma is shown. MDM2 is expressed in the nucleus, while HUWE1 is undetectable. The error bars indicate 100 μm.

whether HUWE1 protein levels are diminished (and MDM2 levels are upregulated) upon acquisition of resistance to HER2 inhibitors, as observed in the cell line model [6].

In contrast to breast cancer, liposarcoma, especially the well-differentiated subtype, is often associated with amplification of MDM2 (**Table 6**; also see [13]). Interestingly, HUWE1 protein was only detected in less than 40% of MDM2-positive liposarcoma cases (**Table 6**). Our gene expression analysis using independently generated, publically available gene expression datasets showed that gene expression patterns do not reflect the unique protein expression patterns of MDM2 and HUWE1 in breast cancer ($MDM2^{low}$/$HUWE1^{high}$) and in liposarcoma ($MDM2^{high}$/$HUWE1^{low}$) (**Tables 4**, **5**, **7**, and **8**). Therefore, it is likely that a post-transcriptional/post-translational mechanism plays a role in creating the differential MDM2/HUWE1 protein expression patterns in these cancer tissues. As we showed previously [6], one of such mechanisms may be regulation of HUWE1 protein stability by MDM2. If MDM2 protein expression or its activity is low, HUWE1 protein would become stable, leading to the accumulation of HUWE1 protein (**Figure 3**). Conversely, high levels of MDM2 protein expression or its activity would result in degradation of HUWE1 protein (**Figure 3**). It remains to be fully elucidated what factor(s), positively or negatively, regulates the ligase activity of MDM2 towards HUWE1. In this regard, it is interesting to note that in contrast to nuclear localization of MDM2, HUWE1 was detected mostly in the cytoplasm (**Table 1**; **Figures 1** and **2**). Whether this differential subcellular localization of MDM2 and HUWE1 is a consequence of MDM2-mediated degradation of HUWE1 or of regulation by other factors should also be subjected to further scrutiny.

The role of HUWE1 in normal and cancer cells is not fully understood. This is partly because HUWE1 targets diverse cellular substrates,

Table 7. MDM2/HUWE1 gene expression in liposarcoma (GEO)

	MDM2			HUWE1		
	Fold Change	T-score	*P*-value	Fold Change	T-score	*P*-value
WD vs. ML	3.6	8.74	6.47E-12	1	0.59	>0.1
WD vs. PL	3.64	8.99	4.43E-12	1.04	2.88	0.006
DD vs. ML	5.78	8.6	1.77E-10	0.99	-0.77	>0.1
DD vs. PL	5.86	8.74	1.55E-10	1.02	1.22	>0.1
DD vs. WD	1.61	-1.93	0.06	0.98	-1.53	>0.1

WD: well-differentiated liposarcoma, ML: Myxoid liposarcoma, PL: Pleomorphic liposarcoma, DD: de-differentiated liposarcoma.

Table 8. MDM2/HUWE1 gene expression in well-differentiated liposarcoma (GEO)

		HUWE1 mRNA	
		Low	High
MDM2 mRNA	Low	12	14
	High	14	12

X-squared = 0.076923, df = 1, *p*-value = 0.7815 (Pearson's Chi-squared test with Yates' continuity correction).

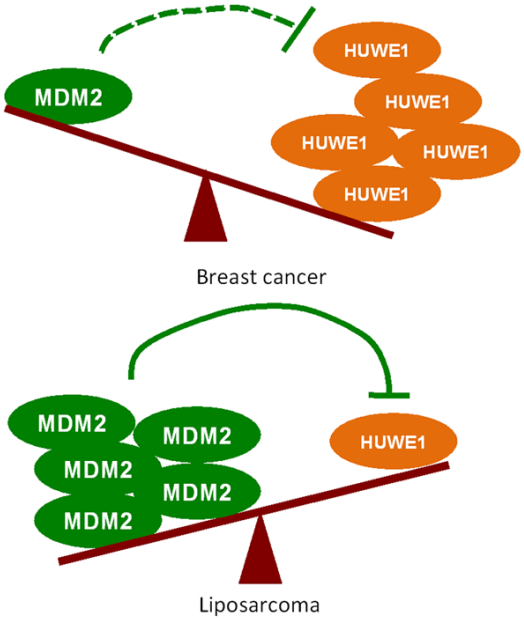

Figure 3. The schematic diagram of the current model. In breast cancer (top panel), MDM2 is not highly expressed. Consequently, one of its substrates, HUWE1, is stabilized and accumulates. In liposarcoma (bottom panel), MDM2 is often overexpressed, leading to ubiquitination and degradation of HUWE1. As a consequence, HUWE1 protein levels become low.

including p53 [8]. Consistent with the role of HUWE1 as an ubiquitin E3 ligase for p53, *Huwe1*-null mice are embryonically lethal because of fatal p53 activation [15]. However, skin-specific Huwe1 knockout in mice did not cause significant p53 activation in the Huwe1-deleted tissues [16]. In contrast, B cell-specific Huwe1 knockout mice resulted in apoptosis, which was rescued by co-knockout of *p53* [17]. Thus, the function of HUWE1 as an E3 ligase for p53 may be tissue-specific or context-dependent. It should be noted that both HUWE1 and MDM2 are ubiquitin E3 ligases for p53. Moreover, DNA damage promotes degradation of MDM2, which precedes stabilization and activation of p53 [18]. It may be of particular interest to investigate how MDM2-mediated regulation of HUWE1 protein stability contributes to the steady-state p53 levels as well as p53 activation in response to cellular stress.

Other substrates of HUWE1 include MYC, MCL-1, and regulators of DNA damage/repair signaling pathways such as BRCA1 [7, 9, 10, 12]. How MDM2 regulation of HUWE1 affects the abundance of each of these HUWE1 substrates and how it might impact cell fates remains to be elucidated. Answers to these questions would help to understand the role of HUWE1 as well as p53-independent roles of MDM2 in normal and cancer cells.

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Disclosure of conflict of interest

None.

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