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Distinct promoter usage of mdm2 gene in human breast cancer

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Abstract. Human breast cancers, especially estrogen receptor α (ERα)-positive ones, often overexpress the oncoprotein MDM2 without mdm2 gene amplification. The mdm2 gene is transcribed into two different mRNAs, namely L-mdm2 and S-mdm2, which are generated from promoters P1 (constitutive) and P2 (regulated by tumor suppressor p53), respectively. To cast light on the mechanisms of MDM2 overexpression, we measured the expression levels of these mdm2 mRNAs using RT-PCR analysis in three human breast cancer cell lines and 15 breast cancer samples obtained from surgery. ERα-positive MCF-7 cells, which possess wild-type p53, displayed dominant expression of S-mdm2. In contrast, two other cell lines with mutant p53, T47-D (ERα-positive) and MDA-MB-231 (ERα-negative), showed almost equivalent expression of L-mdm2 and S-mdm2. Treatment of 17ß-estradiol (E2) significantly enhanced the expression of S-mdm2 but not that of L-mdm2 in MCF-7. Among 6 breast cancer samples regarded as ERα-positive with wild-type p53, 5 samples showed increased expression of S-mdm2. Expression of S-mdm2 was stimulated in 2 ERα-positive samples with mutant p53. In contrast, 4 of 5 samples which express mutant p53 without ERα showed poor expression of S-mdm2. There is a tendency that ERα-positive breast cancers with wild-type p53 preferably use P2 promoter for the expression of mdm2, possibly through E2-induced accumulation of p53. However, wild-type p53 and ERα are not necessarily enough for the utilization of S-mdm2. Tumors with mutant p53 also showed expression of S-mdm2 in some cases. These results strongly suggest that other factor(s) is also implicated in the promoter usage of mdm2 gene in human breast cancer tissues.

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Key words: mdm2, promoter, estrogen receptor, p53, breast cancer

Introduction

Since the mdm2 gene is frequently amplified in certain types of cancers, including osteosarcoma (1-4), and shows oncogenic activity in NIH3T3 cells when exogenously introduced (5), it is considered to be an oncogene. The human mdm2 gene encodes a polypeptide consisting of 491 amino acids that contains a binding domain for the tumor suppressor p53 (5-8). MDM2 binds to the N-terminal region of p53 and inhibits its transcriptional activity by concealing the trans-activation domain (1,6). In addition, MDM2 promotes a rapid degradation of p53 via a ubiquitin-proteasome pathway (9,10). On the other hand, the mdm2 gene is transcriptionally regulated by p53. Therefore, MDM2 is thought to be a negative feedback regulator of this oncosuppressor (6,11).

Mdm2 transcripts appeared to be generated from two different promoters (Fig. 1) (12,13). Promoter P1 locates upstream of the translation start site, and promoter P2 resides within the first intron of the gene (12). The two mdm2 mRNAs, designated as L-mdm2 and S-mdm2, transcribed from P1 and P2, respectively, have differences only at the 5' untranslated region, so that the resulting portions are identical. Importantly, p53 protein specifically binds to a response element adjacent to the transcription initiation site of S-mdm2 and stimulates the transcription from P2 without influencing the activity of the upstream promoter, P1 (13). Overexpression of MDM2 proteins is often observed in human tumor cells containing wild-type p53, which activates the P2 promoter to generate S-mdm2 mRNA (14). S-mdm2 mRNA is shown to be more efficiently translated than L-mdm2 *in vitro* and *in vivo* (14). In addition, the 5' leader sequence of L-mdm2 is only sufficient to transfer translation repression to a reporter gene, while that of S-mdm2 allows efficient translation of the gene (15).

Although mdm2 gene amplification is uncommon in breast cancers, the level of its mRNA and/or protein is upregulated in about 30-40% of breast cancer specimens (16-18). It is well known that the MDM2 protein is expressed at higher levels in estrogen receptor α (ERα)-positive breast cancers than in ERα-negative ones (19). We have recently presented evidence that overexpressed MDM2 protein may provide a growth advantage to ERα-positive breast cancer (20). In addition, we have reported recently that MDM2 enhances the transcriptional function of ERα in a ligand-dependent manner

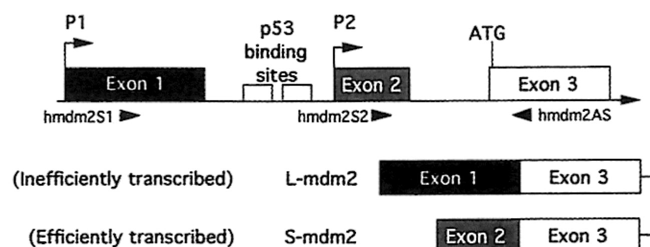

Figure 1. Schematic illustration of the 5' portion of the human mdm2 gene and the two different transcripts. L-mdm2 and S-mdm2 are generated from promoters 1 (P1) and 2 (P2), respectively. Arrowheads indicate the positions of the primers used for the RT-PCR. Bent arrows indicate the positions of the two transcription start sites.

through direct interaction with ERα, but not through the modification of p53 (21). These findings lead us to elucidate the mechanism of mdm2 overexpression, especially in ERα-positive tumors. Although the positive effect of ERα expression on the mdm2 mRNA transcription is well-documented (22,23), the usage of promoters on mdm2 gene in human breast cancer has not yet been investigated.

In the present study, we analyzed, for the first time, the distinct promoter usage of the mdm2 gene in human breast cancer cell lines and tissues. Breast cancers with ERα thereby showed the tendency of increased transcription from P2 promoter. On the other hand, unexpectedly, there was no correlation between promoter usage and the p53 mutation status.

Materials and methods

Cell culture. Human mammary tumor cell lines (MCF-7, T47-D, MDA-MB-231) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 5 units/ml penicillin, and 5 μg/ml streptomycin (Sigma) at 37˚C in a humidified atmosphere of 5% CO_2 in air. For experiments evaluating the effect of estradiol (E2), cells were cultured in phenol red-free DMEM (PRF-DMEM, Gibco BRL) containing 10% FBS stripped off steroids by dextran-coated charcoal (DCC-FBS).

Tissue samples. Human primary breast cancer specimens from 15 female patients were obtained during surgical treatment at Saitama Cancer Center Hospital, Japan and mRNA and DNA were extracted, as described (24). Clinicopathological findings of the samples are summarized in Table I.

Preparation of RNA and semi-quantitative RT-PCR. Total RNAs were prepared from culture cells (2-5x10^6 cells) and approximately 0.1 g human breast cancer tissue according to the method of Chomczynski and Sacchi (25). Semi-quantitative RT-PCR was carried out using a GeneAmp RNA PCR kit ver. 2.1 (Takara Shuzo Co., Ltd., Tokyo), as previously described (26). Oligonucleotide primers used are as follows: hMDM2S1, AGG AAA CTG GGG AGT CTT G; hMDM2S2, ATT GGA GGG TAG ACC TGT G; hMDM2AS, CCT CTT TCA TAG TAT AAG TGT C for L- and S-mdm2 (Fig. 1); hMDM2 cds S, AGT TGA ATC TCT CGA CTC AG and hMDM2 cds AS, CCA ACA TCT GTT GCA ATG TG for

Table I. Clinicopathological findings and data for p53 mutation on each exon, ERα protein amount and the mdm2 mRNA S/L value in 15 breast cancer samples.

Sample no.	Age	pTNM[a]	Clinical stage[b]	Histology[c]	p53 status	ERα (fmol/mg protein)	mdm2 S/L arbitrary unit
1	72	$T_2N_1M_0$	IIB	Inv. Duc. Sol	mt (exon 7)	0	0.132
2	43	$T_2N_1M_0$	IIB	Inv. Duc. Sol	mt (exon 7)	0	0.053
3	84	$T_2N_1M_0$	IIB	Inv. Duc. Sol	mt (exon 5)	0	0.058
4	82	$T_2N_1M_0$	IIB	Inv. Duc. Sol	mt (exon 5)	0	0.037
5	49	$T_{4a}N_1M_0$	IIIB	Inv. Duc. Pap	mt (exon 5)	0	0.68
6	45	$T_3N_1M_0$	IIIA	Inv. Duc. Sol	mt (exon 5)	8	0.602
7	35	$T_{1b}N_0M_0$	I	Inv. Duc. Sci	mt (exon 4)	10	0.287
8	48	$T_{1c}N_0M_0$	I	Non. Duc.	ND	15	0.709
9	63	$T_2N_1M_0$	IIB	Inv. Duc. Sci	wt	15	0
10	34	$T_{4a}N_3M_0$	IIIB	Inv. Duc. Sol	wt	16	0.654
11	44	$T_2N_0M_0$	IIA	Inv. Duc. Sci	wt	18	0.03
12	69	$T_2N_1M_0$	IIB	Inv. Duc. Sci	ND	130	0.26
13	60	$T_{2c}N_0M_0$	IIA	Inv. Duc. Pap	wt	130	0.089
14	53	$T_{1b}N_0M_0$	I	Inv. Duc. Sci	wt	200	0.893
15	47	$T_3N_1M_0$	IIIA	Inv. Duc. Pap	wt	240	0.709

mt, mutation; wt, wild-type; ND, not determined; [a]Pathological classification by UICC. [b]Stage grouping by UICC. [c]Inv. Duc., invasive ductal cancer; Non. Duc., non-invasive ductal cancer; Sol, solid tubular; Pap, papillo tubular; Sci, scirrhous.

total mdm2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as described previously (27). Two µg of total RNA were reverse transcribed to synthesize cDNA using random nonamers at 42˚C. One tenth of synthesized cDNA was subjected to PCR amplification with specific primers (0.2 µg each) and 3 µCi of [α-^{32}P]dCTP (3000 Ci/mmol) in 50 µl mixtures consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.2 mM dNTPs (dATP, dTTP, dGTP, dCTP). PCR comprised 26 cycles for GAPDH, 32 cycles for L- and S-mdm2 and 27 cycles for total mdm2 mRNA with denaturing at 95˚C for 1 min, annealing at 62˚C (66˚C for L- and S-mdm2) for 1 min and extension at 72˚C for 1 min in each cycle using a GeneAmp™ PCR System 9600 (Perkin-Elmer, Cetus). Cycle numbers were optimized in several experiments with determination of linear phase PCR reaction. The PCR products were then subjected to 5% polyacrylamide gel-electrophoresis. The radioactivity of the labeled PCR products from human breast cancer specimens was quantified by Fuji Bio-Image Analyzer BAS2000 (Fuji Film Co., Ltd., Tokyo). For the quantification of total mdm2 mRNA in 15 clinical samples, loading amounts of PCR products to polyacrylamide gel were normalized with reference to GAPDH values.

ERα protein level. ERα enzyme immuno-assay was performed using ER-EIA kits (Abbott). ERα values <5 fmol/mg protein were considered as negative.

p53 mutation analysis. Genomic DNAs from 13 out of the 15 breast cancer samples were prepared and examined for p53 alterations (exons 4-8 and 10) using the PCR-single-strand conformation polymorphism (PCR-SSCP) method (28). PCR reactions were performed essentially as described previously (26) with reaction mixtures containing 50 ng of genomic DNA, 10 pmoles of each pair of primers, 0.2 mM dNTPs, 1.0 U *Taq* polymerase (Boehringer Mannheim), 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride and 1.5 mM magnesium chloride in the presence of 2.5 µCi of [α-^{32}P]dCTP. Primer sets used were described previously (29). After initial denaturation (for 2 min at 94˚C), 30 cycles were carried out as follows: 94˚C for 30 sec, an adequate annealing temperature for 30 sec and 72˚C for 30 sec in GeneAmp PCR system 9600 (Perkin-Elmer Corp.). PCR products were denatured at 98˚C for 5 min, and applied to 4% non-denaturing polyacrylamide gels with 10% glycerol, and electrophoresed at 22˚C. After the electrophoresis, gels were vacuum-dried and exposed to X-ray film. Both normal and abnormal DNA fragments were eluted from the dried gels and reamplified by PCR with the same set of primers and PCR conditions. The reamplified DNAs were sequenced using dRhodamine terminator cycle sequencing kit (Applied Biosystem Inc.) and ABI PRISM 377 (Perkin-Elmer Co.).

Results

Detection of mdm2 transcripts from two different promoters in human breast cancer cell lines. By RT-PCR analysis with the indicated primers shown in Fig. 1, we evaluated the expression of L-mdm2 and S-mdm2 mRNAs, which originated from P1 and P2 promoters, respectively, in three different human breast cancer cell lines, MCF-7, T47-D and MDA-MB-231, in the absence or presence of 10 nM E2. Among these cell lines, ERα is expressed in MCF-7 and T47-D, but not in MDA-MB-231. Wild-type p53 is expressed only in MCF-7 but two others have mutant one. Fig. 2A shows the representative results of RT-PCR analysis for L- and S-mdm2, together with control GAPDH. The densitometric value of each band is measured by densitometer, then the value of S-mdm2 was divided with that of L-mdm2 mRNAs (S/L). Mean S/L value from three independent experiments are presented in Fig. 2B. Among the three cell lines, MCF-7 showed higher S/L value as compared with other cells in the absence of steroid hormones (Fig. 2, p<0.01, t-test). Addition of E2 significantly increased the S/L value in MCF-7 (Fig. 2, p<0.05, t-test). In T47-D cells, S/L-mdm2 value was not

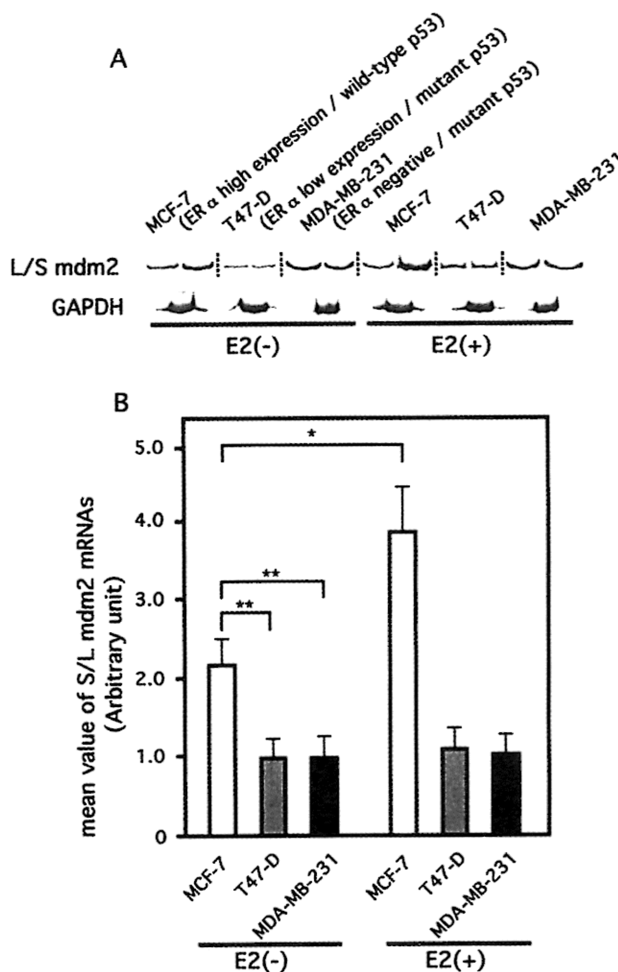

Figure 2. Expressions of L- and S-mdm2 mRNAs in three human breast cancer cell lines detected by semi-quantitative RT-PCR. (A), L- and S-mdm2 derived from MCF-7, T47-D and MDA-MB-231 in the presence (+) or absence (-) of 10 nM E2. Total RNAs were taken at 24 h and semi-quantitative RT-PCR was performed. Products labeled with [^{32}P] were autoradiographed. The results shown are representative of three independent experiments. GAPDH was shown as an internal control. (B), The mean value of S-mdm2 divided with that of L-mdm2 mRNAs (S/L-mdm2 value) were calculated from three independent experiments. Bars indicate SD. *p<0.05; **p<0.01.

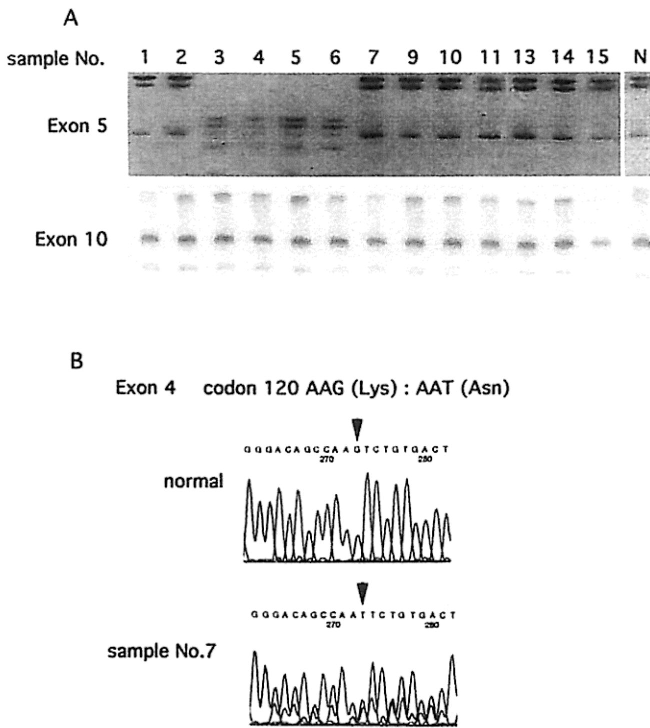

Figure 3. Mutation analysis of the p53 gene. (A), PCR-SSCP analysis of breast cancer specimens. Exons 4 to 8 and 10 were examined. Electrophoretic patterns for exons 5 and 10 are shown. (B), Direct sequencing of exon 4 of the p53 gene. Exon 4 of sample no. 7 has a point mutation (arrowheads) at codon 120 of p53, resulting in a substitutional change of an amino acid from lysine to asparagine.

significantly affected by E2. Little change was observed in the S/L-mdm2 value in MDA-MB-231 regardless of E2 supplementation (Fig. 2). These findings suggest that breast cancer with ERα and wild-type p53, such as MCF-7 cells, may express MDM2 protein effectively through E2-induced accumulation of S-mdm2 mRNA.

Mutation analysis of the p53 gene and measurement of ERα expression in human breast cancer samples. Since more than 40% of human breast cancers possess mutations on p53 gene which may alter its transcriptional activity (30), we next analyzed exon mutational status of p53 gene in 13 breast cancer specimens. Exons from 4 to 8 and 10 of the p53 gene in 13 samples were analyzed using PCR-SSCP. Fig. 3A shows representative results for exon 5 and 10. Since amplified exon 5 cDNA fragments of samples 3, 4, 5 and 6 showed different SSCP patterns as compared with that from normal tissue (Fig. 3A, upper panel), those were recognized as mutants. Aberrant bands were not observed for exon 10 (Fig. 3A, lower panel). No mutation on p53 gene was found in exons 6 and 8 by PCR-SSCP (data not shown). Since detection of p53 mutations in exon 4 with SSCP was difficult, direct sequencing of the PCR products was performed. Sample 7 thereby showed a point mutation at codon 120, resulting in substitutional change of lysine to asparagine (Fig. 3B). ERα levels of individual samples measured by enzyme-linked immuno-assay are shown in Table I. Based on these results,

Table II. Relationship between ERα expression and p53 gene status in human breast cancer samples.

ERα	p53 status		p-value[a]
	Wild-type	Mutant	
Negative (<5 fmol/mg protein)	0	5	0.016
Positive (≥5 fmol/mg protein)	6	2	

[a]Fisher's exact probability test.

the relation between ERα protein level and p53 status is summarized in Table II. The data demonstrate a negative correlation with statistical significance (p=0.016, Fisher's exact probability test), in line with previous findings (31,32).

The relationship among L- and S-mdm2 transcripts, p53 and ERα in human breast cancer samples. Analysis of L- and S-mdm2 mRNA expression in 15 human breast cancer samples including the samples determined for their p53 mutation status was performed by the same procedure as for cell lines. Fig. 4 shows the representative results of RT-PCR analysis of L-, S- and total mdm2, together with control GAPDH mRNA. Although total amount of mdm2 mRNA was considerably constant but showed fluctuation among the samples, L- and S-mdm2 showed more prominent variety in expression patterns. The S/L-mdm2 value of each sample from three independent experiments ranged from 0 (no S-mdm2 mRNA could be detected) to 0.893 (Table I). S- and L-mdm2 mRNAs were also examined in the normal ductal part of 15 cancer samples. Signals of both types of mdm2 mRNAs were detected in 7 normal samples by our RT-PCR analysis, and the value of S/L-mdm2 never exceeded 0.2 among the samples tested (data not shown). Therefore, 0.2 is designated as the cut-off value for the estimation of upregulation of S-mdm2 transcript. The correlation between the S/L-mdm2 value and p53 mutation status was hardly observed (Fig. 5A; p=0.496, Student's t-test), although p53 positively promotes S-mdm2 expression in several human cancers *in vitro* and *in vivo* (14,33). Next, to analyze whether expression levels of ERα was correlated with mdm2 promoter usage or not, the S/L-mdm2 values were plotted in a scatter graph with ERα protein expression levels (Fig. 5B). Seven of 10 samples which regarded as ERα-positive by EIA showed increased expression of S-mdm2. In contrast, high expression of S-mdm2 was seen in only 1 of 5 ERα-negative tumors. These results indicate that ERα expressing tumors, generally possess wild-type p53 like MCF-7, have a tendency to utilize P2 promoter more preferably than those without ERα. Though the analyzed cases were limited, this showed a broader significance (p=0.067). However, 2 samples of ERα-positive tumors with mutant p53 also showed increased usage of P2 promoter, and 1 sample of ERα-negative tumor with p53 mutation could utilize the P2 promoter. Therefore, there is a possibility that other transcriptional factor(s) is also involved in regulation of the P2 promoter.

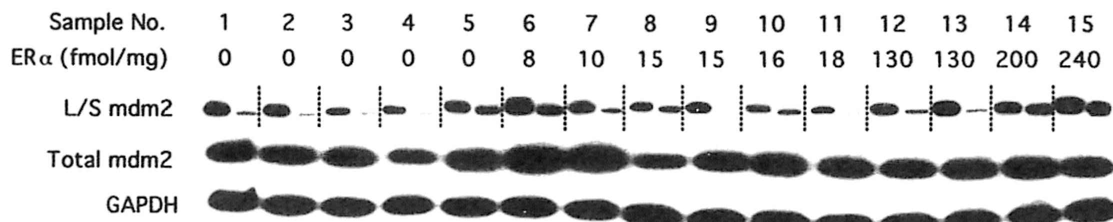

Figure 4. Expressions of L-, S- and total mdm2 mRNAs in human breast cancers from 15 resected specimens detected by semi-quantitative RT-PCR. Total RNAs were taken from each sample, and semi-quantitative RT-PCR was performed. Products labeled with [^{32}P] were autoradiographed. Three independent assays were performed and representative results are shown. Expression levels of ERα protein are also given for each sample. GAPDH was used as an internal control.

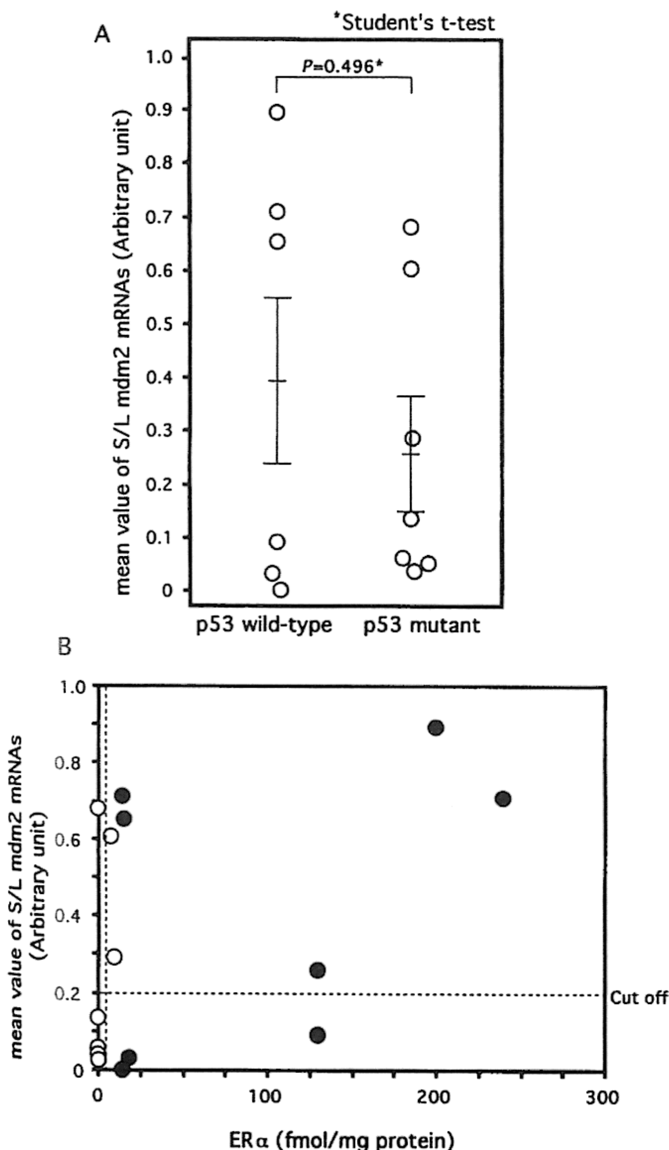

Figure 5. Analysis of the relationship between the mean value of S/L-mdm2 mRNAs determined by semi-quantitative RT-PCR with p53 status or ERα expressions in human breast cancer specimens. (A), Comparison of the p53 gene mutation status with the mean value of S/L-mdm2 mRNAs. Statistical significance was not revealed (p=0.496, Student's t-test). Bars indicate SE. (B), Correlation between expression levels of ERα and the mean value of S/L-mdm2 mRNAs. Open circles are the samples with mutant p53 and closed ones are those with wild-type p53. A horizontal broken line indicates the cut-off value determined using normal mammary tissue samples, and vertical one is for ERα-positivity.

Discussion

We have analyzed how mdm2 mRNA is upregulated both in human breast cancer cell lines and the clinical breast cancer specimens. First, we demonstrated different usage of P1 and P2 promoters of the mdm2 gene in various breast cancer cell lines. The human mdm2 gene is transcribed from different promoters, namely P1 and P2. Transcription from P1 is not affected by p53 but is considered to contribute to a constitutive expression. On the other hand, transcription from P2 is induced by binding of p53 to its response element residing in intron 1 of the gene (13,34). MCF-7 cells which expressed relatively high level of ERα and wild-type p53 displayed significantly high value of S/L-mdm2 as compared with two other cell lines expressing mutant p53 in a steroid hormone-stripped medium. Furthermore, E2 significantly increased the S/L-mdm2 value only in MCF-7 cells. However, in MDA-MB-231 (ERα-negative/p53 mutant) and T47-D (ERα-positive/p53 mutant), E2 failed to increase the S/L-mdm2 value. It is well known that E2 causes the accumulation of p53 protein in MCF-7 cells (20). Therefore, these results lead us to propose the possible mechanism that in ERα-positive breast cancers E2 accumulates the p53 protein and then stimulates transcription of mdm2 through p53 responsive promoter (P2).

Secondly, to ascertain whether the proposed mechanism can be applied to clinical breast cancer or not, we also examined expressions of L- and S-mdm2, p53 status and amounts of ERα in tissues obtained from breast cancer patients. As expected, S-mdm2 tended to be higher in ERα-positive cases than in ERα-negative ones (Fig. 5B). Especially, samples 14 and 15 which were at premenopausal states exhibited considerably high S/L-mdm2 values indicating that tumor expressing high level of ERα may utilize P2 promoter predominantly. Since S-mdm2 mRNA has been shown to be approximately 8-fold more efficiently translated into protein than L-mdm2 *in vitro* (14), frequent overexpression of MDM2 protein in ERα-positive breast cancers could be due to enhanced expression of S-mdm2. On the other hand, samples with undetectable expression of ERα, all of which had mutant p53, showed generally low S/L-mdm2 value. The modulation of mdm2 transcription or the feedback loop between p53 and MDM2 may not be preserved strictly in these cases. Furthermore, our analysis did not reveal any correlation between the S/L-

mdm2 value and p53 status (Fig. 5A), suggesting that other mechanism(s) must be involved in the regulation independent of p53 in some cases.

It has been documented that p53 mutations correlate well with negative ERα status in breast cancers (31,32,35). Mutations of p53 are detected in more progressed or late staged cancer samples (36). The p53 protein overexpression generally means mutation on p53. Overexpressed mdm2 correlates to worse prognosis. A recent report also suggested that mdm2 gene amplification and p53 mutation on exon 5 or 7 caused poor prognosis in node-negative patients (37). Therefore, P1-induced transcriptional mechanism and/or other transcriptional factors, which may be closely involved in cancer progression at the late stage, should be analyzed more carefully.

We showed P1 and P2 promoters of the mdm2 gene to be differently regulated in breast cancers with regard to ERα status rather than p53. The existence of wild-type p53 is not necessary to induce upregulation of mdm2 mRNA through p53 responsive promoter (P2) in breast cancer. It is apparent that mdm2 is not only regulated by p53 but also influenced by other factor(s) which may relate to cancer progression in each stage. More detailed analysis of this mechanism will be required for clearer explanation in breast cancer progression. In order to elucidate this question, we are now analyzing all promoter regions of mdm2 gene for finding the critical sequence for transcription and for cloning the novel factor which binds to those sequences.

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