Molecular Alterations of *h-warts/LATS1* Tumor Suppressor in Human Soft Tissue Sarcoma

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SUMMARY: *h-warts/LATS1* is a human homolog of the *Drosophila* *warts* tumor suppressor gene, which functions as a component of the mitotic apparatus. *LATS1*-deficient (*Lats1*^{-/-}) mice have been reported to develop ovarian stromal tumors and soft tissue sarcomas. We investigated the status of the *h-warts/LATS1* in 50 human soft tissue sarcomas to address its potential function as a tumor suppressor in human sarcoma development. In our RT-PCR assay, 7 (14%) (3 of 4 myxoid liposarcomas, 3 of 7 leiomyosarcomas, 1 of 9 malignant fibrous histiocytomas) of 50 sarcomas examined had no or a reduced expression of the *h-warts/LATS1*, indicating down-regulated gene expression. We further analyzed alterations of the *h-warts/LATS1* in these seven sarcomas. Using microsatellite markers for chromosome 6q23-25.1, to which the *h-warts/LATS1* is localized, an allelic loss of this locus was detected in one leiomyosarcoma, in which a missense point mutation of the *h-warts/LATS1* was detected by PCR single-strand conformation polymorphism. Clusters of CpG dinucleotides in a 5' putative promoter region were hypermethylated in the other six sarcomas. Our data suggest that the molecular alterations of the *h-warts/LATS1* could be of pathologic importance in human sarcomagenesis. (*Lab Invest* 2002, 82:1427-1435).

Tumor suppressor genes are those that, when functioning normally, prevent the development of tumors; losses of their normal functions (inactivation) are required for tumorigenesis. Allelic losses, gene mutations, and cryptic deletions are often detected as mechanisms involved in the inactivation of tumor suppressor genes. In addition, hypermethylation of promoter regions of genes is considered another important mechanism for down-regulation or "silencing" of the genes. Tumor suppressor genes can be subdivided into two groups: genes that directly regulate tumor growth by inhibiting cell proliferation or promoting cell death ("gatekeepers") and genes whose inactivation causes genetic instability, which in turn leads to mutations that promote tumor growth ("caretakers") (Kinzler and Vogelstein, 1998). *p53*, *RB-1*, *WT1*, *NF1*, *APC*, *VHL*, *BRCA1*, *BRCA2*, *E-CAD*, *p16^{INK4A}*/*p14^{ARF}*, and *p15^{INK4B}* genes are now included as members in the growing list of human tumor suppressor genes (Hooper, 1998).

The *warts* (also referred to as *LATS*) was identified based on its ability to act as a tumor suppressor gene in *Drosophila*. A human homolog of this gene, *h-warts/LATS1*, has recently been isolated (Nishiyama et al,

1999; St John et al, 1999; Tao et al, 1999). *h-warts/LATS1* encodes a putative serine/threonine kinase, which is phosphorylated in a cell cycle-dependent manner and forms a complex with CDC2 in early mitosis, suggesting its function as a negative regulator of CDC2/cyclin complexes during cell division (Hirota et al, 2000; Nishiyama et al, 1999; Tao et al, 1999). Notably, *h-warts/LATS1*-deficient (*Lats1*^{-/-}) mice spontaneously develop ovarian stromal tumors and soft tissue sarcomas and have a significant susceptibility to induction of the latter tumors (St John et al, 1999). Except for *p53*-deficient or *p16^{INK4A}*-deficient mice (Serrano et al, 1996; Williams and Jacks, 1996), few animal models lacking tumor suppressor genes have been reported to be sensitive to sarcoma development. Besides, the status of the *h-warts/LATS1* in human neoplasms has not been investigated, except for one recent study on gene mutations in human breast cancer (Morinaga et al, 2000).

In this study, the role of *h-warts/LATS1* as a human suppressor gene was assessed by investigating its molecular alterations in human soft tissue sarcomas.

Results

Genomic Structure of *h-warts/LATS1*

As a tool that should help in the design of primers for the gene expression analysis and in the search for mutations of the *h-warts/LATS1*, we first undertook characterization of its genomic structure. After the PCR and subsequent DNA sequencing, we identified seven junctions between different exons and the 5'-uncoding region within the examined sequence of the *h-warts/LATS1* mRNA (Fig. 1, Table 1). The sizes of the exons ranged from 148 to 1514 bp. However, the

DOI: 10.1097/01.LAB.0000032381.68634.CA

Received July 2, 2002.

This work was supported in part by a 2000 Grants-in-Aid for Scientific Research (C) (No. 12670184) and Encouragement of Young Scientists (No. 12770102) from the Japan Society for the Promotion of Science and by a grant for cancer research from the Fukuoka Cancer Society, Japan.

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Figure 1.

Schematic representation of the *h-warts/LATS1* and its approximate chromosomal position. Names of neighboring microsatellite markers examined in this study are italicized. *Open boxes* (uncoding region) and *closed boxes* (coding region) represent exons of the *h-warts/LATS1*. CpG dinucleotides (*vertical lines*) are clustered in the 5' region including a putative promoter of the *h-warts/LATS1*.

Table 1. Genomic Structures of *h-Warts/LATS1*

Intron-exon boundaries				
No.	5' Position of exon ^a	3' of the previous intron	5' exon ... 3' exon (size)	5' of the next intron
1	—	... aca gta agt agc cag	TTA TTA CTT TAA CGT ... TGC TCT CCC CTC CAG (1155 bp)	gta ggt ccg ggg ctg ...
2	419	... ttc ttt ctt tta cag	AGT TAA TTA TTT ATA ... GCT GGA TTT GAT GAG (488 bp)	gta aga atg ttt aaa ...
3	897	... ttc aat tca tat cag	GAT ATG GTT ATA CAA ... CCA GCA TGA AAC CAG (148 bp)	gtg att aat caa ata ...
4	1045	... ctc tta taa cca tag	GGA ATG TGC AGC AAT ... AAT GAA ATG ATG CGG (1514 bp)	gta aaa cct ttt aaa ...
5	2559	... agt tta ttt gcc tag	GTT GGA TTA TCT CAA ... AGT ACT ATC AGA GTG (588 bp)	gta agt aaa ctc ata ...
6	3142	... tct ttt gtt gtg cag	GTG ACC ATC CAC GGC ... TGT TGC TAC GAA CAG (183 bp)	gta aag cac ttc ttt ...
7	3325	... act ttg tta ttt tag	GAT ACA CAC AGT TGT ... GAA ACA CAA ATG AAG (107 bp)	gta agg tat ggc atc ...
8	3432	... aaa aaa tct tta aag	GTT ATC AAC TGG CAA ... (3' boundary, not determined)	

^a Position defined according to the sequence of *Homo sapiens* LATS homolog 1 mRNA (GenBank no. NM_004690).

structure of the remaining 3' tail following the serine/threonine kinase domain was not determined in this study. All the intronic donor and acceptor splice sites conformed to the consensus GT-AG mRNA splice sites (Table 1). The sequence coding the putative serine/threonine kinase domain spanned four distinct exons.

Gene Expression of *h-warts/LATS1* in Soft Tissue Sarcomas

In our multiplex PCR using cDNAs from the 50 tumor samples, 6 (2 leiomyosarcomas, 3 myxoid liposarcomas, and 1 malignant fibrous histiocytoma [MFH]) had no detectable gene expression of the *h-warts/LATS1* (Fig. 2, Table 2). In addition, another leiomyosarcoma

had unequivocally reduced gene expression (Fig. 2). Identical results were obtained in another PCR using a different primer set of the *h-warts/LATS1* (Fig. 2).

Analyses of *h-warts/LATS1* Alterations

To determine the molecular mechanisms of the down-regulated *h-warts/LATS1* expression in the seven sarcomas, allelic losses of 6q23-25.1 were first analyzed using five microsatellite markers for chromosome 6q23-25.1. All seven sarcomas were informative for this analysis, and an allelic loss of this chromosomal locus was identified in the leiomyosarcoma with reduced gene expression in RT-PCR (Fig. 3).

Figure 2.

Gene expressions of soft tissue sarcomas analyzed by RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the sequences flanking exons 4 to 5 (upper panel) and 5 to 6 (lower panel) of the *h-warts/LATS1*. Six sarcomas (cases 3, 5, 6, 7, 10, and 12) have no detectable expression of the *h-warts/LATS1*, and one (case 11) has reduced gene expression compared with that of GAPDH. Lanes 1 to 4 = malignant fibrous histiocytomas; lanes 5 to 8 = myxoid liposarcomas; lanes 9 to 12 = leiomyosarcomas; lane 13 = synovial sarcoma; PBC = peripheral blood mononuclear cells, M = 100-bp DNA ladder.

Table 2. Gene Expression of *h-Warts/LATS1* in 50 Soft Tissue Sarcomas

Tumor	No. of cases examined	No. of cases with down-regulated gene expression
MFH	9	1
Leiomyosarcoma	7	3
Liposarcoma, well differentiated	6	0
Liposarcoma, myxoid	5	3
Dermatofibrosarcoma protuberans	4	0
Extraskeletal Ewing sarcoma/PNET	3	0
Synovial sarcoma	3	0
Liposarcoma, dedifferentiated	3	0
Extraskeletal myxoid chondrosarcoma	1	0
Clear cell sarcoma	1	0
Epithelioid sarcoma	1	0
Angiosarcoma	1	0
Alveolar soft part sarcoma	1	0
Malignant peripheral nerve sheath tumor	1	0
Malignant hemangiopericytoma	1	0
Rhabdomyosarcoma, alveolar	1	0
Rhabdomyosarcoma, embryonal	1	0
Malignant mesenchymoma	1	0
Total	50	7

MFH, malignant fibrous histiocytoma; PNET, peripheral primitive neuroectodermal tumor.

In PCR–single-strand conformation polymorphism (SSCP) for gene mutations, two leiomyosarcomas showed point mutations in the coding regions of the *h-warts/LATS1*: a missense mutation at codon 566 (CAA→CGA, Gln→Arg) and a silent mutation at codon 1002 (GAT→GAC) (Fig. 4). Four sarcomas (2 myxoid liposarcomas, 1 leiomyosarcoma, and 1 MFH) with obvious gene expression in RT-PCR were also included in this analysis, but no gene mutation was identified. In Southern blot analysis of 4 sarcomas lacking gene expression (2 myxoid liposarcoma, 1 leiomyosarcoma, and 1 MFH), no aberrant bands suggesting genomic rearrangements of the *h-warts/LATS1* were found. No genomic amplification or deletion of the *h-warts/LATS1* was identified either.

Although the CpG dinucleotide frequency in the human genome is nearly 0.6%, the 5' region of the *h-warts/LATS1* has clustered CpG dinucleotides, suggesting its 5' regulatory element. Using a promoter scanning program, we found a putative promoter sequence located approximately 250 to 500 bp upstream of exon 1. We therefore investigated the methylation status of this region in 13 sarcomas, including 6 sarcomas with the obvious expression of the *h-warts/LATS1* as controls. Aberrant hypermethylation of the putative promoter of the *h-warts/LATS1* was identified in all but one sarcoma with reduced or no gene expression (Fig. 5). In the six sarcomas with gene expression, the promoter region was not hypermethylated. Summarized results

Table 3. *h-Warts/LATS1* Alterations in the Seven Sarcomas Examined

No.	Tumor	Age/sex	Site	Altered gene expression	LATS1 alterations			
					Allelic loss	Gene mutation	Genomic rearrangement	Methylated 5' CpG
3	MFH	68/M	Thigh (lt.)	+ (undetectable)	—	—	—	+
5	Myxoid liposarcoma	50/M	Thigh (rt.)	+ (undetectable)	—	—	—	+
6	Myxoid liposarcoma	24/F	Thigh (lt.)	+ (undetectable)	—	—	—	+
7	Myxoid liposarcoma	48/F	Abdominal wall	+ (undetectable)	—	—	Not examined	+
10	Leiomyosarcoma	46/M	Thigh (lt.)	+ (undetectable)	—	+ (codon 1002) ^a	—	+
11	Leiomyosarcoma	66/F	Thigh (lt.)	+ (reduced)	+	+ (codon 566) ^b	Not examined	—
12	Leiomyosarcoma	63/M	Groin (rt.)	+ (undetectable)	—	—	Not examined	+

^a Silent mutation.^b Missense mutation (Gln → Arg). **Figure 3.**

An allelic loss of 6q23-25.1 in a leiomyosarcoma (case 11) demonstrated by a microsatellite marker analysis. In the tumor tissue (T), loss of heterozygosity of this locus was shown by all the five markers examined. N = nontumor tissue.

Figure 4.

A, PCR-single-strand conformation polymorphism (SSCP) for gene mutation, showing aberrant bands (arrowheads) in two leiomyosarcomas (T) (cases 10 and 11). N = nontumor tissue of the corresponding case; B, point mutations by sequencing of the PCR products extracted from the aberrant bands. An antisense cDNA sequence is shown in #11.

of the molecular studies of the seven sarcomas examined are shown in Table 3.

Discussion

The tumor suppressor genes are considered to play a crucial role in development and/or progression of a wide variety of human neoplasms, and *p53*, *RB-1*, and *p16^{INK4A}/p14^{ARF}* are the genes frequently investigated in human sarcomas. Abnormalities of the *p53* pathway occur in nearly 50% of human carcinomas and, to a lesser extent, in sarcomas (Greenblatt et al, 1994; Latres et al, 1994; Leach et al, 1993). Aberrations in the *Rb*-cyclin D pathway are common in certain types of sarcomas, such as leiomyosarcoma, myxoid lipo-

sarcoma, and osteosarcoma (Benassi et al, 1999; Dei Tos et al, 1996, 1997). In addition, *p53*-deficient or *p16INK4A*-deficient mice frequently develop spontaneous sarcomas (Serrano et al, 1996; Williams and Jacks, 1996).

The recently identified human homolog of *Drosophila* tumor suppressor *h-warts/LATS1* protein is localized to the mitotic apparatus and interacts with Cdc2 kinase and zyxin, a regulator of actin filament assembly during mitosis (Hirota et al, 2000; Nishiyama et al, 1999; Tao et al, 1999). *h-warts/LATS1* is a member of the subfamily of protein kinases including *dbf2*, *orb6*, *cot-1*, *ndr*, and *kpm*, which are involved in cell cycle regulation and morphogenesis (Hori et al, 2000). Dis-

Figure 5.

A, methylation-specific PCR using genomic DNA modified with bisulfite and methylated (M) and unmethylated (U) primers. B, partial sequence of the PCR product containing CpG dinucleotides unaffected by the chemical modification (underlined), representing methylated CpG. Modified and unmodified (original) sequences without methylated bases of the corresponding region are written.

ruption or abrogation of *h-warts/LATS1* function may lead to an impaired mitotic process, potentially causing chromosomal or genetic instability, and/or dysregulation of the cell cycle by uncontrolled Cdc2/cyclin A function. The *h-warts/LATS1* has been shown to be ubiquitously expressed in normal fetal and adult tissues (St John et al, 1999). Therefore, the *h-warts/LATS1* seems to act as a human tumor suppressor gene, and development of soft tissue sarcomas and ovarian stromal tumors in mice deficient of m-LATS1 protein (Lats1^{-/-}) further reinforces this notion. Moreover, significant susceptibility to soft tissue sarcoma induction by carcinogens in these knockout mice has been documented (St John et al, 1999). However, phenotypic differences may be present between human and such animal models because, for example, mice heterozygous for a null mutation in the *RB-1* do not develop retinoblastoma or osteosarcoma, which are the major heterozygote phenotypes in humans, but develop pituitary tumors (Jacks et al, 1992). Although recent molecular studies have shown that overexpression or ectopic expression of *h-warts/LATS1* significantly suppressed the growth of human tumor cells such as carcinoma cells and glioblastoma cells (Xia et al, 2002; Yang et al, 2001), human mesenchymal tumor cells have not been examined. Thus, we focused our study on human soft tissue sarcomas.

To date, little information regarding the status of the *h-warts/LATS1* in clinical samples of human neoplasms is available. Only a recent molecular analysis by (Morinaga et al, 2000) has shown that no gene mutation was identified in 25 human breast cancer tissues by their RT-PCR-SSCP, and one patient had a loss of heterozygosity of the *h-warts/LATS1*. However, they did not fully address the inactivation of this gene in such a human epithelial malignancy.

When we initiated this study, the genomic structure of the *h-warts/LATS1* had not been well characterized.

We examined intron-exon boundaries by comparing genomic DNA and cDNA sequences spanning 75% of *h-LATS1* mRNA or 95% of its coding sequence recorded in GenBank. Seven junctions between different exons were identified in the genomic region analyzed, and one of them was located in its 5'-uncoding region. This genomic characterization of the *h-warts/LATS1* facilitated the design of primers in our RT-PCR for gene expression.

In this study, various molecular approaches were conducted to investigate the prevalence of the inactivated or down-regulated *h-warts/LATS1* as well as their underlying mechanisms in human soft tissue sarcomas. We found that 14% of the soft tissue sarcomas examined were shown to have down-regulated gene expression, suggesting a biologic role of the *h-warts/LATS1* as a human tumor suppressor gene.

Like other tumor suppressor genes, the inactivation of the *h-warts/LATS1* potentially involves three major mechanisms: allelic losses or deletions of the gene, gene mutations, and hypermethylation of its promoter region. In the current analysis, only one leiomyosarcoma with reduced gene expression demonstrated an allelic loss of 6q23-25.1. The low frequency of allelic alterations in our series might reflect the fact that aberrations of 6q have been only rarely detected in human soft tissue sarcomas. A recent study using comparative genomic hybridization showed DNA copy number changes in 6q in leiomyosarcomas, although they were mostly gains (El-Rifai et al, 1998). Interestingly, deletion of 6q has been demonstrated in three myxoid/round cell liposarcomas with t(12;16) (Altunoguz et al, 1995). Because the *h-warts/LATS1* alterations were almost confined to leiomyosarcoma and myxoid liposarcoma in our study, the molecular or genetic changes involving the *h-warts/LATS1* may be specifically associated with these sarcoma types.

Table 4. Sequences of Primers Used for PCR, RT-PCR, and Methylation-Specific PCR

Name of primer set (position ^a)	Forward primer (5'–3')	Reverse primer (5'–3')	Size of PCR product (bp)	Annealing temperature (° C)
LATS1–1 (380–534)	CTG TCC AGG AGC TCT GCT CT	ATG TAG CCC ACA CGA AGG AC	155	56
LATS1–2 (515–700)	GTC CTT CGT GTG GGC TAC AT	TCA GCC TTA GCA GCA TCA GA	186	56
LATS1–3 (681–875)	TCT GAT GCT GCT AAG GCT GA	CAA GTC TTG AAG CAT TTG TGG A	195	56
LATS1–4 (871–1051)	ACT TGC AAG CTG CTG GAT TT	ACA TTC CCT GGT TTC ATG CT	181	56
LATS1–5 (1032–1231)	AGC ATG AAA CCA GGG AAT GT	GGG TGA GCT TGA ACA AAT GC	200	56
LATS1–6 (1229–1419)	CCC TAG CAA CGG ACA GAG AG	CAG GTG GGA CAG GAG AGA TT	191	56
LATS1–7 (1402–1598)	TCT CCC GAA TCT CTC CTG TC	ACC ATT CTG CAT TCC AGG TC	197	56
LATS1–8 (1570–1746)	CAT CAG GGA GAC CTG GAA TG	ATG ACG AAG GAG CAG CAG AT	177	56
LATS1–9 (1727–1915)	ATC TGC TGC TCC TTC GTC AT	GGT TGC CAT GTA GGG ATT TC	188	56
LATS1–10 (1913–2116)	ACC TAA CAT ACC AGT GAG GTC	GGC TGT GGT ATC CAA GAA GG	204	56
	AAA			
LATS1–11 (2103–2305)	TGG ATA CCA CAG CCA ATT CA	CTT GGC TGA TCC TCT TTG CT	203	56
LATS1–12 (2285–2465)	AGC AAA GAG GAT CAG CCA AG	AAA TGC TTG AGG AGA ATA ACT	181	56
	TTG A			
LATS1–13 (2460–2657)	GCA TTT AAA TTC TTT ATG GAG CAA C	AGA CTT GTC CAT TTT AGC CCT TT	198	56
LATS1–14 (2628–2828)	CGT CTT AAA AGG GCT AAA ATG AGC G	TTC AGC CAG GAT ATC TCT C	201	56
LATS1–15 (2710–2997)	AGA TAT CCT GGC TGA AGC TGA CAA C	CTG CAC AGG TAA GTT CTG C	188	56
LATS1–16 (2985–3230)	ACC TGT GCA GTT GAA AGT GTT TAA C	TGG CTT CAG TCT GTC TCC A	245	56
LATS1–17 (3209–3421)	TGG AGA CAG ACT GAA GCC ATT GTT A	TCT AAT GGT GTT TGT GCC A	213	56
LATS1–18 (3401–3607)	GGC ACA AAC ACC ATT AGA AAC CTC A	AGG TCA CTG GAG AAG TCA A	207	56
LATS1–M ^b	T <u>CG</u> TTT TGT <u>CGT</u> TTA GGT TGG	<u>CGA</u> <u>CGT</u> AAT AAC <u>GAA</u> <u>CGC</u> CTA	132	56
LATS1–U ^b	TAG GTT GGA GTG <u>TGG</u> TGG <u>TG</u>	CCC <u>AAC</u> <u>ATA</u> <u>ATA</u> <u>ACA</u> <u>AAC</u> ACC T	121	53
GAPDH (586–1037)	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452	56

^a Position defined according to the sequences of *Homo sapiens* LATS homolog 1 mRNA (GenBank no. NM_004690) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (#M33197).

^b Sequence differences between modified primers and unmodified DNA are in boldface type, and differences between methylated/modified and unmethylated/modified are underlined.

In our PCR-SSCP for gene mutations, a missense mutation was detected in one leiomyosarcoma with an allelic loss of 6q23-25.1. Although such a mutation does not seem to result in down-regulation of gene expression, the product of the mutated gene may alter its biologic property, potentially leading to the inactivation of the *h-warts/LATS1*.

Methylation of cytosine residues in CpG islands is an important mechanism through which genes can be differently transcribed, and abnormal methylation patterns effect either activation (hypomethylation) or silencing (hypermethylation) of genes. Methylation status of 5' promoter regions of tumor suppressor genes such as the *p16^{INK4A}/p14^{ARF}* has been recently assessed in various human malignancies. Hypermethylation-induced inactivation of the tumor suppressor genes has been detected in variable proportions of human sarcomas; for example, the prevalence of the hypermethylated *p16^{INK4A}/p14^{ARF}* has varied, ranging from 0% to more than 50% in osteosarcoma (Benassi et al, 1999; Wei et al, 1999).

Because our molecular analyses for allelic losses, gene mutation, and genomic rearrangements failed to identify abnormalities potentially leading to the down-regulation of the *h-warts/LATS1* expression in six of the seven sarcomas, we then performed methylation-specific PCR of the 5' putative promoter region containing CpG islands to verify hypermethylation-induced inactivation of the *h-warts/LATS1* in the sarcomas. As we expected, hypermethylation of this region was identified in the six sarcomas without gene expression in RT-PCR, supporting an alternative mechanism in inactivation of the *h-warts/LATS1*. Although unmethylated sequences were also amplified by our PCR in these sarcomas, it is unknown if they were a result of incomplete hypermethylation of the *h-warts/LATS1* or contamination of non-neoplastic tissue in the samples examined.

In conclusion, molecular abnormalities of the *h-warts/LATS1* in subsets of human soft tissue sarcomas suggest its functional role as a human tumor suppres-

sor. Although clinical significance of the *h-warts/LATS1* remains to be determined, our findings could expand the variety of tumor-associated gene alterations involved in human mesenchymal malignancies.

Materials and Methods

Fifty cases of soft tissue sarcomas were retrieved from the files of the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan. Diagnoses of the tumors were established by the combination of morphologic, immunohistochemical, and clinical features. Molecular findings such as the presence of tumor-specific fusion gene transcripts were referred to in the diagnoses of selected tumors (SYT-SSX in synovial sarcoma, *EWS-FLI1/ERG* in the Ewing/PNET family of tumors, *TLS-CHOP* in myxoid liposarcoma, and *TAFI168-CHN* in extraskeletal myxoid chondrosarcoma). Genomic DNA and total RNA were extracted from frozen tumor tissues and peripheral blood mononuclear cells (PBMC) provided by a healthy volunteer according to standard procedures. In a microsatellite marker analysis, DNA was isolated separately from neoplastic and non-neoplastic areas of formalin-fixed, paraffin-embedded tumor tissues by microdissection of tissue sections lightly stained with hematoxylin.

PCR, RT-PCR, and DNA Sequencing

After a treatment of DNase I (Life Technologies, Inc., Rockville, Maryland), 1 to 2 μ g of the extracted total RNA was reverse transcribed into cDNA using a random primer and SuperScript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. In the molecular analyses of this study, standard PCR was performed using 0.5 μ l of template DNA or cDNA, 2.5 U of *Taq* DNA polymerase (AmpliTaQ Gold; Perkin Elmer, Norwalk, Connecticut), 1.5 mM $MgCl_2$, 10 $\times$ PCR buffer (Perkin Elmer), 200 mM deoxynucleotide triphosphates, and 0.2 mM each of forward and reverse primers. A PC-801 thermal cycler (Astec Co., Shimen, Japan) was used for the PCR amplification. PCR products were cloned into a pCR2.1 vector using a TA cloning kit (Invitrogen, San Diego, California) and sequenced by an automated sequencing system (ALFexpress DNA sequencer; Pharmacia Biotech, Uppsala, Sweden).

Genomic Characterization of *h-warts/LATS1*

Based on the reported sequence of *h-warts/LATS1* mRNA (GenBank Accession No. NM_004690), 18 sets of primers (Table 4) were designed to amplify segments ranging from 155 to 245 bp in length and spanning most (positions 380 to 3607) of the mRNA sequence including a 5'-uncoding region (positions 380 to 548) and the putative serine/threonine kinase domain (positions 2661 to 3575). Using the genomic DNA and cDNA of PBMC, multiple PCR was performed to find junctions between different exons under the conditions of 94° C for 9 minutes followed by

40 cycles at 94° C for 30 seconds, 56° C for 30 seconds, and 72° C for 45 seconds, and final extension at 72° C for 10 minutes. In the segments with no detectable bands by the standard PCR of genomic DNA, long-range PCR using 5 U of TaKaRa LA *Taq* polymerase (Takara Shuzo Co., Ohtsu, Japan) was performed to more effectively amplify this region of the genomic DNA. The exact positions of the junctions between exons and sequences at the intron-exon boundaries were determined by DNA sequencing of the PCR products by referring to unpublished human DNA sequences of chromosome 6, which had been submitted directly to GenBank (Accession Nos. AL583963 and AL358852).

Expression Analysis of *h-warts/LATS1*

RT-PCR was performed to amplify both *h-warts/LATS1* and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcripts in the same microtubes. Two sets of primers (*LATS1*-13 and 16) (Table 4) flanking the regions including the junctions between different exons were selected to differentiate PCR products from amplified genomic DNA contamination. The PCR conditions were mostly the same as described above. In repeated preliminary RT-PCR, the number of cycles for the PCR reaction was chosen to remain within the exponential phase of the reaction and to avoid exceeding the limit of comparable intensities of the staining with ethidium bromide after gel electrophoresis. The tumors with no or unequivocally less intense bands compared with reference bands of GAPDH were regarded as down-regulated *h-warts/LATS1* expressions.

Microsatellite Marker Analysis of 6q23-25.1

The following five dinucleotide (CA) repeat markers, listed in order from centromere to telomere, were analyzed by PCR: D6S1587, D6S471, D6S978, D6S1564, and D6S1687. The sequences of these satellite markers are distributed within a region of approximately 16 Mbp in length at chromosome 6q23-25.1, to which the *h-warts/LATS1* is localized (Nishiyama et al, 1999). The sequences of the PCR primers for these markers are available from the Genome Database (<http://www.gdb.org>). Forty cycles for the PCR reaction were used in this assay. The PCR products were loaded onto polyacrylamide gels and then stained with silver (Bio-Rad, Hercules, California).

Mutation Analysis of *h-warts/LATS1*

To screen gene mutations in most of the coding sequence of the *h-warts/LATS1*, SSCP was analyzed after the PCR using genomic DNA or cDNA with the above 18 sets of primers. The PCR products were heat denatured and loaded onto polyacrylamide gels at 15° C for 90 minutes with a peltier temperature-regulated electrophoresis unit (Genephor; Pharmacia Biotech). The gels were visualized with silver, and aberrant bands were removed from the gels. Gene mutations were further confirmed by DNA sequencing

of the extracted PCR products from the aberrant bands. DNA with the same alteration in different clones was considered to be mutated.

Southern Blot Analysis for Gene Rearrangement

Ten micrograms of high molecular weight DNA of tumor tissues were digested with 100 U of restriction enzymes (*EcoRI*, *HindIII*, *HaeIII*) and loaded onto 1% agarose gels. After treatment with 0.25 M HCl, the digested DNA was transferred to positively charged nylon membranes (Amersham Pharmacia Biotech, Tokyo, Japan) and hybridized with an alkaline phosphatase-labeled probe for *h-warts/LATS1* cDNA flanking the serine/threonine kinase domain (980 bp) that was generated by PCR. Detection of hybridized signals was made by an enhanced chemiluminescence detection system (AlkPhos Direct; Amersham Pharmacia Biotech).

Methylation Analysis of CpG Islands in *h-warts/LATS1* Promoter

To identify a putative promoter sequence in the 5' region of the *h-warts/LATS1*, a genomic sequence (approximately 2 kb in length) including a part of exon 1 was analyzed by a computer-based online program (PROSCAN Ver 1.7, <http://bimas.dcrn.nih.gov/molbio>) (Prestridge, 1995). The methylation status of CpG dinucleotides (islands) in the 5' putative promoter region of the *h-warts/LATS1* was assessed by methylation-specific PCR using primer sets for methylated and unmethylated sequences of genomic DNA modified with bisulfite (CpGenome DNA Modification Kit; Invitrogen, Purchase, New York) (Herman et al, 1996). Sequences of the primers used are shown in Table 4. The modified DNA was amplified by the PCR under the conditions of 40 cycles of 94° C for 15 seconds, 56° C (methylated) or 53° C (unmethylated) for 15 seconds, and 72° C for 30 seconds. Sequences of the PCR products were confirmed by DNA sequencing.

Acknowledgements

The authors thank Drs. K. Yokoyama (National Kokura Hospital) and M. Ushijima (Kitakyushu Municipal Hospital) for providing tumor tissues.

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