LATS2 Is a Tumor Suppressor Gene of Malignant Mesothelioma

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Abstract

Malignant mesothelioma (MM) is an aggressive neoplasm associated with asbestos exposure. We carried out genome-wide array-based comparative genomic hybridization analysis with 14 MM cell lines. Three cell lines showed overlapping homozygous deletion at chromosome 13q12, which harbored the LATS2 (large tumor suppressor homolog 2) gene. With 6 other MM cell lines and 25 MM tumors, we found 10 inactivating homozygous deletions or mutations of LATS2 among 45 MM tumors. LATS2 encodes a serine/threonine kinase, a component of the Hippo tumor-suppressive signaling pathway, and we transduced LATS2 in MM cells with its mutation. Transduction of LATS2 inactivated oncprotein YAP, a transcriptional coactivator, via phosphorylation, and inhibited MM cell growth. We also analyzed LATS2 immunohistochemically and found that 13 of 45 MM tumors showed overlapping homozygous deletion at chromosome 13q12, which harbored the NF2 (neurofibromatosis type 2) tumor suppressor genes. LATS2 and recent comprehensive analyses have identified other candidate cancer-associated genes responsible for MM development, progression, and poor outcome (7–10).

The NF2 gene, which encodes Merlin, is inactivated in 40% to 50% of MM tumors (11–13). Transduction of NF2 into MM cells was shown to inhibit cell proliferation and invasiveness of MM cells (14, 15). Mouse models with nf2 allele loss have been shown to enhance mesothelioma development after asbestos exposure (16, 17). Mesothelioma also develops with a high incidence in NF2/Arf conditional knockout mice (18). However, it remains unclear whether MM tumors without an NF2 mutation express functional Merlin or the tumor-suppressive activity of Merlin is inactivated by other mechanisms. In this regard, possible involvement of the increased expression of CPI-17, a regulator of Merlin, or the upregulation of microRNA that might target NF2 has been suggested (19, 20).

The mammalian Hippo cascade, which was initially identified via genetic studies in Drosophila, is one of the possible downstream signaling cascades of Merlin and Expanded (21–25). This pathway controls tissue growth by inhibiting cell proliferation and by promoting apoptosis. The components of this pathway include SAV1 (also called WW45), MST (Drosophila Hippo), and LATS family members, which ultimately phosphorylate and inactivate the YAP transcription coactivator. YAP, a candidate oncogene, was shown to be amplified in human cancers (26, 27). We previously reported amplification of the chromosomal 11q22 region including YAP in a subset of MM specimens and a positive role of YAP in MM cell proliferation (28).

In this study, we carried out array-based comparative genomic hybridization (CGH) and sequencing analyses and found that 10 of 45 MM had an inactivating homozygous deletion or mutation of LATS2. Furthermore, we showed that transduction of LATS2 induced phosphorylation of YAP and inhibited MM cell growth. Our results suggest that the Merlin-Hippo pathway is frequently inactivated in MM cells and that LATS2 is a TSG of MM.
Materials and Methods

Cell lines and primary specimens of malignant mesothelioma

Fourteen Japanese MPM (malignant pleural mesothelioma) cell lines, including ACC-MESO-1, -4, Y-MESO-8D, -9, -12, -14, -21, -22, -25, 26B, -27, -28, -29, and -30, were established in our laboratory as reported previously and described elsewhere, and the cells at 10 to 15 passages were used for assays (29, 30). Four MPM cell lines, including NCI-H28, NCI-H2052, NCI-H2373, and MSTO-211H, and one immortalized mesothelial cell line, Met-5A, were purchased from the American Type Culture Collection (ATCC) and cells at 3 to 5 passages were used after receiving from ATCC. NCI-H290 and NCI-H2452 were the kind gifts of Dr. Adi F. Gazdar. All MPM cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1× antibiotic-antimycotic (Invitrogen) at 37°C in a humidified incubator with 5% CO2. MeT-5A was cultured according to ATCC instructions. MM tissue samples from patients treated at Aichi Cancer Center Hospital, Nagoya University Hospital, Japanese Red Cross Nagoya First Hospital, Toyota Kosei Hospital, and Kasugai City Hospital were obtained according to the Institutional Review Board – Toyota Kosei Hospital, and Kasugai City Hospital were obtained according to the Institutional Review Board–approved protocol for each and the written informed consent from each patient. The human mesothelioma tissue array with 19 MM samples was also used (US Biomax Inc.).

Preparation of DNA and RNA

Genomic DNA was extracted using a standard phenol-chloroform method (31). Total RNA was prepared using RNeasy Plus RNA extraction kit (Qiagen K.K.) according to chloroform method (31). Total RNA was prepared using 19 MM samples was also used (US Biomax Inc.). from each patient. The human mesothelioma tissue array with obtained according to the Institutional Review Board – Toyota Kosei Hospital, and Kasugai City Hospital were

Oligonucleotide array CGH analysis

All microarrays were used as Agilent 244K whole human genome microarrays, with an average distance of 6.4 kb between each probe (array G411B sourced from the NCBI genome Build 36; Agilent Technologies). Comparison genomic DNA was obtained commercially (Promega) and matched for sex. The methods for labeling, hybridization, and scanning using a G2505B Agilent DNA microarray scanner (Agilent Technologies) were conducted according to the manufacturer’s protocol. Random-primed, first-strand cDNA was synthesized from 3 μg of total RNA, using Superscript II, according to the manufacturer’s instructions (Invitrogen).

Plasmid and lentiviral vector

The cDNA fragments of wild-type or mutant LATS2 were amplified by PCR, using PrimeSTAR Max DNA polymerase (Takara Bio), and introduced into the pFLAG-CMV2 expression vector (Sigma) with an infusion cloning system (Clontech), thereby fusing these cDNAs with the FLAG sequence. The sequences of all constructs were confirmed. To generate LATS2-expressing lentiviral vector, cDNA coding for the human LATS2 tagged with FLAG was amplified by PCR and cloned in the pLil3.7 lentiviral vector. NF2 expression vectors were described previously (28). RNA interference vectors to generate lentiviruses that transcribe short hairpin (sh)-RNA were prepared as described previously (32). sh-LATS2-RNA interference vector (sh-LATS2) contained a target sequence of the hairpin loop of LATS2 (5’-GGACCTCCTGCTGATTTA-3’). A control shRNA vector for luciferase (Sh-Luc), which contained a target sequence for luciferase (5’-CGTACGCGGAATTCTC-3’), was described previously (32).

Cell proliferation assays

A total of 1.0 × 10⁶ and 2.0 × 10⁵ cells were seeded onto flat-bottomed 24- and 12-well plates, respectively. Cells were transduced with lentiviral vectors at the multiplicity of infection of 5,
incubated for an additional 6 hours, and then changed with RPMI 1640 medium with 5% or 1% FCS. Cells were incubated for an additional 90 hours. Each viral transduction was applied to triplicate wells for cells. Cell numbers were counted under a light microscope every 24 hours. Calorimetric assays were carried out with the addition of 100 μL of TetraColor One (Seikagaku), containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonylphenyl)-2H-tetrazolium, monosodium salt, and 1-methoxy-5-methylphenazinium methylsulfate as electron carrier, in each well and then incubating at 37°C for 1 hour. Absorbance was read at 450 nm with a multiplate reader.

Growth inhibition was expressed as a mean ratio of absorbance reading from treated versus untreated cells.

**Immunofluorescent microscopic analysis**

Cells were fixed in 4% paraformaldehyde for 20 minutes on ice, followed by permeabilization with PBS containing 0.3% Triton X-100 for 3 minutes. Slides were blocked in PBS containing 3% goat normal serum for 20 minutes at room temperature (RT). Samples were stained with primary antibodies (mouse anti-YAP antibody, 1 μg/mL), containing 3% goat normal serum for 20 minutes at room temperature (RT). Samples were stained with primary antibodies (mouse anti-YAP antibody, 1 μg/mL) and/or sequencing analyses. We found that 2 tumors had significant loss (their log2 ratio values were −2.5 and −1.6, respectively), which indicated homozygous deletion, and 1 had a somatic mutation at the intron 6–exon 7 boundary (c2676-3C>A), which caused an aberrant transcript (data not shown). Thus, we considered that 3 (12%) of 25 had a genetic alteration of LATS2 to inactivate. Furthermore, 5 tumors had a loss of LATS2 with array CGH analysis, which also suggested a possibility of LATS2 inactivation in the other allele, though we could not determine any of them as a homozygous deletion because of the contamination of normal cell populations in the tumors (data not shown).

**SAV1 is homozygously deleted in one MM cell line**

SAV1 was reported to be deleted in renal cancer cell lines (33). Array CGH analysis detected a homozygous deletion at chromosome 14q22 in Y-MESO-28, and PCR analysis confirmed complete deletion of exons 1 and 2 of SAV1 (data not shown). However, sequencing analysis of SAV1 did not detect mutations in other cell lines.

**Comparison of inactivation status of Merlin, LATS2, and SAV1 in MM cell lines**

We analyzed the mutation and expression status of NF2 and compared the inactivating status among Merlin, SAV1, and LATS2 (Fig. 3 and Table 1). Among 20 MM cell lines, 15 (75%) showed inactivation for 1 of the 3 genes, with 3 (Y-MESO-14, Y-MESO-26B, and NCI-H2052) having inactivation of 2 genes. Because 2 MM cell lines (Y-MESO-28 and Y-MESO-8D) without NF2 mutation did not express Merlin, 16 (80%) cell lines were considered to be inactivated in the Merlin-Hippo pathway.

**Dysregulation of Merlin-Hippo signaling in MM cells**

When cells grow and become confluent, cell surface signals transmit via the Hippo signaling pathway and activated LATS phosphorylates YAP, a transcriptional coactivator (25). The phosphorylated YAP (inactivated form as a transcriptional coactivator) is translocated to the cytoplasm, which results in cell contact inhibition (25). To determine whether the
inactivated Merlin-Hippo signaling pathway in MM cells could
be reactivated, we transduced NF2 or LATS2 expression con-
structs. NF2 transduction in NCI-H290 cells with NF2 deletion
induced YAP phosphorylation (Fig. 4A). In contrast, NF2
transduction in Y-MESO-14 that has both NF2 and LATS2
mutations did not induce YAP phosphorylation, suggesting
that LATS2 was necessary to transmit a growth inhibitory
signal from Merlin to YAP (Fig. 4A). Furthermore, we carried
out a knockdown experiment with a shRNA expression vector
of LATS2 and tested whether YAP phosphorylation in NCI-
H290 cells could be blocked when wild-type NF2 was trans-
duced (Supplementary Fig. 1). We found that LATS2 knock-
down significantly blocked phosphorylation of YAP with NF2
transduction, suggesting that LATS2 is a crucial mediator of
Merlin-Hippo signaling and that LATS1 might have only a
minor role, if any, in the phosphorylation of YAP in MM cells.

We also confirmed that transduction of the wild-type LATS2
induced phosphorylation of YAP in MM cells with LATS2
deletion (Fig. 4B). However, the mutant LATS2 (LATS2-delEx6)
construct, which deleted exon 6, did not induce YAP phos-
phorylation, indicating that the mutant detected in the
Y-MESO-30 cell line was functionally inactive.

**Figure 1.** Deletion of chromosome 13q12.11 region in MM cells. A, 3 cell lines showed a high-level loss with multiple probes, indicating a homozygous
deletion. Y-MESO-14 and Y-MESO-27 had a deletion of exon 3–8 and Y-MESO-21 had a deletion of exon 1–2 of LATS2. B, FISH analysis detected
loss of the LATS2 region (RP11-23H13 probe, green signal) but not 13q telomeric region (RP11-1148G1, red signal) in Y-MESO-14 cell line.
LATS2 acts as a growth suppressor in MM cells

To determine whether the regulation of YAP by cell density was abrogated in MM cells, we then analyzed the change of cellular localization of YAP with immunocytochemistry. All 3 MM cell lines with LATS2 mutation showed nuclear accumulation of YAP even at high cell density, whereas MeT-5A, immortalized, nonmalignant mesothelial cells, showed nuclear accumulation at low cell density but presented cytoplasmic translocation at high cell density (Fig. 4C). As expected, Western blot analysis showed that the subcellular localization change of YAP in MeT-5A according to high cell density was accompanied with a significant increase in phosphorylated YAP (Fig. 4D). In contrast, the basal levels of YAP phosphorylation in MM cells were low and there was only a modest increase in YAP phosphorylation levels (Fig. 4D).

To determine whether LATS2 has a growth-suppressive activity, we transduced the both wild-type and mutant LATS2 constructs in MM cells. Transduction of the wild-type, but not the mutant, LATS2 inhibited cell density of MM cells with LATS2 mutation, indicating that LATS2 acts as a growth suppressor in MM cells in vitro (Fig. 5A and Supplementary Fig. 2A). We also carried out anchorage-independent colony formation and Transwell migration assays and found that LATS2 transduction in MM cell lines with LATS2 mutation inhibited both activities in these cell lines (Fig. 5B and C and Supplementary Fig. 2B).

Finally, we carried out a knockdown experiment of LATS2 in MeT-5A cells to determine whether silencing of LATS2 promotes cell growth of nonmalignant mesothelial cells. LATS2 knockdown significantly decreased YAP phosphorylation status and slightly increased YAP protein level (Supplementary Fig. 3A). We found that silencing of LATS2 increased the cell proliferation of MeT-5A cells under low serum condition (Supplementary Fig. 3B and C), but the colony formation in soft agar was not enhanced (data not shown). These results suggested that LATS2 was involved in the regulation of cell proliferation of nonmalignant mesothelial cells as well as MM cells.

Immunohistochemical analysis of LATS2 and YAP in primary MMs

To determine whether immunostaining can be useful to detect LATS2 inactivation status in MMs, we carried out immunohistochemical analysis with anti-LATS2 antibody.
(Fig. 6). The 2 MM tumors with homozygous deletion detected by array CGH analysis showed negative or only weak staining of LATS2, suggesting that weak (1+) signals might be caused by nonspecific staining. Among 45 cases, 2 showed negative and 11 showed weak staining of LATS2, whereas 32 had moderate or strong staining of LATS2, suggesting that 13 (29%) of 45 primary MMs had down-regulation of LATS2.

We also carried out immunohistochemical analysis to determine how frequently primary MMs show YAP activation (Fig. 6). Among 45 cases, 36 showed positive staining for YAP and 33 tumors showed stronger or equal staining of YAP in the nucleus.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NF2†</th>
<th>LATS2</th>
<th>SAV1</th>
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<tbody>
<tr>
<td>NCI-H290</td>
<td>HD</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NCI-H2373</td>
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<td>+</td>
</tr>
<tr>
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<tr>
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<td>+</td>
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<tr>
<td>Y-MESO-29</td>
<td>+</td>
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</tbody>
</table>

Abbreviation: HD, homozygous deletion.
†Mutation status of NF2 in 9 cell lines (ACC-MESO-1, -4, Y-MESO-8D, NCI-H28, -H290, -H2052, -H2373, -H2452, and MSTO-211H) was previously described (11, 29, 30), and the one in the other 11 cell lines was analyzed in the present study.
‡Silenced expression.

Table 1. Inactivation of NF2, LATS2, and SAV1 in MM cell lines

Figure 3. Western blot analysis of Merlin, SAV1, and LATS2. LATS2 protein was undetectable in 5 cell lines harboring a homozygous deletion or a premature termination, although faint nonspecific bands with slower mobility than the wild-type LATS2 were observed (indicated by asterisks). Aberrant short LATS2 proteins were detected in MSTO-211H and Y-MESO-30 (indicated by "S"). Expression of β-actin was used as the control.
than in cytoplasm, indicating constitutive YAP activation in more than 70% of primary MMs (Supplementary Table 1).

We finally studied the relation of LATS2 expression with YAP activation status (Supplementary Table 2). Among 13 tumors with negative or weak LATS2 expression, 11 had stronger or equal staining of YAP in the nucleus, suggesting that negative or weak LATS2 may be an indicator of YAP activation.

Discussion

In the present study, we showed that LATS2 was genetically inactivated in 7 of 20 MM cell lines and 3 of 25 primary tumors. We found that MM cells with LATS2 mutation showed constitutive activation of YAP with underphosphorylation, regardless of high cell density, whereas YAP in nonmalignant mesothelial cells was phosphorylated and inactivated at high cell density. We further showed that transduction of LATS2 into MM cells with LATS2 mutation induced phosphorylation of YAP, which resulted in suppression of MM cell proliferation and anchorage-independent growth. Our study indicates that LATS2 may be a TSG in MM cells.

Merlin is a membrane–cytoskeleton-associated protein with an FERM (Four-point-one, Ezrin, Radixin, and Moesin) domain, and is known to interact with 34 proteins, including CD44, ERM (ezrin radixin moesin) proteins, and PAK1 (p21-activated kinase 1; ref. 34). The prevalence of NF2 mutations in sporadic tumors, especially schwannomas, meningiomas, and MMs, suggest that Merlin has a relatively broad tumor suppressor function (35, 36). Merlin and the ERM proteins have been suggested to function to both stabilize the membrane–cytoskeleton interface and to organize the distribution of, and signaling by, membrane receptors (37). Merlin exerts inhibitory effects on multiple mitogenic signaling pathways such as RAS-ERK, PI3K-AKT, and mTOR. A recent study also indicated that a closed, growth-inhibitory form of Merlin accumulates in...
the nucleus, binds to the E3 ubiquitin ligase CRL4\textsuperscript{DCAP1}, and suppresses its activity (38). In addition to these pathways, the Hippo pathway is thought to be one of the downstream signaling pathways of Merlin, which is regulated via signaling with cell–cell adhesion, cell–cellular matrix, or other cell membrane receptors with binding of extracellular ligands (21). Dysregulation of the Hippo pathway causes an increase in organ size both in Drosophila and in mammalians (22). The recent findings indicated that a variety of human malignancies, such as homozygous deletion of SAV1 in renal cancer cell lines (33) and hypermethylation of MST in soft tissue sarcoma (39), have alterations in each component. Overexpression of YAP was reported in hepatocellular carcinomas (40) and colonic and lung adenocarcinomas (41). In our previous study, we also reported YAP amplification in a subset of MM cells (28). Regarding LATS2, downregulation of LATS2 was reported to be correlated with poor prognosis of leukemia (42) and missense mutation was also reported in lung cancer (43). However, null status of LATS2 such as by homozygous deletion or nonsense mutation was not reported in these malignancies.

Why only 40% to 50% of MMs have NF2 mutation and the rest do not has been a long-standing enigma. The representative hypotheses for them are that MM tumors without an NF2 mutation may not express functional Merlin, or that the other molecules of Merlin-associated signaling cascades are altered. Supporting the former hypothesis, one study indicated that Merlin was phosphorylated on Ser518 if present and functionally inactivated in MM cells with elevated CPI-17, a cellular inhibitor of myosin phosphatase MYPT1-PP1 \textsuperscript{d} (19), and the other showed that upregulation of microRNAs, such as hsa-miR-885-3p, might target NF2 (20). Meanwhile, our data may explain the latter hypothesis, indicating that one of the major downstream pathways of Merlin can be inactivated with an LATS2 or SAV1 mutation. We think of the idea that LATS2 is a TSG of MM is supported by the evidence that the mutation frequency of LATS was in 22% (10 of 45 MMs including 20 cell lines and 25 primary tumors), and that the
characteristics of inactivation mechanisms were direct and robust by a homozygous deletion, small deletion, or nonsense mutation. To our knowledge, our study is the first to show such frequent genetic inactivation of the \textit{LATS2} gene in any human malignancy.

Interestingly, several MM cell lines show inactivation of both \textit{NF2} and \textit{LATS2}. This is in contrast to our hypothesis that the functional link between the Merlin and Hippo pathway was direct and that inactivation of each gene might be sufficient for the inactivation of the Merlin-Hippo cascade in MM cells. Indeed, although underphosphorylated, active YAP in the Y-MESO-14 cell line that had both gene inactivations was not downregulated by phosphorylation when the wild-type \textit{NF2} gene was transduced (Fig. 4A), the cell growth of this cell line was suppressed (data not shown). In this regard, Merlin has been clearly shown to inhibit mTORC1 pathway in MM cells, with Merlin-negative MM cells displaying unregulated mTORC1 signaling and also an enhanced growth-inhibitory effect of rapamycin, an mTORC1 inhibitor (44, 45). Thus, growth suppression in Y-MESO-14 cells was likely to be induced via such signaling cascades but not via the Hippo signaling. This suggests another possibility that the main roles of Merlin for tumor-suppressive activity in MM cells reside outside the Hippo pathway regulation, that the functional link between the Merlin and Hippo pathway in MM cells is not as direct as expected, and that the simultaneous inactivation of Merlin and Hippo pathway inactivation may merely enhance MM cell growth. Furthermore, some MM cell lines with \textit{NF2} mutation but not \textit{LATS2} mutation also showed significant phosphorylation levels of YAP, especially at

![Figure 6. Immunohistochemical analyses of LATS2 and YAP in 45 primary MMs. Representative cases of LATS2 and YAP staining are presented. Case KD1074 with LATS2 homozygous deletion and case KD1067 with LATS2 deletion showed negative (0) and weak (1+) LATS2 staining, respectively. Both cases showed stronger staining of YAP in the nucleus. Meanwhile, cases KD1072 and KD1077 without LATS2 deletion showed moderate (2+) and strong (3+) LATS2 staining, respectively. N, nucleus; C, cytoplasm.](image-url)
confluence, suggesting that the Hippo pathway in MM cells can also be activated in a Merlin-independent manner (data not shown). Thus, more detailed mechanisms of the tumor-suppressive pathways in which Merlin and LATS2 are involved must be elucidated in future studies.

The mutation frequency of LATS2 in MM cell lines was higher than the one in primary tumors. Because most primary MM tumors contain abundant normal cells, the sensitivities of detection of allelic loss or point mutation in primary tumors are expected to be lower than those in cell lines. In this regard, among 5 primary tumors that we evaluated to have at least an allelic loss, there might be cases of homozygous deletion. Indeed, 3 of 4 tumors with an allelic loss showed the weak intensity of LATS2 staining comparable with a tumor with homozygous deletion. Indeed, 3 of 4 tumors with an evaluated to have at least an allelic loss, there might be cases of homozygous deletion. Indeed, 3 of 4 tumors with an allelic loss showed the weak intensity of LATS2 staining comparable with a tumor with homozygous deletion, which suggested that both alleles of LATS2 might be inactivated in these tumors. Thus, although the mutation frequency of 12% for LATS2 in primary tumors was low compared with that of 35% in cell lines, we thought that one reason for this was the lower sensitivity of mutation detection. However, we could not exclude another possibility, namely, that the difference in mutation frequencies was due to selection pressure during establishment of cell lines.

In conclusion, we showed that the tumor-suppressive Hippo signaling pathway can be inactivated by LATS2 mutation in MM cells and that LATS2 may play a critical role in regulating cell proliferation and/or survival in MM cells and nonmalignant mesothelial cells. Our result may also suggest that inhibition of activated YAP or transcription factors may serve to develop a more effective target therapy for patients with MM in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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