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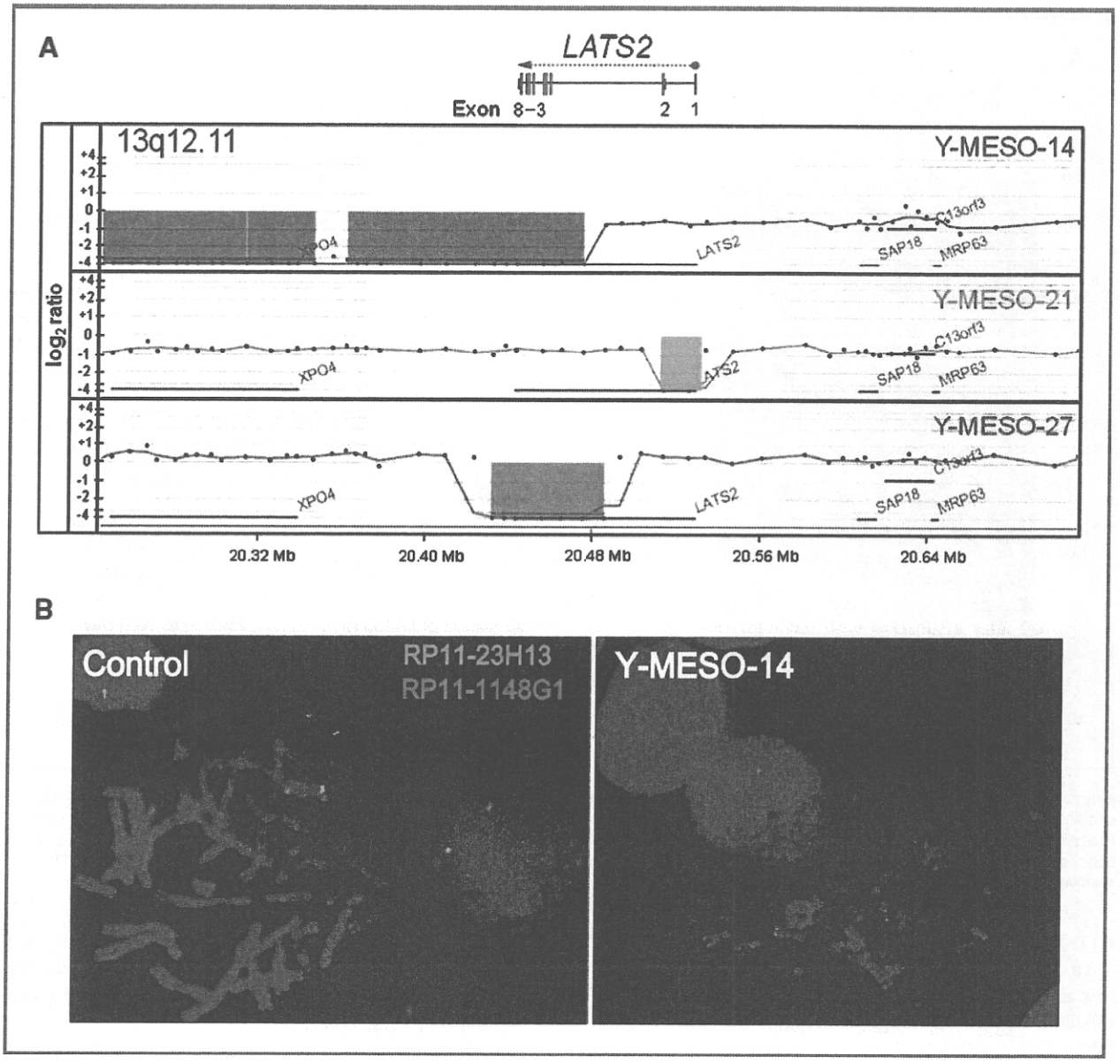

Figure 1. Deletion of chromosome 13q12.11 region in MM cells. A, 3 cell lines showed a high-level loss with multiple probes, indicating a homozygous deletion. Y-MESO-14 and Y-MESO-27 had a deletion of exon 3–8 and Y-MESO-21 had a deletion of exon 1–2 of *LATS2*. B, FISH analysis detected loss of the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

inactivated Merlin-Hippo signaling pathway in MM cells could be reactivated, we transduced *NF2* or *LATS2* expression constructs. *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 transduced (Supplementary Fig. 1). We found that *LATS2* knockdown 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 phosphorylation, indicating that the mutant detected in the Y-MESO-30 cell line was functionally inactive.

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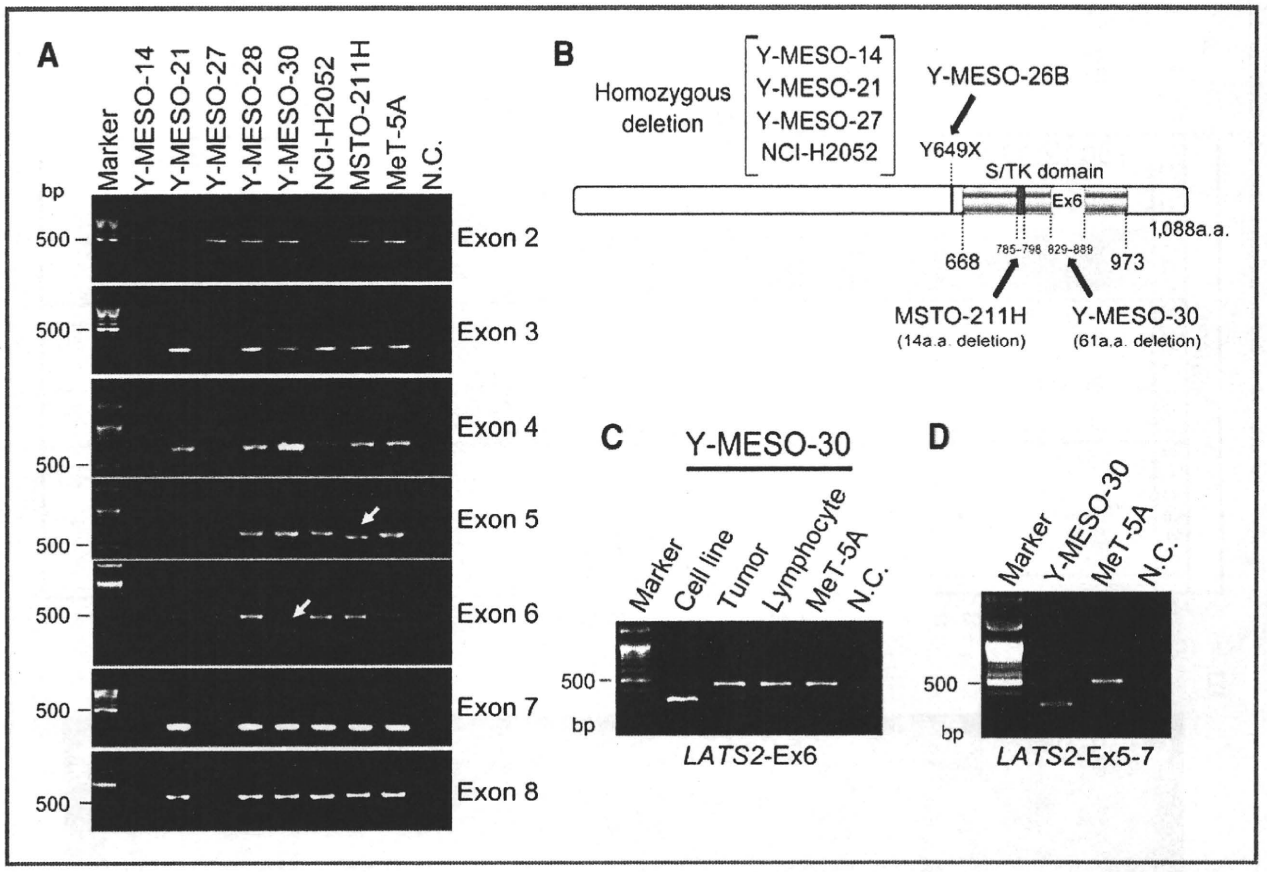

Figure 2. Genetic inactivations of *LATS2* in MM cells. A, genomic PCR analysis detected homozygous deletion in Y-MESO-14, -21, -27, and NCI-H2052, and shorter fragments in Y-MESO-30 and MSTO-211H cell lines (arrows). B, a diagram of 7 *LATS2* inactivating mutations in MM cell lines. C, genomic PCR analysis detected a shorter product by 125 bp in the Y-MESO-30 cell line and its corresponding tumor but not in lymphocytes. This deletion disrupted the donor site of the exon 6 boundary (14 bp in exon 6 and 111 bp in intron 6). D, RT-PCR analysis covering exon 5–7 detected a shorter fragment skipping exon 6, which caused the deletion of 61 amino acids coded by 183 nucleotides of exon 6.

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 proliferation 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

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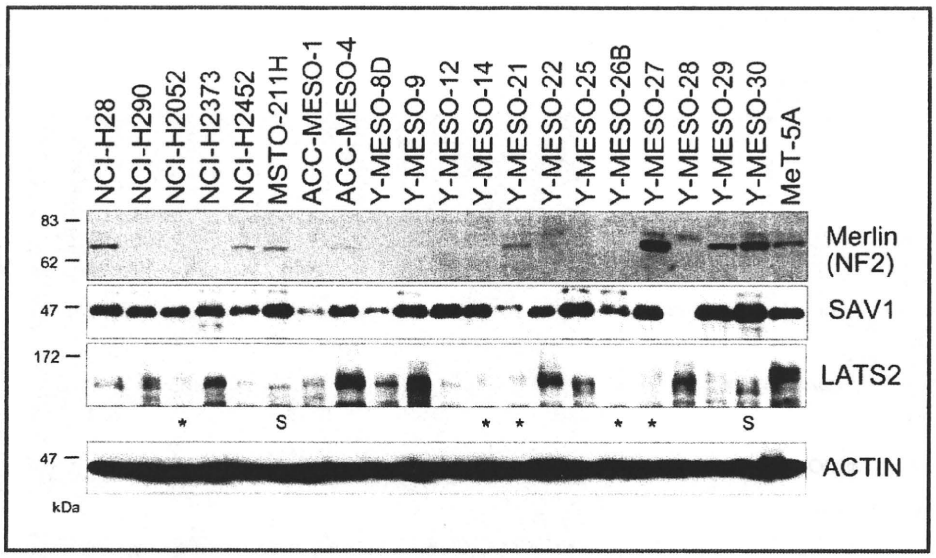

Figure 3. Western blot analysis of Merlin, SAV1, and LATS2. LATS2 protein was undetectable in 5 cell lines harboring a homozygous deletion or a premature termination, although faint nonspecific bands with slower mobility than the wild-type LATS2 were observed (indicated by asterisks). Aberrant short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

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

Cell line	*NF2*[a]	*LATS2*	*SAV1*
NCI-H290	HD	+	+
NCI-H2373	HD	+	+
ACC-MESO-1	Q389X	+	+
Y-MESO-9	NM_000268:c.527_528del2	+	+
Y-MESO-12	HD	+	+
Y-MESO-22	HD	+	+
Y-MESO-25	NM_000268:c.532_571del40	+	+
Y-MESO-14	Q196X	HD	+
Y-MESO-26B	HD	Y649X	+
NCI-H2052	R341X	HD	+
Y-MESO-21	+	HD	+
Y-MESO-27	+	HD	+
Y-MESO-30	+	NM_014572:c.2652_2665+111del125	+
MSTO-211H	+	NM_014572:c.2355_2396del42	+
Y-MESO-28	–[b]	+	HD
Y-MESO-8D	–[b]	+	+
NCI-H28	+	+	+
NCI-H2452	+	+	+
ACC-MESO-4	+	+	+
Y-MESO-29	+	+	+

Abbreviation: HD, homozygous deletion.

[a]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.

[b]Silenced expression.

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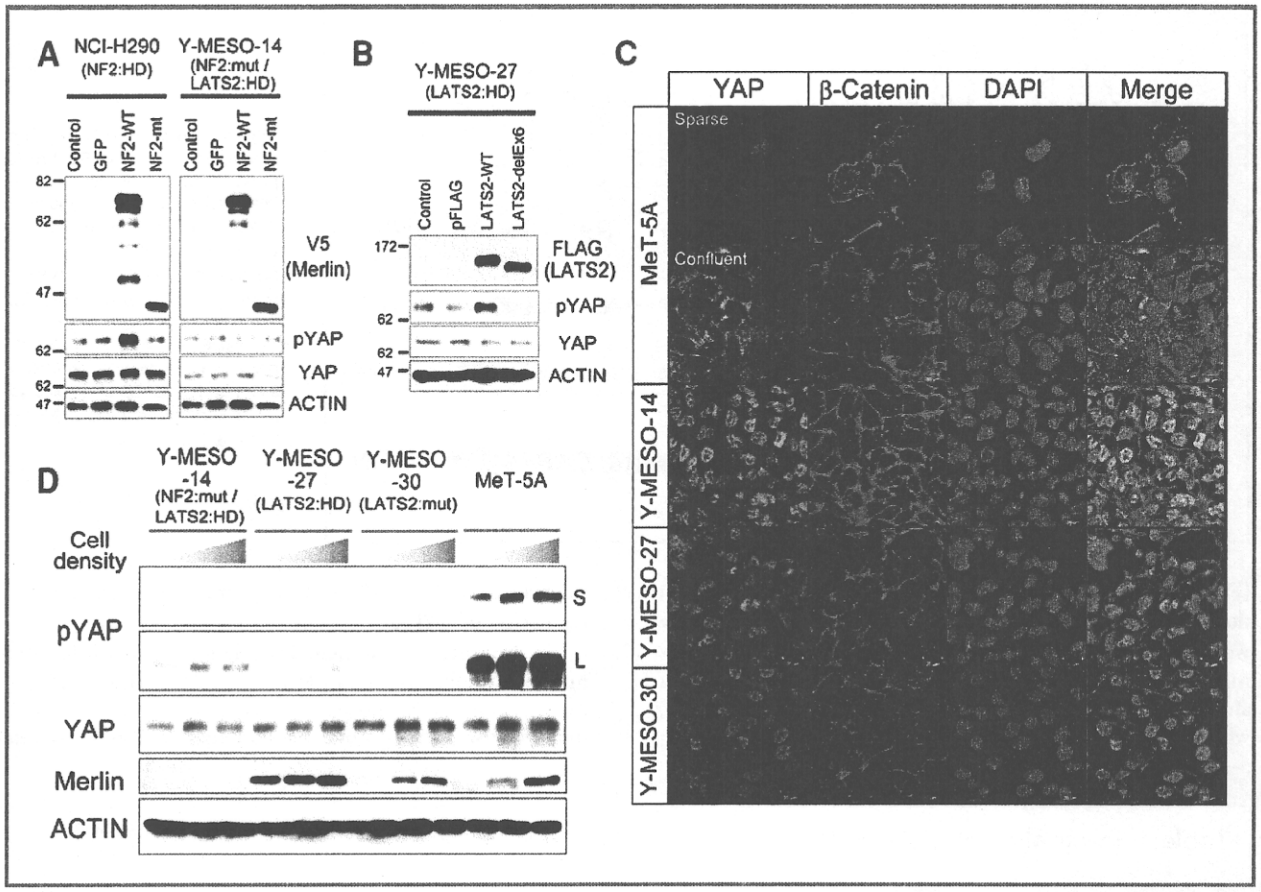

Figure 4. Inactivation of the Merlin-Hippo signaling pathway in MM cells. A, wild-type *NF2* (NF2-WT), but not truncated *NF2* (NF2-mt), induced phosphorylation of YAP in NCI-H290 cells with *NF2* homozygous deletion (HD). In contrast, wild-type *NF2* did not induce phosphorylation of YAP in Y-MESO-14 cells with both *NF2* mutation and *LATS2* HD. B, wild-type *LATS2*, but not mutant *LATS2* with exon 6-deletion (LATS2-delEx6), induced phosphorylation of YAP in Y-MESO-27 cells with *LATS2* HD, indicating that this aberrant form detected in Y-MESO-30 was kinase dead. C, MeT-5A showed YAP translocation in the cytoplasm at high cell density, whereas YAP in MM cells remained in the nucleus at high cell density. D, YAP phosphorylation according to the increasing cell density was induced in MeT-5A, whereas the basal levels of phospho-YAP (pYAP) were low at low cell density in the 3 MM cell lines. Only a modest increase in YAP phosphorylation was observed according to the higher cell density. S, short exposure; L, long exposure.

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

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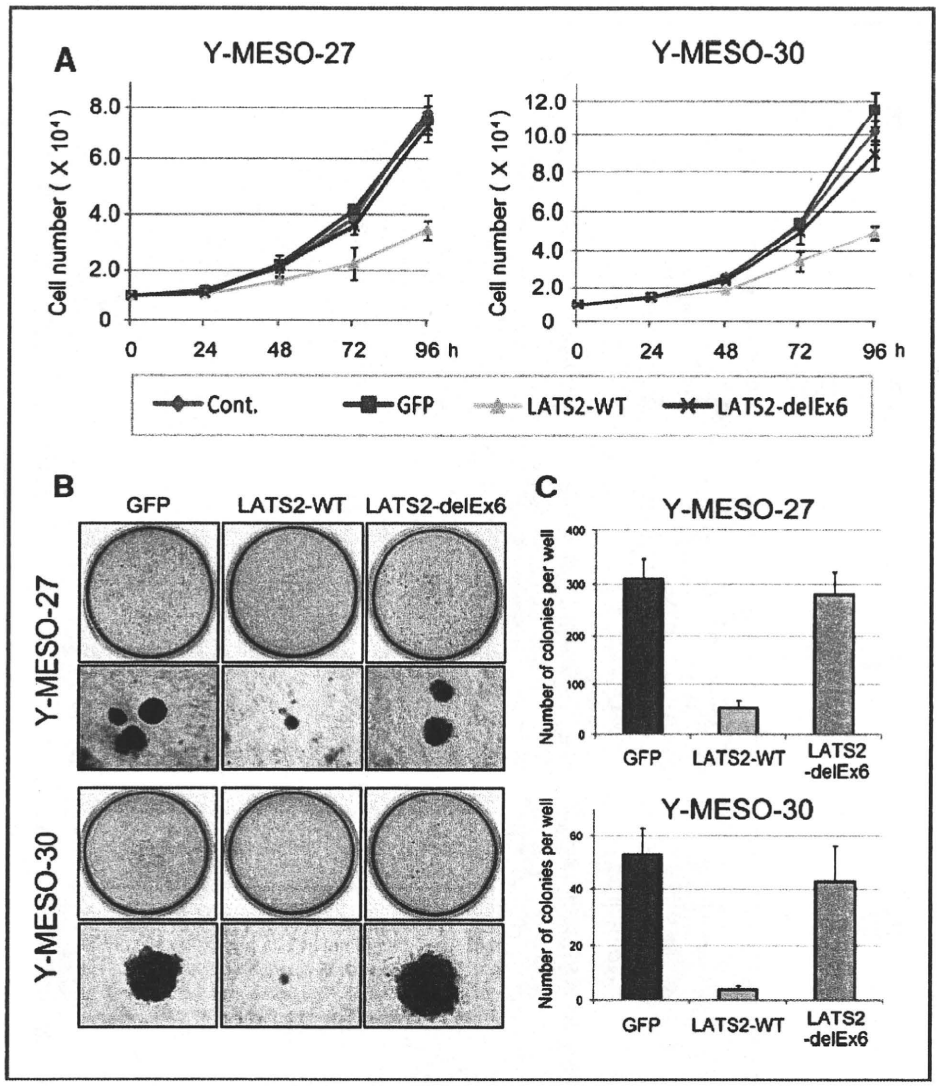

Figure 5. *LATS2* acts as a TSG in MM cells. A, inhibition of cell proliferation of Y-MESO-27 and Y-MESO-30 cells by re-expression of *LATS2*. Cells were transduced with wild-type *LATS2* (LATS2-WT), mutant *LATS2* (LATS2-delEx6), or GFP viruses (GFP), or uninfected (Cont.). Cell numbers were counted every 24 hours. Points, mean; bars, SD. B, re-expression of LATS2-WT in MM cell lines suppressed anchorage-independent colony formation. Representative results of the Y-MESO-27 and Y-MESO-30 cell lines are shown (top) with higher magnifications of their representative colonies (bottom). C, the numbers of colonies in the triplicate experiments are presented. Columns, mean; bars, SD.

the nucleus, binds to the E3 ubiquitin ligase $CRL4^{DCAF1}$, 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-PP1δ (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

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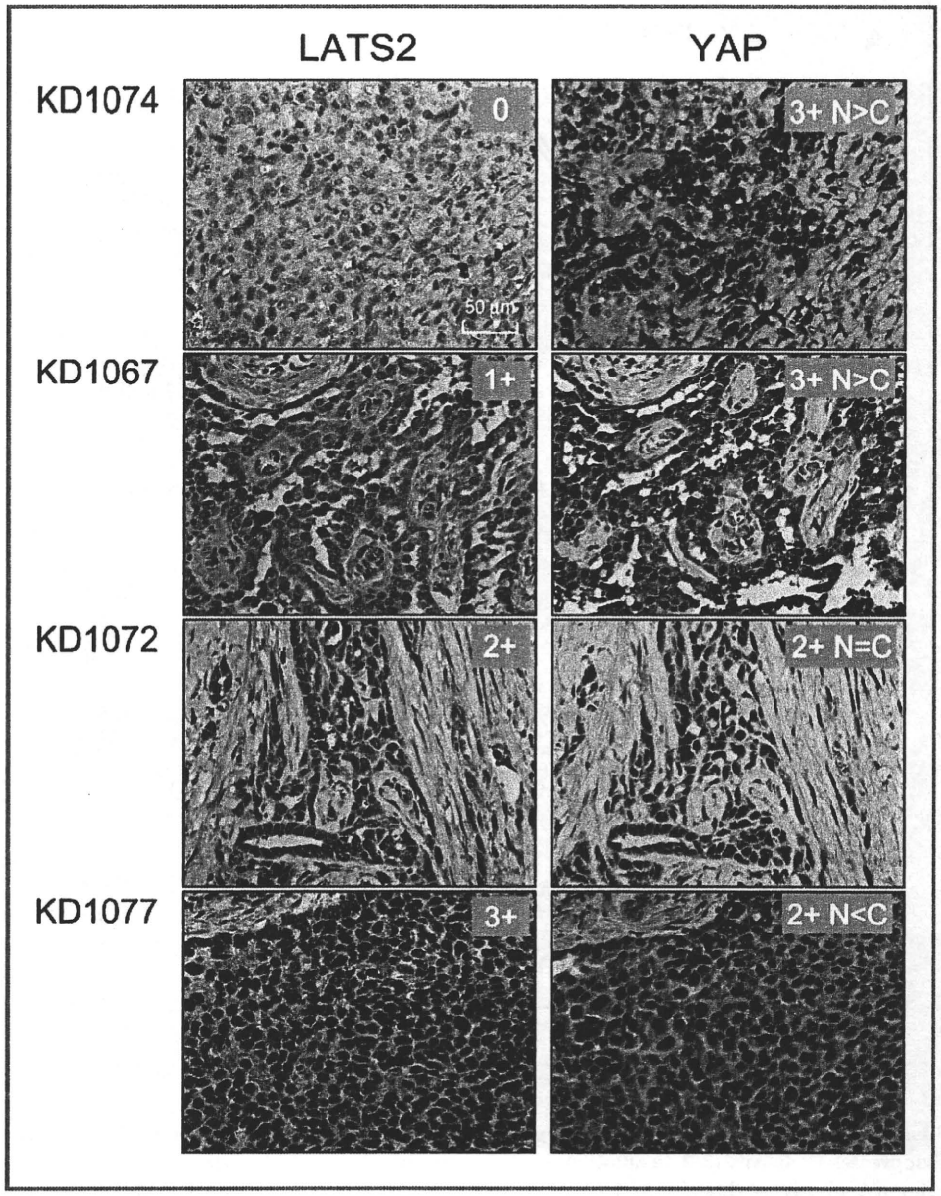

Figure 6. Immunohistochemical analyses of LATS2 and YAP in 45 primary MMs. Representative cases of LATS2 and YAP staining are presented. Case KD1074 with *LATS2* homozygous deletion and case KD1067 with *LATS2* deletion showed negative (0) and weak (1+) LATS2 staining, respectively. Both cases showed 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.

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 *LATS2* gene in any human malignancy.

Interestingly, several MM cell lines show inactivation of both *NF2* and *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 *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 *NF2* mutation but not *LATS2* mutation also showed significant phosphorylation levels of YAP, especially at

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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, 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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References

1. Pass HI, Vogelzang N, Hahn S, Carbone M. Malignant pleural mesothelioma. Curr Probl Cancer 2004;28:93–174.
2. Robinson BW, Lake RA. Advances in malignant mesothelioma. N Engl J Med 2005;353:1591–603.
3. Yang H, Testa JR, Carbone M. Mesothelioma epidemiology, carcinogenesis, and pathogenesis. Curr Treat Options Oncol 2008;9:147–57.
4. Ramos-Nino ME, Testa JR, Altomare DA, Pass HI, Carbone M, Bocchetta M, et al. Cellular and molecular parameters of mesothelioma. J Cell Biochem 2006;98:723–34.
5. Fennell DA, Gaudino G, O'Byrne KJ, Mutti L, van Meerbeeck J. Advances in the systemic therapy of malignant pleural mesothelioma. Nat Clin Pract Oncol 2008;5:136–47.
6. Tsao AS, Wistuba I, Roth JA, Kindler HL. Malignant pleural mesothelioma. J Clin Oncol 2009;27:2081–90.
7. Sugarbaker DJ, Richards WG, Gordon GJ, Dong L, De Rienzo A, Maulik G, et al. Transcriptome sequencing of malignant pleural mesothelioma tumors. Proc Natl Acad Sci U S A 2008;105:3521–6.
8. Ivanov SV, Miller J, Lucito R, Tang C, Ivanova AV, Pei J, et al. Genomic events associated with progression of pleural malignant mesothelioma. Int J Cancer 2009;124:589–99.
9. Gray SG, Fennell DA, Mutti L, O'Byrne KJ. In arrayed ranks: array technology in the study of mesothelioma. J Thorac Oncol 2009;4:411–25.
10. Bueno R, De Rienzo A, Dong L, Gordon GJ, Hercus CF, Richards WG, et al. Second generation sequencing of the mesothelioma tumor genome. PLoS One 2010;5:e10612.
11. Sekido Y, Pass HI, Bader S, Mew DJ, Christman MF, Gazdar AF, et al. Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. Cancer Res 1995;55:1227–31.
12. Bianchi AB, Mitsunaga SI, Cheng JQ, Klein WM, Jhanwar SC, Seizinger B, et al. High frequency of inactivating mutations in the neurofibromatosis type 2 gene (NF2) in primary malignant mesotheliomas. Proc Natl Acad Sci U S A 1995;92:10854–8.
13. Cheng JQ, Lee WC, Klein MA, Cheng GZ, Jhanwar SC, Testa JR. Frequent mutations of NF2 and allelic loss from chromosome band 22q12 in malignant mesothelioma: evidence for a two-hit mechanism of NF2 inactivation. Genes Chromosomes Cancer 1999;24:238–42.
14. Xiao GH, Gallagher R, Shetler J, Skele K, Altomare DA, Pestell RG, et al. The NF2 tumor suppressor gene product, Merlin, inhibits cell proliferation and cell cycle progression by repressing cyclin D1 expression. Mol Cell Biol 2005;25:2384–94.
15. Poulikakos PI, Xiao GH, Gallagher R, Jablonski S, Jhanwar SC, Testa JR. Re-expression of the tumor suppressor NF2/Merlin inhibits invasiveness in mesothelioma cells and negatively regulates FAK. Oncogene 2006;25:5960–8.
16. Fleury-Feith J, Lecomte C, Renier A, Matrat M, Kheuang L, Abramowski V, et al. Hemizygosity of Nf2 is associated with increased susceptibility to asbestos-induced peritoneal tumours. Oncogene 2003;22:3799–805.
17. Altomare DA, Vaslet CA, Skele KL, De Rienzo A, Devarajan K, Jhanwar SC, et al. A mouse model recapitulating molecular features of human mesothelioma. Cancer Res 2005;65: 8090–5.
18. Jongsma J, van Montfort E, Vooijs M, Zevenhoven J, Krimpenfort P, van der Valk M, et al. A conditional mouse model for malignant mesothelioma. Cancer Cell 2008;13:261–71.
19. Thurneysen C, Opitz I, Kurtz S, Weder W, Stahel RA, Felley-Bosco E. Functional inactivation of NF2/Merlin in human mesothelioma. Lung Cancer 2009;64:140–7.
20. Guled M, Lahti L, Lindholm PM, Salmenkivi K, Bagwan I, Nicholson AG, et al. CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma-A miRNA microarray analysis. Genes Chromosomes Cancer 2009;48:615–23.
21. Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, et al. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat Cell Biol 2006;8:27–36.

Published OnlineFirst January 18, 2011; DOI:10.1158/0008-5472.CAN-10-2164

22. Edgar BA. From cell structure to transcription: Hippo forges a new path. Cell 2006;124:267–73.
23. Pan D. Hippo signaling in organ size control. Genes Dev 2007;21:886–97.
24. Saucedo LJ, Edgar BA. Filling out the Hippo pathway. Nat Rev Mol Cell Biol 2007;8:613–21.
25. Zhao B, Lei QY, Guan KL. The Hippo-YAP pathway: new connections between regulation of organ size and cancer. Curr Opin Cell Biol 2008;20:638–46.
26. Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, et al. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc Natl Acad Sci U S A 2006;103:12405–10.
27. Zender L, Spector MS, Xue W, Flemming P, Cordon-Cardo C, Silke J, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell 2006;125:1253–67.
28. Yokoyama T, Osada H, Murakami H, Tatematsu Y, Taniguchi T, Kondo Y, et al. YAP1 is involved in mesothelioma development and negatively regulated by Merlin through phosphorylation. Carcinogenesis 2008;29:2139–46.
29. Usami N, Fukui T, Kondo M, Taniguchi T, Yokoyama T, Mori S, et al. Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients. Cancer Sci 2006;97:387–94.
30. Taniguchi T, Karnan S, Fukui T, Yokoyama T, Tagawa H, Yokoi K, et al. Genomic profiling of malignant pleural mesothelioma with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including JUN amplification on 1p32. Cancer Sci 2007;98:438–46.
31. Goto Y, Shinjo K, Kondo Y, Shen L, Toyota M, Suzuki H, et al. Epigenetic profiles distinguish malignant pleural mesothelioma from lung adenocarcinoma. Cancer Res 2009;69:9073–82.
32. Kawaguchi K, Murakami H, Taniguchi T, Fujii M, Kawata S, Fukui T, et al. Combined inhibition of MET and EGFR suppresses proliferation of malignant mesothelioma cells. Carcinogenesis 2009;30:1097–105.
33. Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber DA, et al. Salvador promotes both cell cycle exit and apoptosis in *Drosophila* and is mutated in human cancer cell lines. Cell 2002;110:467–78.
34. Scoles DR. The Merlin interacting proteins reveal multiple targets for NF2 therapy. Biochim Biophys Acta 2008;1785:32–54.
35. Baser ME. The distribution of constitutional and somatic mutations in the neurofibromatosis 2 gene. Hum Mutat 2006;27:297–306.
36. McClatchey AI, Giovannini M. Membrane organization and tumorigenesis—the NF2 tumor suppressor, Merlin. Genes Dev 2005;19:2265–77.
37. McClatchey AI, Fehon RG. Merlin and the ERM proteins—regulators of receptor distribution and signaling at the cell cortex. Trends Cell Biol 2009;19:198–206.
38. Li W, You L, Cooper J, Schiavon G, Pepe-Caprio A, Zhou L, et al. Merlin/NF2 suppresses tumorigenesis by inhibiting the E3 ubiquitin ligase CRL4(DCAF1) in the nucleus. Cell 2010;140:477–90.
39. Seidel C, Schagdarsurengin U, Blumke K, Wurl P, Pfeifer GP, Hauptmann S, et al. Frequent hypermethylation of MST1 and MST2 in soft tissue sarcoma. Mol Carcinog 2007;46:865–71.
40. Xu MZ, Yao TJ, Lee NP, Ng IO, Chan YT, Zender L, et al. Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. Cancer 2009;115:4576–85.
41. Steinhardt AA, Gayyed MF, Klein AP, Dong J, Maitra A, Pan D, et al. Expression of Yes-associated protein in common solid tumors. Hum Pathol 2008;39:1582–9.
42. Kawahara M, Hori T, Chonabayashi K, Oka T, Sudol M, Uchiyama T. Kpm/Lats2 is linked to chemosensitivity of leukemic cells through the stabilization of p73. Blood 2008;112:3856–66.
43. Strazisar M, Mlakar V, Glavac D. LATS2 tumour specific mutations and down-regulation of the gene in non-small cell carcinoma. Lung Cancer 2009;64:257–62.
44. Lopez-Lago MA, Okada T, Murillo MM, Socci N, Giancotti FG. Loss of the tumor suppressor gene NF2, encoding Merlin, constitutively activates integrin-dependent mTORC1 signaling. Mol Cell Biol 2009;29:4235–49.
45. James MF, Han S, Polizzano C, Plotkin SR, Manning BD, Stemmer-Rachamimov AO, et al. NF2/Merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. Mol Cell Biol 2009;29:4250–61.

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Phase I study of intravenous ASA404 (vadimezan) administered in combination with paclitaxel and carboplatin in Japanese patients with non-small cell lung cancer

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ASA404 (5,6-dimethylxanthenone-4-acetic acid, vadimezan), a flavone-8-acetic acid analogue, is a novel tumor-vascular disrupting agent. In this study, the safety and tolerability, pharmacokinetics and pharmacodynamics of ASA404 in combination with standard therapy of paclitaxel and carboplatin (P/C) were assessed. A total of 15 Japanese patients with stage IV advanced non-small cell lung cancer were enrolled and P/C plus ASA404 at three dose levels (600–1800 mg/m^2) was administered every 3 weeks. Dose limiting toxicities were observed in two patients during Cycle 1 of ASA404 treatment (Grade 3 febrile neutropenia at ASA404 1200 mg/m^2 and Grade 3 QT prolongation at ASA404 1800 mg/m^2) and the incidence of dose limiting toxicity was ≤1/3. The most frequently reported adverse events were injection site pain, peripheral sensory neuropathy, alopecia, neutropenia, nausea, anorexia and arthralgia, which were similar to those seen in previous Phase I/II studies. Pharmacokinetic analysis revealed the plasma area under the curve (AUC) of total ASA404 increased in a mostly dose-proportional manner within the dose range investigated. Administration of ASA404 raised plasma 5-hydroxyindole-3-acetic acid level dose-dependently by 116 and 204% after 1200 and 1800 mg/m^2 doses, respectively. Partial response was observed in four patients (27%), and seven patients (47%) exhibited stable disease. Overall, the safety and preliminary efficacy profiles were comparable to those seen in non-Japanese patients in previous Phase I and Phase II studies, and support the further evaluation of ASA404 (1800 mg/m^2) in Phase III studies in combination with P/C in Japanese patients with advanced non-small cell lung cancer. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2010.01839.x, 2011)

Worldwide, over 1.3 million people are diagnosed each year with lung cancer, with over 1.1 million deaths.[1,2] Non-small cell lung cancer (NSCLC) accounts for 87% of all lung cancers, with most patients diagnosed at advanced stages for which the 5-year survival rate is poor (<5%).[3,4] Lung cancer is also a disease that predominantly affects the elderly, with most cases (85%) occurring in patients over 60 years of age.[5] In Japan alone, the number of newly diagnosed patients with NSCLC reached 85 000 by 2005, and 45 927 Japanese men and 17 307 women died from the disease in 2006.[6,7] Current standard treatments are usually platinum-based combination therapies, and platinum-taxane regimens predominate in Japan (>55%).[8] These treatment regimens extend survival but are rarely curative,[9,10] and there remains a need for more effective and better tolerated therapies.

Like all solid tumors, lung tumors depend upon a functional vascular supply to meet demand for oxygen and nutrients required for growth and development.[11] Furthermore, a high level of vascularity in peripheral lung tumor tissue has been shown to correlate with lung cancer progression.[12] Microvessel density in lung cancers is also a prognostic indicator of metastasis and poor survival.[13–15] The vasculature of solid tumors is typified by aberrant vessels, the unique characteristics of which present an opportunity for selective therapeutic intervention.[16,17] ASA404 (vadimezan), a flavone-8-acetic acid analogue, is a tumor-vascular disrupting agent (tumor-VDA) that selectively targets the immature and rapidly proliferating endothelial cells of established tumor vasculature.[18] ASA404 induces rapid tumor endothelial cell apoptosis and a cascade of events that induces a sustained effect on tumor blood flow, causing hypoxia, vascular failure, and inflammatory responses.[18–22] These effects lead to extensive tumor necrosis,[23] although a viable rim of surviving cells remains at the tumor periphery when the agent is used alone.[24] The activity of ASA404, which causes necrosis at the tumor core, may thus be maximized by combining it with chemotherapeutic agents, which often have greatest effect at the tumor periphery. Preclinical studies on ASA404 combined with various chemotherapeutic agents have demonstrated enhanced anti-tumor activity compared with chemotherapy alone. This synergy is most notable with taxanes,[25–27] and studies in combination with paclitaxel in human NSCLC xenografts have produced tumor cures.[25,28]

Three Phase I clinical studies with ASA404 established a maximum tolerated dose of 3700 mg/m^2, with dose-limiting toxicities (DLTs) occurring at doses of 4900 mg/m^2.[29–31] Side effects were relatively mild, and only transient, with moderate cardiac changes occurring at higher doses.[29,30] Visual disturbances were also reported in these studies but only at the highest doses (≥2400 mg/m^2).[29–31] A dose of 1200 mg/m^2 was selected for a randomized Phase II study to determine the feasibility of combining ASA404 with paclitaxel and carboplatin (P/C). The study (n = 37) examined the potential for pharmacokinetic (PK) interactions between components of this regimen and evaluated its safety and efficacy in patients with previously untreated advanced NSCLC.[32] The ASA404 combination improved a range of efficacy endpoints compared with P/C alone—most notably overall survival (14.0 months in the P/C + ASA404 [1200 mg/m^2] group vs 8.8 months for P/C alone). The risk of death was reduced by 27% (hazard ratio [HR] = 0.73, 95% confidence interval [CI]: 0.39, 1.38) with a response rate of 31 vs 22% for P/C alone.[32] A single-arm extension of this study (n = 29) subsequently elevated the ASA404 dose to 1800 mg/m^2, which was well tolerated, and a

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median survival of 14.9 months was demonstrated.[33] Safety and efficacy endpoints were also similar in both squamous and non-squamous patients.[34] Phase II evaluations have shown that ASA404 is a promising addition to standard NSCLC chemotherapy but this must be confirmed in a larger prospective study. The Phase III study of ASA404 as a first-line treatment for NSCLC in combination with P/C (ATTRACT-1) was halted following interim data analysis showing futility. However, no safety concerns were identified.[35]

The primary objective of this open-label, non-randomized, sequential dose escalation Phase I study was to assess the safety profile and tolerability of ASA404 when administered in combination with fixed doses of P/C in Japanese patients with previously untreated, stage IIIb/IV advanced NSCLC. Secondary objectives were to characterize the PK profile in Japanese patients, to assess pharmacodynamic (PD) effects and evaluate preliminary anti-tumor activity.

Materials and Methods

Patient population. Japanese patients ≥20 years of age with newly diagnosed, histologically or cytologically confirmed, stage IIIb/IV NSCLC were eligible for inclusion. Other requirements were that patients had no prior treatment for stage IIIb/IV disease (prior neoadjuvant or adjuvant chemotherapy within 6 months was allowed), World Health Organization (WHO) performance status (PS) of 0–1 and life expectancy ≥12 weeks. Eligible patients could have either squamous or non-squamous histology.

Specific criteria for exclusion were: symptomatic central nervous system (CNS) metastases requiring treatment; second primary cancer with the exception of non-melanoma skin cancer or cervical cancer *in situ*; radiotherapy (unless palliative) within 4 weeks; major surgery within 4 weeks; prior exposure to tumor-VDAs or other vascular targeting agents; pleural effusion requiring drainage; hemoptysis associated with NSCLC; long QT syndrome; myocardial infarction within 12 months; poorly controlled angina pectoris; ventricular tachycardia; history of ventricular fibrillation or torsades de pointes and use of medication known to prolong the QT interval.

Dosing and administration. The dose of ASA404 was selected based on the current data from clinical studies held in western countries. ASA404 was administered at doses of 600, 1200 and 1800 mg/m^2 as a 20 min intravenous (IV) infusion following infusion of paclitaxel (200 mg/m^2 IV over 3 h) and carboplatin (IV over 30 mins at a plasma AUC of 6 mg/ml × min) on day 1 of every cycle. Each treatment cycle span was 21 days and study treatment was administered for six cycles, although responding patients could proceed beyond six cycles. The initial dose of ASA404 was 600 mg/m^2, followed by dose escalation to 1200 mg/m^2, and then to 1800 mg/m^2. Evaluation at each dose level was performed based on data from at least three patients during cycle 1. If a DLT was confirmed during cycle 1 in one of the first three patients, the dose cohort was expanded by three additional patients for evaluation. Intra-patient dose escalation was not permitted for any patient. If the probability of DLT incidence was ≤1/3, escalation to the next dose level was performed. The recommended dose for the next phase was set as the highest achievable dose with a DLT incidence ≤1/3.

Safety assessments. The safety population comprised all patients who had received at least one dose of ASA404 and had at least one post-baseline safety assessment after drug administration. The dose-determining population included all patients from the safety population who either completed minimum safety evaluation requirements or discontinued due to DLT in cycle 1. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events, version 3.0 (Japanese version). A DLT was defined as a study drug-related adverse event (AE) including cardiac toxicity, QT prolongation, Grade 4 neutropenia (for >7 days), Grade 4 thrombocytopenia and persistent CNS toxicity, including ophthalmic toxicity, or abnormal laboratory value, occurring during cycle 1. Resumption of drug administration followed if dose modification criteria were met. No study drug dose reduction was permitted.

All patients were followed for AEs and serious AEs (SAEs) for 4 weeks following the last dose of ASA404. Patients whose treatment was permanently discontinued due to an AE or abnormal laboratory value were followed at least once a week for 4 weeks. SAEs or events that had a suspect relationship to study drug, were followed at 4 week intervals, until resolution or stabilization of the event, whichever came first. Visual disturbances were assessed in all patients at baseline and end of treatment.

Pharmacokinetic and pharmacodynamic analyses. To characterize the PK profile of ASA404, plasma concentrations of total (sum of plasma protein bound and unbound) ASA404 were measured during cycle 1 (immediately prior to infusion, <1 min before the end of infusion, and at 0.5, 1, 2, 4, 6, 24 and 48 h time points after infusion) and cycles 2–6 (immediately prior to infusion, <1 min before the end, and 1 and 4 h after infusion). Free (protein unbound) plasma ASA404 concentrations were also measured in cycle 1 only. Previous studies have shown that co-administration of ASA404 does not fundamentally alter the PK parameters of either paclitaxel or carboplatin.[32]

Urinary excretion was determined at cycle 1 day 1 (prior to infusion, start of infusion to 6 h post-infusion, and 6–24 h post-infusion) and cycle 1 day 2 (24–48 h post-infusion). Concentrations of ASA404 in plasma and urine were determined by liquid chromatography/tandem mass spectroscopy. Pharmacokinetic parameters were calculated by a non-compartmental method using WinNonlin Professional Edition (Pharsight, St. Louise, MO, USA) by lead PK analyst.

Plasma was also collected to evaluate the PD of ASA404 and determine whether markers predictive of activity could be defined. The ASA404-induced vascular damage biomarker, 5-hydroxyindole-3-acetic acid (5-HIAA) was determined, together with the angiogenesis markers, vascular endothelial growth factor (VEGF), placental growth factor, soluble VEGF receptors-1 and -2 (sVEGFR-1 and -2) and basic fibroblast growth factor. These samples were taken on cycle 1 day 1 at pre-study treatment, post-P/C dosing but pre-study drug infusion, 4 h post-study drug infusion and 24 h post-study drug infusion; and on cycle 2 day 1, cycle 4 day 1 and cycle 6 day 1 at pre-study treatment, post-carboplatin but pre-study drug infusion, and 1 h post-study drug infusion. In addition, a single blood collection was taken at the end of treatment visit.

Efficacy assessments. Efficacy was determined using the full analysis population of all patients who had received at least one dose of ASA404 according to the intention to treat principle. Tumor response was assessed in patients with measurable disease at baseline according to response evaluation criteria in solid tumors (RECIST) criteria, performed within 28 days before start of treatment. Tumor assessment was every 6 weeks and at the end of study. Best overall response in each patient was evaluated as complete response, partial response, progressive disease or stable disease. The objective response rate in this study was evaluated as the number of patients with complete response or partial response.

Results

Accrual and patient characteristics. A total of 15 patients with NSCLC were recruited and baseline characteristics are shown in Table 1. The majority of patients (60%) had adenocarcinoma, 13% had squamous cell carcinoma, and all patients had stage IV

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Table 1. Patient baseline characteristics

	P/C + ASA404 600 mg/m^2 n = 3	P/C + ASA404 1200 mg/m^2 n = 6	P/C + ASA404 1800 mg/m^2 n = 6	All subjects n = 15
Sex n (%)				
Female	1 (33)	5 (83)	2 (33)	8 (53)
Male	2 (67)	1 (17)	4 (67)	7 (47)
Age (years)				
Mean ± SD	57.3 ± 12.66	62.8 ± 6.27	60.2 ± 2.56	60.7 ± 6.62
WHO PS n (%)				
0	1 (33)	0 (0)	3 (50)	4 (27)
1	2 (67)	6 (100)	3 (50)	11 (73)
Histology/cytology n (%)				
Adenocarcinoma	1 (33)	4 (67)	4 (67)	9 (60)
Squamous cell carcinoma	0 (0)	1 (17)	1 (17)	2 (13)
Other	2 (67)	1 (17)	1 (17)	4 (27)
Stage n (%)				
Stage III	0 (0)	0 (0)	0 (0)	0 (0)
Stage IV	3 (100)	6 (100)	6 (100)	15 (100)
Prior antineoplastic therapy	1 (33)	2 (33)	0 (0)	3 (20)

P/C, paclitaxel and carboplatin; SD, standard deviation; WHO PS, World Health Organization Performance Status.

disease. WHO performance status (PS) was predominantly PS = 1 (73%). Approximately 70% of patients received a third course of ASA404 treatment.

Safety. Two patients exhibited DLT during cycle 1 of ASA404 treatment: one of six patients at a dose of 1200 mg/m^2 had Grade 3 febrile neutropenia and one of six patients at a dose of 1800 mg/m^2 had Grade 3 QT prolongation (the event resolved by day 6 of cycle 1; patient was discontinued).

The AEs observed were as expected for this population and for this class of drug,[29–33] and were experienced by all patients. The most frequently reported AEs by system organ class (SOC) were blood and lymphatic disorders, general disorders and administration site conditions, and nervous system disorders (n = 15, all 100% incidence). Blood and lymphatic disorders (n = 15, 100%) consisted of neutropenia including one neutrophil count decreased (n = 13, 87%), anemia (n = 9, 60%), thrombocytopenia (n = 4, 27%), and lymphopenia (n = 3, 20%). The most frequently reported Grade 3 or 4 hematological abnormality as a laboratory parameter was reduction in absolute neutrophils. The most common AEs of any grade were injection site pain, peripheral sensory neuropathy and alopecia, each occurring in 14 (93%) patients across all doses (Table 2). These events were mostly Grade 1 or 2, and resolved without treatment or concomitant medications. Other frequently occurring AEs were neutropenia, including one neutrophil count decreased (n = 13), anorexia (n = 12), arthralgia (n = 12), and nausea (n = 12). Grade 3 febrile neutropenia was reported as DLT in one patient, but Grade 3 or 4 AEs occurred with low frequency (<15%) with the exception of neutropenia (87% over all doses, including reduced neutrophil count and febrile neutropenia) and are presented in Table 3.

Serious adverse events occurred in six patients, five of which occurred during the study, with none occurring at the lowest dose level of 600 mg/m^2, four at 1200 mg/m^2 and two at 1800 mg/m^2. All these SAEs occurred in one patient each, were not clustered in any particular primary system organ class and consisted of hemorrhagic enterocolitis, femoral neck fracture, pneumonia, pharyngitis and tumor hemorrhage. Seven patients discontinued the study due to AEs (three at the 1200 mg/m^2 dose and four at 1800 mg/m^2). No deaths were reported during the study, but one patient died more than 40 days after the last administration of ASA404 1800 mg/m^2 due to acute myocardial infarction.

Electrocardiogram (ECG)-evaluated QT prolongation occurred in three patients and T-wave inversion in one patient treated with ASA404. QT prolongation was reported in two patients who received P/C + ASA404 1800 mg/m^2, one of which was Grade 3 and judged as a DLT. However, both events were

Table 2. Frequently occurring (≥33% in all patients) adverse events of any grade, regardless of study drug relationship by preferred term

Adverse events	P/C + ASA404 600 mg/m^2 n = 3 (n, %)	P/C + ASA404 1200 mg/m^2 n = 6 (n, %)	P/C + ASA404 1800 mg/m^2 n = 6 (n, %)	All patients n = 15 (n, %)
Alopecia	2 (67)	6 (100)	6 (100)	14 (93)
Injection site pain	2 (67)	6 (100)	6 (100)	14 (93)
Peripheral sensory neuropathy	3 (100)	5 (83)	6 (100)	14 (93)
Neutropenia	3 (100)	6 (100)*	4 (67)	13 (87)
Anorexia	3 (100)	5 (83)	4 (67)	12 (80)
Arthralgia	3 (100)	5 (83)	4 (67)	12 (80)
Nausea	3 (100)	5 (83)	4 (67)	12 (80)
Fatigue	2 (67)	4 (67)	4 (67)	10 (67)
Anemia	2 (67)	4 (67)	3 (50)	9 (60)
Constipation	2 (67)	2 (33)	4 (67)	8 (53)
Myalgia	2 (67)	2 (33)	4 (67)	8 (53)
Diarrhea	2 (67)	2 (33)	1 (17)	5 (33)

*Including decreased neutrophil count. P/C, paclitaxel and carboplatin.

Table 3. Grade 3 or 4 adverse events, regardless of study drug relationship by preferred term

Adverse events	P/C + ASA404 600 mg/m^2 n = 3 (n, %)	P/C + ASA404 1200 mg/m^2 n = 6 (n, %)	P/C + ASA404 1800 mg/m^2 n = 6 (n, %)	All patients n = 15 (n, %)
Neutropenia	3 (100)	6 (100)*	4 (67)	13 (87)
Anemia	0 (0)	1 (17)	1 (17)	2 (13)
Anorexia	0 (0)	1 (17)	1 (17)	2 (13)
QT Prolongation	0 (0)	0 (0)	1 (17)	1 (7)
Enterocolitis hemorrhagic	0 (0)	1 (17)	0 (0)	1 (7)
Febrile neutropenia	0 (0)	1 (17)	0 (0)	1 (7)
Femoral neck fracture	0 (0)	1 (17)	0 (0)	1 (7)
Hyponatremia	0 (0)	1 (17)	0 (0)	1 (7)
Lymphopenia	0 (0)	1 (17)	0 (0)	1 (7)
Peripheral sensory neuropathy	0 (0)	0 (0)	1 (17)	1 (7)
Pneumonia	0 (0)	0 (0)	1 (17)	1 (7)

*Including decreased neutrophil count. P/C, paclitaxel and carboplatin.

asymptomatic and resolved within a short period (1 and 4 days, respectively) without treatment. There were no other cardiac events in this study.

No ophthalmic abnormalities were reported at ASA404 doses of 600 and 1200 mg/m^2; two incidences of dyschromatopsia occurred at the higher dose of 1800 mg/m^2. Visual acuity changes were not reported at any of the ASA404 dose levels used in this study.

Efficacy. The best overall response by investigator assessment was partial response, which was observed in four patients (27%), and seven patients (47%) exhibited stable disease. Response data are presented in Table 4.

Pharmacokinetics. Mean plasma concentration–time profiles of total and free ASA404 in cycle 1 are shown in Fig. 1, and a summary of PK parameters is given in Table 5. After reaching C_{max} at the end of infusion, plasma total ASA404 concentrations decreased biphasically over time at a dose of 600 mg/m^2, becoming more monophasic at higher doses, probably due to saturation of plasma protein binding. C_{max} increased dose proportionally from 600 to 1200 mg/m^2 but less than dose proportionally from 1200 to 1800 mg/m^2 (Fig. 2). The plasma AUC of total ASA404 was essentially dose-proportional over the range investigated (Fig. 2). Systemic clearance of total ASA404 decreased slightly with increasing dose from 3.88 L/h at 600 mg/m^2 to 2.87 and 2.78 L/h at doses of 1200 and 1800 mg/m^2, respectively (Table 5). Systemic clearance of free ASA404 was 20- to 50-fold higher than that of total ASA404 and the distribution volume of free ASA404 was 20- to 40-fold greater than that of total ASA404. Plasma protein binding of ASA404 was >93% and the free fraction in plasma decreased at lower total drug concentrations, indicating that protein binding is saturable. Mean urinary excretion of unchanged ASA404 was 6, 2 and 3% of the administered dose for 600, 1200 and 1800 mg/m^2, respectively, and mean renal clearance was 0.2, 0.07 and 0.08 L/h, respectively. Plasma concentrations of ASA404 did not alter at each cycle, indicating that there was no accumulation of ASA404 over repeated 3-weekly dosing, consistent with its observed short elimination half-life of 4.61–7.04 h.

Biomarker changes. *Effect of ASA404 on plasma 5-HIAA levels.* Plasma levels of the vascular damage PD biomarker 5-HIAA after infusion of P/C + ASA404 were evaluated on day 1 of cycle 1, 2, 4 and 6. Levels of 5-HIAA did not change after paclitaxel or carboplatin infusion, but were elevated 4 h after infusion of ASA404 at 1200 and 1800 mg/m^2, and were still

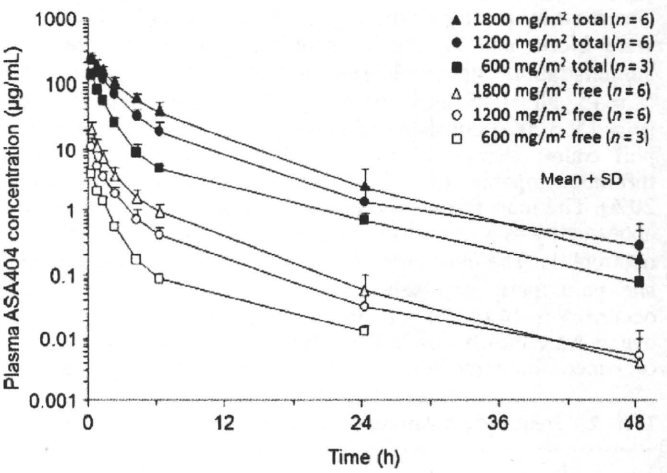

Fig. 1. Mean plasma concentration–time profiles of total and free ASA404 in cycle 1 of treatment; log-linear plot. SD, standard deviation.

Table 4. Best overall response observed by investigator assessment and RECIST criteria

Best overall response	P/C + ASA404 600 mg/m^2 n = 3 (n, %)	P/C + ASA404 1200 mg/m^2 n = 6 (n, %)	P/C + ASA404 1800 mg/m^2 n = 6 (n, %)	All patients n = 15 (n, %)
Complete response	0 (0)	0 (0)	0 (0)	0 (0)
Partial response	0 (0)	3 (50)	1 (17)	4 (27)
Stable disease	3 (100)	2 (33)	2 (33)	7 (47)
Progressive disease	0 (0)	1 (17)	3 (50)	4 (27)

P/C, paclitaxel and carboplatin; RECIST, response evaluation criteria in solid tumors.

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Table 5. Pharmacokinetic parameters

PK parameter		P/C + ASA404 600 mg/m^2 n = 3 (mean ± SD)	P/C + ASA404 1200 mg/m^2 n = 6 (mean ± SD)	P/C + ASA404 1800 mg/m^2 n = 6 (mean ± SD)
Total ASA404	T_{max} (h)	0.32 (0.28–0.33)†	0.31 (0.28–0.37)†	0.33 (0.30–0.35)†
	C_{max} (μg/mL)	137 ± 9.5	224 ± 33.8	247 ± 34.1
	$AUC_{0\text{-}inf}$ (μg*h/L)	260 ± 25.9	650 ± 97.2	1040 ± 137
	$t_{1/2}$ (h)	7.04 ± 1.06	5.86 ± 2.59	4.61 ± 1.07
	CL (L/h)	3.88 ± 0.77	2.87 ± 0.57	2.78 ± 0.55
	V_{ss} (L)	14.4 ± 3.12	11.7 ± 3.42	12.9 ± 4.93
Free ASA404	T_{max} (h)	0.32 (0.28–0.33)†	0.31 (0.28–0.37)†	0.33 (0.30–0.35)†
	C_{max} (μg/mL)	3.69 ± 0.74	11.0 ± 4.17	20.2 ± 6.03
	$AUC_{0\text{-}inf}$ (μg*h/L)	5.87 ± 0.97	18.7 ± 3.25	37.6 ± 9.46
	$t_{1/2}$ (h)	5.83 ± 0.13	5.68 ± 2.77	5.24 ± 1.92
	CL (L/h)	176 ± 53.7	101 ± 24.6	80.4 ± 28.2
	V_{ss} (L)	517 ± 171	318 ± 81.2	292 ± 227

†, median (range). AUC, area under the curve; CL, clearance; C_{max}, maximum plasma concentration; P/C, paclitaxel and carboplatin; PK, pharmacokinetic; SD, standard deviation; T_{max}, time to maximum plasma concentration; V_{ss}, distribution volume at steady state.

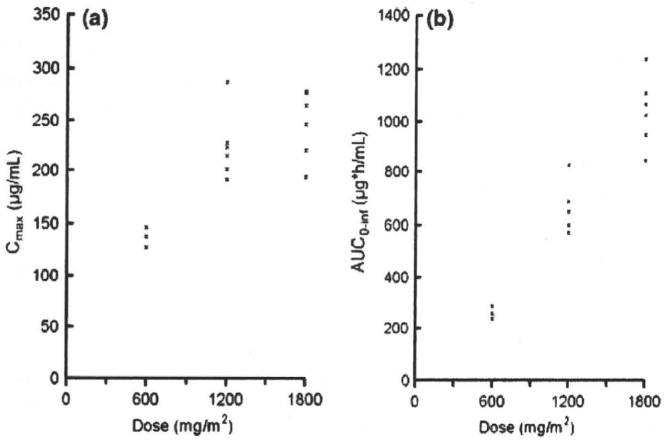

Fig. 2. Non-linear dose-exposure relationships for ASA404 at each dose level for (a) C_{max} and (b) area under the curve (AUC).

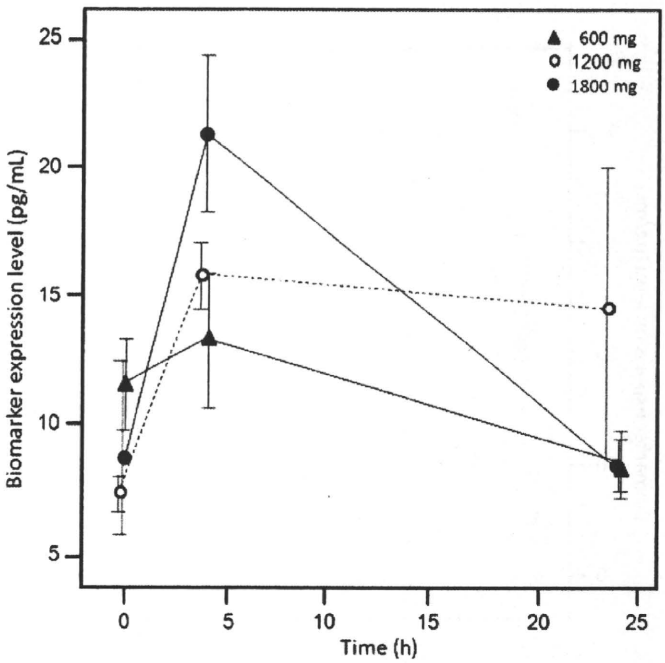

Fig. 3. Rapid dose-dependent induction of plasma 5-hydroxyindole-3-acetic acid levels after ASA404 infusion at cycle 1 day 1.

higher than baseline at 24 h post-infusion despite a post-4 h decline. Induction of 5-HIAA was dose-dependent, with increases of 116 and 204% over pre-infusion levels for 1200 and 1800 mg/m^2 dosing, respectively (Fig. 3). Induction of 5-HIAA was also observed 1 h after ASA404 infusion at all doses in cycles 2, 4 and 6. These results were in agreement with previous studies in which ASA404 caused dose-dependent acute vascular disruption and induction of plasma 5-HIAA.

Effect of ASA404 on plasma angiogenesis marker levels. No obvious changes in plasma basic fibroblast growth factor, VEGF, placental growth factor or sVEGFR-2 were observed, and sVEGFR-1 levels were highly variable.

Effect of ASA404 on plasma inflammatory cytokine and von Willebrand factor levels. On cycle 1 day1, increased plasma levels of the inflammatory cytokines interleukin-8 and monocyte chemotactic protein-1 were observed 4 and 24 h after ASA404 infusion (Fig. 4). Levels of von Willebrand factor increased 4 and 24 h after ASA404 infusion at the 1800 mg/m^2 dose, and 24 h after ASA404 infusion at the1200 mg/m^2 dose (Fig. 5).

Discussion

This single-arm, open-label study evaluated the addition of the flavonoid tumor-VDA ASA404 at three doses (600, 1200 and 1800 mg/m^2) to standard P/C therapy in 15 Japanese patients with stage IV advanced NSCLC. A total of 15 patients were treated with ASA404, and three patients (two patients at 600 mg/m^2 and one patient at 1200 mg/m^2) completed six cycles of treatment. Approximately 70% of patients received cycle three of ASA404 treatment and the median cumulative dose was similar within each dose level (6100–7600 mg). A total of 12 patients (80%) were discontinued from the study due to AEs (n = 7) or progressive disease (n = 5). All dose levels were well tolerated when administered every 3 weeks, and the incidence of DLTs was ≤1/3. This safety profile was comparable to that seen in non-Japanese patients in previous Phase I and Phase II studies.[29–33] The most frequently occurring AEs regardless of causality were injection site pain, peripheral sensory neuropathy, alopecia and neutropenia. Cardiac events were not evident in this study with the exception of QT prolongation, which, although reported in three patients, did not require therapeutic intervention and could be managed through study drug discontinuation alone. No new ophthalmological abnormalities were observed in the Japanese patients in this study, suggesting

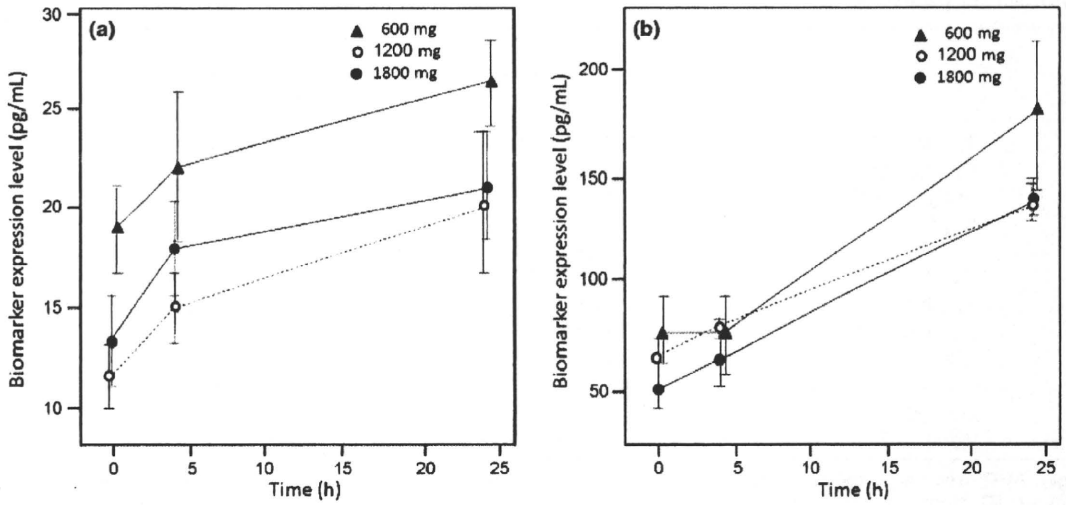

Fig. 4. Effect of ASA404 on levels of plasma inflammatory cytokines, (a) interleukin-8 and (b) monocyte chemotactic protein-1.

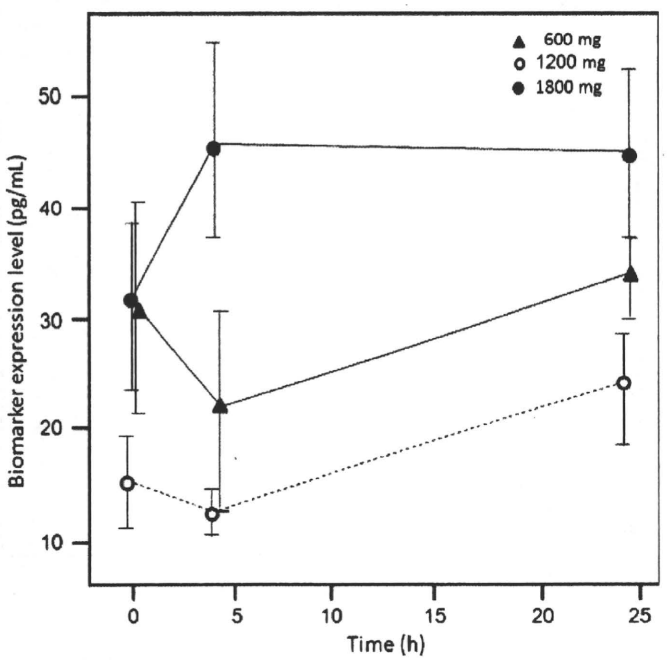

Fig. 5. Effect of ASA404 on plasma von Willebrand factor levels.

that ASA404 at dose levels up to 1800 mg/m^2 can be combined with P/C in this population without the ophthalmic AEs seen at higher doses in previous studies in non-Japanese patients.

PK data indicated saturation of systemic clearance as well as protein binding over the dose range investigated and was consistent with observations in non-Japanese patients.[30] Plasma half-life was consistent with previous studies and confirmed the observed non-accumulation following repeated dosing schedules. PD studies revealed that the pattern of changes seen in plasma angiogenesis markers after ASA404 infusion were markedly different from that seen with anti-angiogenic compounds, for which the typical response is an acute plasma VEGF and placental growth factor increase and sVEGFR-2 decrease.[36,37] The current study indicates that ASA404 has a distinctly different mechanism of action from anti-angiogenic agents with regard to these growth factors. In addition, acute induction of the inflammatory cytokines interleukin-8 and monocyte chemotactic protein-1 was observed after ASA404 infusion. This is in agreement with previous findings that ASA404 may indirectly lead to induction of inflammatory cytokines, vascular damage, and the release of von Willebrand factor.

Although the study was limited to 15 patients, tumor responses were noted, and combined with the observed safety profile, the results support the further evaluation of ASA404 at a dose of 1800 mg/m^2 in Phase III studies in combination with P/C in Japanese patients with advanced NSCLC.

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Disclosure Statement

Hiromi Tanii is an employee of Novartis. Michael M. Shi is an employee of Novartis, holds stocks in Novartis and receives Research funding. Ken Kobayashi is an employee of Novartis and holds stocks in Novartis.

References

1 Ferlay J. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; **18**: 581–92.
2 Parkin DM, Bray F, Ferlay J *et al.* Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
3 Govindan R. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the Surveillance, Epidemiologic, and End Results Database. *J Clin Oncol* 2006; **24**: 4539–43.
4 Subramanian J, Govindan R. Lung Cancer. In: Govindan R, ed. *The Washington Manual of Oncology*. Philadelphia: Lippincott Williams and Wilkins, 2008; 141.
5 Shibuya K. Global and regional estimates of cancer mortality and incidence by site: II. results for the global burden of disease 2000. *BMC Cancer* 2002; **2**: 1–26.
6 Oshima A, Kuroishi T, Tajima K. *Trends of incidence rates of cancer in Japan. White Book of Cancer Statistics: Incidence Death and Prognosis.* Tokyo: Shinohara-shinsha, 2005.
7 Toyoda Y, Nakayama T, Ioka A *et al.* Trends in lung cancer incidence by histological type in Osaka, Japan. *Jpn J Clin Oncol* 2008; **38**: 534–9.
8 Vansteenkiste J, Koester M, Lange M *et al.* First-line treatment patterns in advanced NSCLC in western Europe (EU), the United States, and Japan including a comparative analysis of younger versus elderly patients. *J Clin Oncol* 2009; **27**: (15 Suppl.): 8053 [abstract].
9 NCCN. *Clinical Practice Guidelines in Oncology, v2.* NCCN, INC., 2009.

doi: 10.1111/j.1349-7006.2010.01839.x

10 NSCLC Meta-Analyses Collaborative Group. Chemotherapy in addition to supportive care improves survival in advanced non-small-cell lung cancer: a systematic review and meta-analysis of individual patient data from 16 randomized controlled trials. *J Clin Oncol* 2008; **26**: 4617–25.
11 Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**: 4–6.
12 Ushijima C, Tsukamoto S, Yamazaki K *et al.* High vascularity in the peripheral region of non-small cell lung cancer tissue is associated with tumor progression. *Lung Cancer* 2001; **34**: 233–41.
13 Cox G, Walker RA, Andi A *et al.* Prognostic significance of platelet and microvessel counts in operable non-small cell lung cancer. *Lung Cancer* 2000; **29**: 169–77.
14 Fontanini G, Lucci M, Vignati S *et al.* Angiogenesis as a prognostic indicator of survival in non-small cell lung cancer: a prospective study. *J Natl Cancer Inst* 1997; **89**: 881–6.
15 Meert A-P, Paesmans M, Martin B *et al.* The role of microvessel density on the survival of patients with lung cancer: a systematic review of the literature and meta-analysis. *Br J Cancer* 2002; **87**: 694–701.
16 Konerding MA, Miodonski AJ, Lametschwandtner A. Microvascular corrosion casting in the study of tumor vascularity: a review. *Scanning Microsc* 1995; **9**: 1233–43.
17 Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005; **5**: 423–35.
18 Ching LM, Cao Z, Kieda C *et al.* Induction of endothelial cell apoptosis by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid. *Br J Cancer* 2002; **86**: 1937–42.
19 Baguley BC, Ching LM. DMXAA: an antivascular agent with multiple host responses. *Int J Radiat Oncol Biol Phys* 2002; **54**: 1503–11.
20 Ching LM, Goldsmith D, Joseph WR *et al.* Induction of intratumoral tumor necrosis factor (TNF) synthesis and hemorrhagic necrosis by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in TNF knockout mice. *Cancer Res* 1999; **59**: 3304–7.
21 Ching LM, Zwain S, Baguley BC. Relationship between tumour endothelial cell apoptosis and tumour blood flow shutdown following treatment with the antivascular agent DMXAA in mice. *Br J Cancer* 2004; **90**: 906–10.
22 Zwi L, Baguley B, Gavin J *et al.* Correlation between immune and vascular activities of xanthenone acetic acid antitumor agents. *Oncol Res* 1994; **6**: 79–85.
23 McPhail LD, McIntyre DJ, Ludwig C *et al.* Rat tumor response to the vascular-disrupting agent 5,6-dimethylxanthenone-4-acetic acid as measured by dynamic contrast-enhanced magnetic resonance imaging, plasma 5-hydroxyindoleacetic acid levels, and tumor necrosis. *Neoplasia* 2006; **8**: 199–206.
24 Baguley BC, Wilson WR. Potential of DMXAA combination therapy for solid tumors. *Expert Rev Anticancer Ther* 2002; **2**: 593–603.
25 Kelland LR. Targeting established tumour vasculature: a novel approach to cancer treatment. *Curr Cancer Ther Rev* 2005; **1**: 1–9.
26 Siemann DW, Mercer E, Lepler S *et al.* Vascular targeting agents enhance chemotherapeutic agent activities in solid tumor therapy. *Int J Cancer* 2002; **99**: 1–6.
27 Siim BG, Lee AE, Shalal-Zwain S *et al.* Marked potentiation of the antitumour activity of chemotherapeutic drugs by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA). *Cancer Chemother Pharmacol* 2003; **51**: 43–52.
28 Green C, Griffiths-Johnson D, Dunmore K. Marked potentiation of the *in vivo* antitumor activity of docetaxel in a human prostate cancer xenograft by the vascular targeting agent 5,6 dimethylxanthenone acetic acid, DMXAA. *Proc Amer Assoc Cancer Res* 2005; 2990. [abstract].
29 Jameson MB, Thompson PI, Baguley BC *et al.* Clinical aspects of a phase I trial of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a novel antivascular agent. *Br J Cancer* 2003; **88**: 1844–50.
30 McKeage MJ, Fong P, Jeffery M *et al.* 5,6-Dimethylxanthenone-4-acetic acid in the treatment of refractory tumors: a phase I safety study of a vascular disrupting agent. *Clin Cancer Res* 2006; **12**: 1776–84.
31 Rustin GJ, Bradley C, Galbraith S *et al.* 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a novel antivascular agent: phase I clinical and pharmacokinetic study. *Br J Cancer* 2003; **88**: 1160–7.
32 McKeage MJ, von Pawel J, Reck M *et al.* Randomised phase II study of ASA404 combined with carboplatin and paclitaxel in previously untreated advanced non-small cell lung cancer. *Br J Cancer* 2008; **99**: 2006–12.
33 McKeage MJ, Reck M, Jameson MB *et al.* Phase II study of ASA404 (vadimezan, 5,6-dimethylxanthenone-4-acetic acid/DMXAA) 1800 mg/m^2 combined with carboplatin and paclitaxel in previously untreated advanced non-small cell lung cancer. *Lung Cancer* 2009; **65**: 192–7.
34 McKeage MJ, Jameson MB, AS1404-201 Study Group Investigators. Comparison of safety and efficacy between squamous and non-squamous non-small cell lung cancer (NSCLC) patients in phase II studies of DMXAA (ASA404). ASCO Annual Meeting. Chicago; 2008; 8072 [abstract].
35 Novartis Pharmaceuticals. NCT00662597: a Phase III, randomized, double-blind, placebo-controlled multi-center study of ASA404 in combination with paclitaxel and carboplatin as first-line treatment for locally advanced or metastatic (stage IIIb/IV) non-small cell lung cancer (NSCLC). ATTRACT-1 [cited 4 October 2010], and Available from URL: http://clinicaltrials.gov/ct2/show/NCT00662597?term=ASA404+NSCLC&rank=3
36 DePrimo S, Bello C, Smeraglia J *et al.* Circulating protein biomarkers of pharmacodynamic activity of sunitinib in patients with metastatic renal cell carcinoma: modulation of VEGF and VEGF-related proteins. *J Transl Med* 2007; **5**: 1–11.
37 Rini B, Michaelson M, Rosenberg J *et al.* Antitumor activity and biomarker analysis of sunitinib in patients with bevacizumab-refractory metastatic renal cell carcinoma. *J Clin Oncol* 2008; **26**: 3743–8.

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Phase II study of S-1 monotherapy in platinum-refractory, advanced non-small cell lung cancer

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ABSTRACT

Objective: The aim of this study was to evaluate the efficacy and toxicity of a novel oral 5-fluorouracil formulation (S-1) as second-line therapy after platinum agent chemotherapy for advanced non-small cell lung cancer (NSCLC).
Methods: S-1 was administered orally at a dose of 80 mg/m^2 for 28 days, followed by 14 days of rest (1 cycle); treatment was repeated until disease progression, unacceptable toxicity, or patient refusal.
Results: Of the 46 patients enrolled in this study, 44 were evaluable. Six patients (14%) exhibited a partial response and 28 (64%) showed stable disease. Disease-control rate was 77.3% (34/44) (95% CI, 64.9–89.7%). The overall response rate was 14% (6/44) (95% CI, 3.5–23.8%). Median progression-free survival was 4.2 months. The median survival time was 16.4 months, and the one-year survival rate 60.3%. Grade 3/4 hematological toxicities were minor. All of those adverse reactions were tolerable and reversible.
Conclusion: This study demonstrated the efficacy of S-1 monotherapy as second-line treatment for advanced NSCLC.

1. Introduction

Lung cancer is the most common cancer worldwide, with an estimated 1.2 million new cases globally (12.3% of all cases of cancer) and 1.1 million deaths (17.8% of all cancer deaths) in 2000 [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. For chemotherapy-naive patients with good performance status (PS) at a stage of IIIB (with pleural effusion) or IV, platinum-based chemotherapy offers a modest survival advantage over best supportive care (BSC) alone [2–4]. To improve outcome, however, there is a need for novel regimens. A recent phase III clinical study of conventional second-line therapies reported median survival times (MST) of approximately 6–8 months for docetaxel, gefitinib, and erlotinib [5–8]. On the other hand, in Japanese trials targeting this population have showed more favorable MST of approximately 12–17 months [9,10]. Doublet chemotherapy as second-line treatment for advanced NSCLC significantly elevates response rate and prolongs progression-free survival (PFS), but it is more toxic and does not improve overall survival rate compared to single-agent treatment [11].

S-1 is a novel, orally administered drug that is a combination of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and oteracil potassium (Oxo) in a 1:0.4:1 molar concentration ratio [12]. CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase, which is involved in the degradation of 5-FU, and acts to maintain efficacious concentrations of 5-FU in plasma and tumor tissues. Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, reducing the serious gastrointestinal toxicity associated with 5-FU. The antitumor effect of S-1 has already been demonstrated in a variety of solid tumors such as advanced gastric cancer [13,14], colorectal cancer [15], head and neck cancer [16,17], breast cancer [18], pancreatic cancer [19,20] and biliary tract cancer [21]. A recent phase II study of S-1 for chemo-naive, advanced NSCLC patients yielded promising results, with a response rate of 22.0%, MST of 10.2 months and favorable toxicity profile [22]. S-1 has exhibited promising activity against several tumors. Moreover, treatment with it can be administered on an outpatient basis. Oral agents such as S-1, which feature few adverse events, are appealing in the 2nd or 3rd line setting when patients are in less favorable condition.

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We therefore conducted a prospective study to evaluate the efficacy and feasibility of S-1 monotherapy in patients with progressive, advanced NSCLC under platinum-based, first line chemotherapy.

2. Patients and methods

2.1. Patient eligibility

Patients were required to meet the following eligibility criteria: histologically confirmed NSCLC; at least one measurable lesion; prior platinum-based treatment; Eastern Cooperative Oncology Group performance status of 0–1; age ≥20 years, adequate organ function, defined as a leukocyte count of 3500–12,000 mm^3, neutrophil count of more than 2000 mm^3, platelet count of more than 100,000 mm^3, hemoglobin level of more than 9.0 g/dl, a serum total bilirubin level within 2.0 times the ULN, and serum creatinine level not exceeding the ULN; partial pressure of arterial oxygen ≥60 mm Hg, and an estimated life expectancy of at least 3 months. The exclusion criteria were as follows: pregnancy, serious concomitant disease (brain metastasis, active infection, severe heart disease or uncontrolled diabetes mellitus), concomitant malignancy, pleural effusion requiring treatment, symptomatic cerebral involvement, and obvious interstitial pneumonia or pulmonary fibrosis on chest radiography.

All patients gave written informed consent prior to enrollment in the study, and the protocol was approved by the Institutional Ethics Committee of each participating institution.

2.2. Study treatment

The initial doses of S-1 were assigned on the basis of body surface area (BSA). Accordingly, the patients received one of the following oral doses divided in two and administered daily after meals: 80 mg for patients with BSA < 1.25 m^2, 100 mg for BSA ≥ 1.25 and <1.50 m^2, and 120 mg for BSA ≥ 1.50 m^2. One therapy cycle comprised the administration of single-agent S-1 for 28 consecutive days followed by 14 days of no treatment. This schedule was repeated every 6 weeks until the occurrence of disease progression, unacceptable toxicities, or patient refusal. In the absence of evidence of disease progression, patients were allowed to continue S-1 treatment. A dose reduction of 20 mg/day was recommended if ≥grade 3 hematological or non-hematological toxicity occurred in the previous cycle; dose re-escalation was not allowed. Patients who required more than 4 weeks of rest for recovery from any toxicity other than nausea, vomiting, or anemia, or who required a dose reduction >20 mg/day, were withdrawn from the study.

2.3. Evaluation of response and toxicity

Before entering the study, all patients provided a detailed medical history and underwent a complete physical examination, complete blood cell count, serum chemistry examination, chest computed tomography scan (CT), determination of weight and height, and determination of ECOG PS. A CT scan was performed for tumor assessment within 14 days of initiation of study treatment and was repeated every 1–2 months. Physical examination, symptom evaluation, and routine blood tests and biochemistry blood examination were performed every 2 weeks during the treatment. Objective response was evaluated every 2 months. Ratings of complete response, partial response (PR), stable disease (SD), progressive disease (PD) or not evaluated (NE) were made according to RECIST version 1.0. Toxicity was evaluated every 2 weeks according to the Common Terminology Criteria for Adverse Events (CTCAE), version 3.0.

Table 1
Patient characteristics (n = 44).

Characteristic	No. of patients	%
Sex		
Male	35	79.5
Female	9	20.5
Age, years		
Median	64	
Range	47–79	
ECOG performance status		
0	13	29.5
1	31	70.5
Stage		
IIIB	15	34.1
IV	29	65.9
Histology-no. (%)		
Adenocarcinoma	30	68.2
Squamous cell carcinoma	11	25.0
Large cell carcinoma	1	2.3
Others	2	4.5
First line therapy		
Carboplatin/paclitaxel	33	75.0
Carboplatin/gemcitabine	5	11.4
Carboplatin/vinorelbine	3	6.8
Carboplatin/docetaxel	2	2.3
Cisplatin/docetaxel	1	4.5
Response of first line therapy		
PR	15	34.2
SD	6	13.6
PD	13	29.5
NE	10	22.7

2.4. Study design and statistical analysis

This was a phase II single-arm trial. The primary endpoint was evaluation of the activity, defined as the disease control rate at 8 weeks, of S-1 as a single agent in patients with NSCLC. Disease control rate (DCR) was defined as the percentage of patients without disease progression (CR, PR, or SD) and still on treatment at 8 weeks. DCR was confirmed and sustained 8 weeks or longer. Secondary endpoints included PFS, OS, and safety. PFS was defined as the time from the first day of administration of the study drug to disease progression or death for toxicity or disease progression. OS was defined as the time from the first day of study drug administration to death or last contact.

The primary endpoint of the trial was the DCR (CR + PR + SD according to RECIST criteria) at 8 weeks. The target sample size was 45 patients. Given a threshold DCR of 40% and expected DCR of 60%, the required sample size was estimated to be 39 patients, with $\alpha = 0.05$ and power $\beta = 0.8$.

3. Results

3.1. Patients

From June 2005 to May 2007, 46 patients were enrolled. Of these 46 patients, one was found to be ineligible because of 3rd line treatment, and one other patient withdrew consent. Thus, 44 patients remained in the analysis.

A total of 43 patients had received carboplatin-based chemotherapy, and one patient had received cisplatin-based chemotherapy. Of these 44 patients, one had exhibited CR, 14 PR, 6 SD, and 23 PD. The baseline characteristics of these patients and their tumors are in listed in Table 1.

3.2. Treatments

The 44 patients received a total of 70 cycles of chemotherapy, with a median number of cycles of two (range, 1–7). The dose of S-1 was reduced in 2 patients because of either grade 3 diarrhea

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Table 2
Patient characteristics in relation to the response.

Response rate *n* (%) (2nd line)	
CR	0
PR	6(13.6)
SD	28(63.6)
PD	5(11.3)
NE	5(11.3)
Response rate	13.6 (95% CI, 5.0–20.0%)
Disease control rate	77.3 (95% CI, 64.9–89.7%)

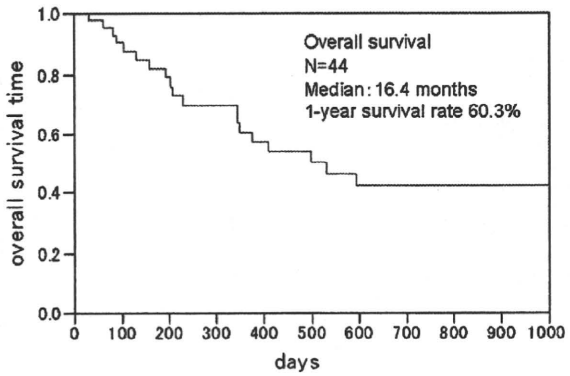

Fig. 1. Overall survival of patient treated with S-1 monotherapy after failure of first-line (platinum-base therapy) treatment.

and abnormal amylase level. The median relative dose intensity for the entire study population was 97.3%, indicating that patient compliance with S-1 chemotherapy was good.

3.3. Efficacy

A total of 44 patients were evaluable for response. Six patients achieved PR, resulting in an overall response rate (RR) of 13.6% (95% CI, 5.0–20%). Twenty-eight patients had SD, yielding an overall DCR of 77.3% (95% CI, 64.9–89.7%). The RR and DCR were evaluated separately from those for the previous first-line chemotherapy (Table 2). At the time of median follow-up interval of 18 months, 20 patients were still alive and censored; MST was calculated for all 44 patients, and OS was 16.4 months, the one-year survival rate 60.3%, and PFS 4.2 months The Kaplan–Meier survival curve is shown in Figs. 1 and 2.

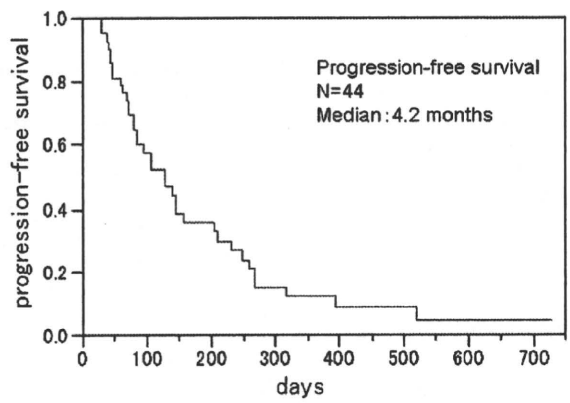

Fig. 2. Progression-free survival of patient treated with S-1 monotherapy after failure of first-line (platinum-base therapy) treatment.

Table 3
Hematologic and non-hematologic toxicities.

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3/4 (%)
Leukopenia	1	1	2	0	4.5
Neutropenia	2	1	2	0	4.5
Anemia	5	1	2	0	4.5
Diarrhea	0	2	1	0	2.3
Amylase	0	0	1	0	2.3
Pulmonary fibrosis	0	0	0	1	2.3

3.4. Safety

All patients were assessed for toxicities, which are listed in Table 3. No grade 4 or more severe hematological toxicities were observed; grade 3 events included two episodes each (4.5%) of leucopenia, neutropenia, and anemia. Non-hematological toxicities included one episode each (2.3%) of grade 3 diarrhea, grade 3 anorexia, grade 3 increase in amylase, and grade 4 interstitial pneumonia, each of which recovered with appropriate treatment. The most common hematological and non-hematological toxicities were mild or moderate. There were no treatment-related deaths directly attributable to S-1 chemotherapy.

4. Discussion

Recently, docetaxel has been demonstrated to be useful as second-line therapy for non-small cell lung cancer (NSCLC). Pemetrexed was then found to yield a response rate, overall survival, and PFS similar to those for docetaxel while producing many fewer adverse events; it has therefore been approved as a regimen for second-line therapy in Europe and the United States. Moreover, EGFR-TKI also became a treatment option for second-line therapy for NSCLC, based on the results of comparative studies with docetaxel and BSC. However, it was also reported that pemetrexed was ineffective against squamous cell carcinoma and that EGFR-TKI exhibited only poor efficacy in a group without EGFR gene mutation (exon 19, exon 21) [23–26].

In addition, the combination chemotherapy using two agents exhibited a better response rate and longer PFS than single-agent chemotherapy. On the other hand, since combination chemotherapy produced more toxicity and did not yield prolongation of overall survival compared with single-agent chemotherapy, single-agent chemotherapy has been recognized as effective as second-line therapy. It has also been reported that response rate with second-line or later chemotherapy worsens each time compared with first-line chemotherapy (first 20.9%, second 16.3%, third 2.3%, fourth 0.0%). Therefore, less toxic and highly tolerable single-agent therapy which can be administered on an outpatient basis without impairing QOL, in addition to demonstrating high response rate, is required [27].

Since it was shown in Phase II studies of S-1 in untreated advanced NSCLC that S-1 has a 22.0% anti-tumor effect with low frequency of serious adverse reactions, the present study was conducted to evaluate the efficacy and safety of S-1 in patients with NSCLC previously treated with platinum-based therapy. The median age of eligible subjects in our study was 64 years, and 70% of them had adenocarcinoma. Of the total of 44 evaluable patients, 6 (14%) were responsive to the treatment. Disease control was achieved in 34 patients (77.3%). Grade 3 or more severe toxicities were infrequently observed, including two episodes each (4.5%) of leucopenia, neutropenia, and anemia as hematological toxicities, and one episode each (2.3%) of diarrhea, nausea/vomiting, anorexia, increase in amylase, and interstitial pneumonia as non-hematological toxicities. Throughout all courses in this study, a total of 16% of the patients discontinued, while 4.5% of them was because of violation of discontinuation criteria. Dose inten-

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sity exhibited a good percentage of 97.3%, however, change of treatment schedule to two-week dosing followed by one-week wash-out and dose adjustment should be considered in some cases. The major treatment category (inpatient/outpatient) was outpatient therapy, suggesting that this treatment can be safely administered in an ambulatory setting as well. OS, one-year survival rate, and PFS with this treatment were 16.4 months, 57%, and 4.2 months, respectively, suggesting that approximately the same results as obtained with other monotherapies can be achieved with S-1 [28].

On the other hand, Yamamoto et al. reported S-1 may be no difference in efficacy according to histological type [29]. Therefore, we compared squamous cell carcinoma with non-squamous cell carcinoma of PFS (Non-sq;3.6M, Sq: 4.6M) and OS (Non-sq;13.6M, Sq: 17.6M), and no significant differences were observed. However, the patient number of the present study is too little to evaluate the histological deviation in the responders to S-1.

In conclusion, S-1 monotherapy appeared to be effective and highly tolerable as second-line therapy in patients with advanced/recurrent NSCLC previously treated with platinum-based therapy. This therapy could be an option for patients wishing to be treated by oral medication or in an ambulatory setting.

Conflicts of interest statement

None declared.

References

[1] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
[2] Stephens R, Fairlamb D, Gower N, Maslove L, Milroy R, Napp V, et al. The Big Lung Trial (BLT): determining the value of cisplatin-based chemotherapy for all patients with non-small cell lung cancer (NSCLC): preliminary results in the supportive care setting. Proc Am Soc Clin Oncol 2002;21:291a (abstr 1161).
[3] Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer—a meta-analysis using updated data on individual patients from 52 randomized clinical trials. BMJ 1995;311:899–909.
[4] Grilli R, Oxman A, Julian J. Chemotherapy for advanced non-small cell lung cancer: how much benefit is enough? J Clin Oncol 1993;11:1866–72.
[5] Shepherd FA, Dancey J, Ramlau R, Mattson K, Gralla R, O'Rourke M, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095–103.
[6] Fossella FV, DeVore R, Kerr RN, Crawford J, Natale RR, Dunphy F, et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-small Cell Lung Cancer Study Group. J Clin Oncol 2000;18:2354–62.
[7] Kim ES, Hirsh V, Mok T, Socinski MA, Gervais R, Wu YL, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer to gefitinib. Lancet 2008;372:1809–18.
[8] Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. Erlotinib in previously treated non-small-cell lung cancer. N Eng J Med 2005;353:123–32.
[9] Maruyama R, Nishiwaki Y, Tamura T, Yamamoto N, Tsuboi M, Nakagawa K, et al. Phase III study V-15-32, of gefitinib versus docetaxel in previously treated Japanese patients with non-small-cell lung cancer. J Clin Oncol 2008;26:4244–52.
[10] Ohe Y, Ichinose Y, Nakagawa K, Tamura T, Kubota K, Yamamoto N, et al. Efficacy and safety of two doses of pemetrexed supplemented with folic acid and vitamin B12 in previously treated patients with non-small cell lung cancer. Clin Cancer Res 2008;14:4206–12.
[11] Di Maio M, Chiodini P, Georgoulias V, Hatzidaki D, Takeda K, Wachters FM, et al. Meta-analysis of single-agent chemotherapy compared with combination chemotherapy as second-line treatment of advanced non-small-cell lung cancer. J Clin Oncol 2009;27:1836–43.
[12] Shirasaka T, Shimamoto Y, Ohshimo H, Yamaguchi M, Kato T, Yonekura K, et al. Development of a novel form of 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor-selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 1996;7:548–57.
[13] Sugimachi K, Maehara Y, Horikoshi N, Shimada Y, Sakata Y, Mitachi Y, et al. An early phase II study of oral S-1 a newly developed 5-fluorouracil derivative for advanced and recurrent gastrointestinal cancers. Oncology 1999;57:202–10.
[14] Koizumi W, Kurihara M, Nakano S, Hasegawa K. Phase II study of S-1, a novel oral derivative of 5-fluorouracil in advanced gastric cancer. Oncology 2000;58:191–7.
[15] Ohtsu A, Baba H, Sakata Y, Mitachi Y, Horikoshi N, Sugimachi K, et al. Phase II study of S-1, a novel oral fluoropyrimidine derivative, in patients with metastatic colorectal carcinoma. Br J Cancer 2000;83:141–5.
[16] Inuyama M, Kida A, Tsukuda M, Kohno N, Satake B. Early phase II study of S-1 in patients with advanced head and neck cancer. Jpn J Cancer Chemother 1998;25:1151–8.
[17] Inuyama M, Kida A, Tsukuda M, Kohno N, Satake B. Late phase II study of S-1 in patients with advanced head and neck cancer. Jpn J Cancer Chemother 2001;28:1381–90.
[18] Saeki T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, et al. A phase II study of S-1 in patients with metastatic breast cancer—A Japanese Trial by the S-1 Cooperative Study Group, Breast Cancer Working Group. Breast Cancer 2004;11:194–202.
[19] Saeki T, Takashima S, Sano M, Horikoshi N, Miura S, Shimizu S, et al. A late phase II clinical study of S-1 in patients with progressed, refractory breast cancer. Jpn J Cancer Chemother 2004;31:539–47.
[20] Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. An early phase II study of S-1 in patients with metastatic pancreatic cancer. Oncology 2005;68:171–8.
[21] Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane. Phase II study of S-1 in patients with advanced biliary tract cancer. Br J Cancer 2004;91:1769–74.
[22] Kawahara M, Furuse K, Segawa Y, Yoshimori K, Matsui K, Kudoh S, et al. Phase II study of S-1 a novel oral fluorouracil in advanced non-small-cell lung cancer. Br J Cancer 2001;85:939–43.
[23] Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, von Pawel J, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 2004;22:1589–97.
[24] Scagliotti GV, Parkih P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol 2008;26:3543–51.
[25] Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Eng J Med 2009;361:947–57.
[26] Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin docetaxel in patients with non-small lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomized phase 3 trial. Lancet Oncol 2010;11:121–8.
[27] Massarelli E, Andre F, Liu DD, Lee JJ, Wolf M, Fandi A, et al. A retrospective analysis of the outcome of patients who have received two prior chemotherapy regimens including platinum and docetaxel for recurrent non-small-cell lung cancer. Lung Cancer 2003;39:55–61.
[28] Totani Y, Saito Y, Hayashi M, Tada T, Kohashi Y, Mieno Y, et al. A phase II study of S-1 monotherapy as second-line treatment for advanced non-small cell lung cancer. Cancer Chemother Pharmacol 2009;64:1181–5.
[29] Yamamoto N, Yamanaka T, Ichinose Y, Kubota K, Sakai H, Genma A, et al. Pooled analysis of S-1 trials in non-small cell lung cancer according to histological type. Anticancer Res 2010;30:2985–90.

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A Phase II Study of Cisplatin and Irinotecan As Induction Chemotherapy Followed by Concomitant Thoracic Radiotherapy with Weekly Low-dose Irinotecan in Unresectable, Stage III, Non-Small Cell Lung Cancer: JCOG 9706

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Objective: It is important to identify optimal regimens of cisplatin-based, third-generation chemotherapy and thoracic radiotherapy for patients with unresectable, Stage III, non-small cell lung cancer.

Methods: Patients with unresectable, Stage III non-small cell lung cancer were treated with the following regimen: cisplatin 80 mg/m^2 on days 1 and 29, with irinotecan 60 mg/m^2 on days 1, 8, 15, 29, 36, and 43 and 30 mg/m^2 on days 57, 64, 71, 78, 85 and 92. Thoracic radiotherapy was started on day 57 at 2 Gy/day (total 60 Gy).

Results: From February 1998 to January 1999, 68 patients were enrolled. Grade 3/4 toxicities during induction chemotherapy primarily included neutropenia (73.5%) and diarrhea (20.6%), while Grade 3/4 toxicities during concomitant thoracic radiotherapy with irinotecan consisted of neutropenia (18.4%), esophagitis (4.1%) and hypoxia (6.5%). There was one treatment-related death due to radiation pneumonitis. The response rate was 64.7% (95% confidence interval, 52.2–75.9%). The median survival time was 16.5 (95% confidence interval, 12.6–19.8) months. The 1- and 2 year survival rates were 65.8% (95% confidence interval, 54.4–77.1%) and 32.9% (95% confidence interval, 21.6–44.1%), respectively. Overall, only 36 (56%) completed both the scheduled chemotherapy and thoracic radiotherapy.

Conclusions: Induction chemotherapy with cisplatin plus irinotecan followed by low-dose irinotecan and concomitant thoracic radiotherapy was feasible according to the prespecified decision criteria in this study for patients with unresectable Stage III non-small cell lung cancer. We did not decide to select this regimen for further investigations because approximately half of the patients completed the scheduled treatment.

Key words: cisplatin – irinotecan – chemoradiotherapy – non-small cell lung cancer