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ORIGINAL ARTICLE

The PCR-invader method (structure-specific 5′ nuclease-based method), a sensitive method for detecting EGFR gene mutations in lung cancer specimens; comparison with direct sequencing

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Abstract

Background Several sensitive assays, including the PCR-invader method (structure-specific 5′ nuclease-based method), have been used to detect EGFR mutations in non-small-cell lung cancer (NSCLC). However, validation has not been reported. We assessed the detection rate of EGFR mutation by the PCR-invader method and direct sequencing using same clinical specimens.

Patients and methods EGFR mutations were analyzed with the PCR-invader method and compared with direct sequencing using paraffin tissues and pleural and pericardial effusions from NSCLC patients. The relationships between the treatment responses and mutations were evaluated retrospectively.

Results Fifty-four samples from 42 NSCLC patients were studied. EGFR mutations were identified in 52% of the patients and 52% of the samples with the PCR-invader method, but only in 43% of the patients and in 35% of the samples by direct sequencing. In the samples obtained from the same patients at different sites and different times, EGFR mutations were coincident in nine out of ten patients by the PCR-invader method but in six out of ten patients by direct sequencing. Seventeen patients with EGFR mutations were treated with gefitinib; the response rate (RR) and disease control rate (DCR) were 41 and 94%, and median treatment duration was more than 6 months. Seven EGFR mutation-negative patients were treated with gefitinib; the RR and DCR were 0 and 14%, and median treatment duration was 1 month.

Conclusion The PCR-invader method was useful for detecting EGFR mutations in clinical lung cancer specimens and is more sensitive than direct sequencing.

Keywords EGFR mutation · Non-small-cell lung cancer · PCR-invader method · Direct sequencing · Validation · Gefitinib

Introduction

Lung cancer is the leading cause of cancer death in the world today. Recent efforts, including large-scale DNA sequencing, indicate that activating mutations in EGFR, BRAF, PI3K, and K-ras genes are generally non-overlapping and identifiable in approximately 40% of non-small-cell lung cancer (NSCLC) [1–4]. EGFR mutation has been reported as a very important factor in decision-making for treatment of NSCLC [3–6]. A recent prospective randomized phase III study (IPASS study) revealed the superiority of gefitinib (an EGFR inhibitor) to standard platinum-based treatment for first line treatment of lung adenocarcinoma, and documented a significantly higher response rate (RR) of 71.2% in EGFR mutation-positive patients but an extremely low RR of 1.1% in mutation-negative patients [5].

Several different EGFR mutation-detection methods are used in daily practical settings and laboratories [7–11], but

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standardization and validation of these methods are needed [12]. The PCR-invader assay (serial invasive signal amplification reaction with structure-specific 5′ nuclease using PCR product) is a sensitive method with which to detect gene mutations such as SNPs [13–17]. To compare the detection rate of EGFR mutation with the PCR-invader method and direct sequencing, which is the current standard [12], we analyzed clinical samples from NSCLC patients.

Patients and methods

Patients with NSCLC whose specimens were available for DNA extraction were eligible. From May 2007 to August 2008, 42 patients provided written informed consent at Yokohama Municipal Citizen's Hospital. Approval for the study was obtained from the institutional review board. Specimens (archived paraffin-embedded tissues, pleural effusions, and pericardial effusions) were obtained by surgery, transbronchial lung biopsy (TBLB), lymph node biopsy, or effusion drainage. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, California, USA). In cases with paraffin-embedded specimens, DNA was extracted with gross dissection with confirmation of adjacent slices having enough (at least 70% of surface area) cancer cells. DNA extraction, the PCR-invader method, and direct sequencing were performed by BML (Tokyo, Japan).

EGFR exons 18, 19, 20, and 21 were amplified by polymerase chain reaction (PCR) using specific primers (Table 1) with Ex *Taq* polymerase (Takara Bio, Shiga, Japan). PCR reaction was performed with the cycles: 94°C 2 min, 96°C 10 s, 65°C 30 s for 50 cycles, 72°C 7 min, 95°C 5 min, 4°C: hold. EGFR mutations were analyzed using the PCR-invader method (Fig. 1) [13–17] and the results were compared with those of direct sequencing. Briefly, in the initial reaction of the PCR-invader method, the target nucleic acid, invader-oligo, and signal probe form a three-dimensional invader structure. A highly specific enzyme (cleavase) recognizes the structure and cleaves the flap portion. In the second reaction, the released flap hybridizes with FRET-probe to make a three-dimensional structure as in the first reaction, and cleavase cleaves it to produce a fluorophore whose signal can be measured [13–17]. The PCR-invader method can only detect known mutations (exon 18: G719A/C/S, exon 19: deletion, exon 20: S768I, exon 21: L858R·L861Q, and known resistant mutation exon 20: T790M) by using probes specific to those mutations (Table 1). The "exon 19 deletion" mutations are in fact several types. The invader assay probes (Table 1) used here for the PCR-invader method are specially constructed to detect three types of "exon 19 deletion" mutations: E746-A750del type1 (DEL1; 2235-2249del GGAATTAAG AGAAGC), E746-A750del type2 (DEL2; 2236-2250del GAATTAAGAGAAGCA), and L747-P753del insS (INS-S). There are other mutations with the "exon 19 deletion", although the frequency is not high. To ensure detection of other types of "exon 19 deletion" mutations, the PCR-invader method included electrophoresis of exon 19 PCR products in the clinical setting. If there were several bands suggesting "exon 19 deletion" but the invader assay probes did not detect the three types of "exon 19 deletion", sequencing followed to elucidate the exact deletion.

Direct sequencing was performed using purified PCR products with a BigDye terminator sequencing kit (Ver. 1.1; Applied Biosystems, California, USA). Sequencing was carried out with the primers as indicated in Table 1. Sequencing was confirmed with forward and reverse reactions. An ABI Prism 3130xl genetic analyzer (Applied Biosystems) was used for analysis.

In patients treated with an EGFR-tyrosine kinase inhibitor (EGFR-TKI), the relationships between the responses (according to RECIST criteria [18]) and EGFR gene mutations were evaluated retrospectively.

To show the sensitivity of the PCR-invader method, sensitivity assay was done using cell lines with known EGFR mutation status, H1650 (exon 19 deletion: DEL1), H1975 (L858R and T790 M), and SK-MES-1 (wild type) [8, 19]. These cell lines were purchased from ATCC. Cell lines were mixed with different ratio, H1650 and SK-MES-1: 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0; H1975 and SK-MES-1: 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0. These cell mixtures were sent to BML (Tokyo, Japan) for the analysis of EGFR mutation by PCR-invader method. BML were not aware of the composition of the cell mixtures.

Results

Characteristics of patients and samples

The median age of the 42 patients was 65 years (range 33–82) (Table 2). All of the patients were Japanese. The histology was adenocarcinoma in 90% of patients, squamous cell carcinoma in 7% of patients. The samples used were archived paraffin-embedded tissues ($n = 49$), pleural effusion specimens ($n = 4$), and pericardial effusion specimens ($n = 1$).

EGFR mutation by the PCR-invader method

EGFR mutations were detected in 22 patients ($n = 22/42$: 52%) and in 28 samples ($n = 28/54$: 52%) by use of the PCR-invader method. Eleven patients (50%) had an exon

Table 1 Primers

Lesion	Name	Sequence	Modification
PCR primers			
Exon 18	EGFRex18-F	GAGAAGCTCCCAACCAAGCTC	
	EGFRex18-R	CAGGGACCTTACCTTATACACCG	
Exon 19	EGFRex19-F	GTCATAGGGACTCTGGATCCCA	
	EGFRex19-R	CAGCAAAGCAGAAACTCACATCG	
Exon 20	EGFRex20-F	GCCTCTCCCTCCCTCCAGGAAG	
	EGFRex20-R	CCGGACATAGTCCAGGAGGCA	
Exon 21	EGFRex21-F	CAGCCAGGAACGTACTGGTG	
	EGFRex21-R	CCACCTCCTTACTTTGCCTCC	
Invader assay probes (primer mixture)			
G719A	sG719A_S1f	CGCGCCGAGGCCCAGCACTTTGATCTT	3′ Amination
	sG719A_S2r	ACGGACGCGGAGGCCAGCACTTTGATCTT	3' Amination
	sG719A_Inv	ACCGTGCCGAACGCACCGGAGT	
G719C	sG719C_S1f	CGCGCCGAGGCCAGCACTTTGATCTTTTT	3′ Amination
	sG719C_S2r	ACGGACGCGGAGACAGCACTTTGATCTTTTTG	3′ Amination
	sG719C_Inv	CGTGCCGAACGCACCGGAGCT	
G719S	asG719S_S1f	CGCGCCGAGGGGCTCCGGTGCG	3′ Amination
	asG719S_S2r	ACGGACGCGGAGAGCTCCGGTGCG	3′ Amination
	asG719S_Inv	CTTGAGGATCTTGAAGGAAACTGAATTCAAAA AGATCAAAGTGCTGT	
"E746-A750del type1"	s746-del1_S1f	CGCGCCGAGGTGCTTCTCTTAATTCCTTGAT	3′ Amination
	s746-del1_S2r	ACGGACGCGGAGTTTGATAGCGACGGGA	3′ Amination
	s746-del1_Inv	CATCGAGGATTTCCTTGTTGGCTTTCGGAGATGTC	
"E746-A750del type2"	s746-del2_S1f	CGCGCCGAGGTTGCTTCTCTTAATTCCTTGA	3′ Amination
	s746-del2_S2r	ACGGACGCGGAGTCTTGATAGCGACGGG	3′ Amination
	s746-del2_Inv	ACATCGAGGATTTCCTTGTTGGCTTTCGGAGATGC	
"L747-P753del insS"	as747-delinsS_S1f	CGCGCCGAGGTTAAGAGAAGCAACATCTCC	3′ Amination
	as747-delinsS_S2r	ACGGACGCGGAGTCGAAAGCCAACAAGG	3′ Amination
	as747-delinsS_Inv	CAGAAGGTGAGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC	
S768I	sS768I_S1f	CGCGCCGAGGCTGGCCATCACGTAG	3′ Amination
	sS768I_S2r	ACGGACGCGGAGATGGCCATCACGTAGG	3′ Amination
	sS768I_Inv	GGCACACGTGGGGGTTGTCCACGT	
T790M	sT790M_S1f	CGCGCCGAGGGGTGATGAGGTGCACGGTG	3′ Amination
	sT790M_S2r	ACGGACGCGGAGATGATGAGGTGCACGGTG	3′ Amination
	sT790M_Inv	GCAGCCGAAGGGCATGAGCTGCT	
L858R	asL858R_S1f	CGCGCCGAGGTGGCCAAACTGCTG	3′ Amination
	asL858R_S2r	ACGGACGCGGAGGGGCCAAACTGCTG	3′ Amination
	asL858R_Inv	CCGCAGCATGTCAAGATCACAGATTTTGGGCC	
L861Q	sL861Q_S1f	CGCGCCGAGGAGTTTGGCCAGCC	3′ Amination
	sL861Q_S2r	ACGGACGCGGAGTGTTTGGCCAGCC	3′ Amination
	sL861Q_Inv	GCATGGTATTCTTTCTCTTCCGCACCCAGCC	
Sequencing primers			
Exon 18	EGFR18F	CATGCCGTGGCTGCTGGTCC	
	EGFR18R	AGTAGATGATGGAAATATACAGCTTGCA	

Table 1 continued

Lesion	Name	Sequence
Exon 19	EGFR19F	CAGATCACTGGGCAGCATGT
	EGFR19R	AGAGCAGCTGCCAGACATGA
Exon 20	EGFR20F	CCCTCCTTCTGGCCACCATGC
	EGFR20R	CCATGGCAAACTCTTGCTATCC
Exon 21	EGFR21F	AGAGCTTCTTCCCATGATGATCTG
	EGFR21R	ACAGCTAGTGGGAAGGCAGC

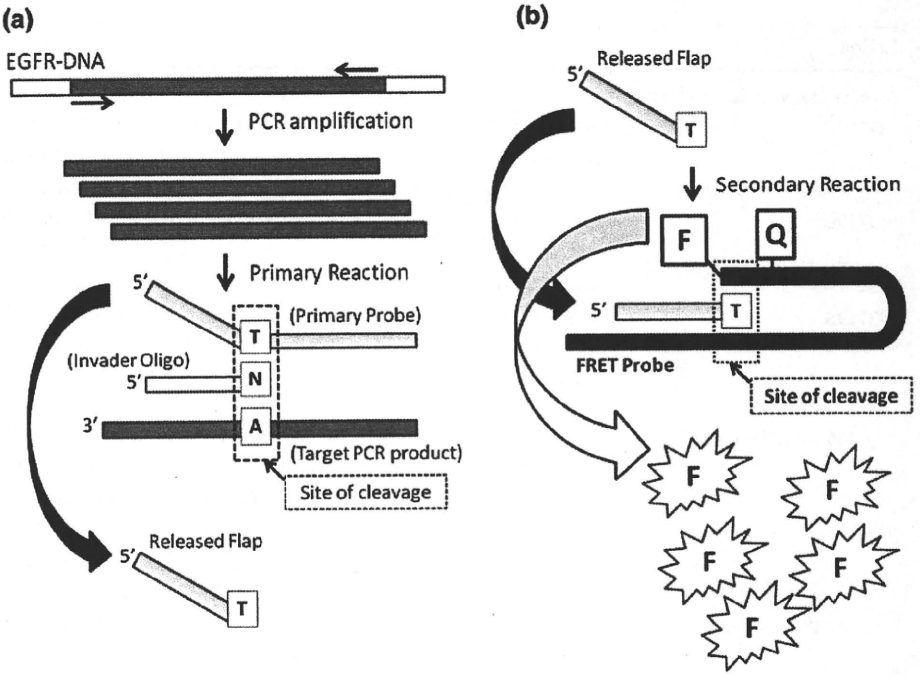

Fig. 1 Schematic illustration of the EGFR-invader method. Target DNA is amplified by multiplex PCR. During the primary reaction, an invader oligo and a matched primary probe are annealed to the target PCR product, overlapping at the mutation position (in this case "A"). The cleavase enzyme recognizes this three-dimensional structure and releases the 5′ flap. If the primary probe does not match the mutation position, cleavase will not act and cleavage of the primary probe will not occur. In the secondary reaction, the 5′ flap anneals to the FRET probe and the second cleavage reaction releases the fluorescent dye (Refs. [15–17]).
F fluorescein, *Q* quencher

Table 2 Baseline patients characteristics ($n = 42$)

Age	Median (range)	65 (33–82)
Sex	Male:female	15:27
Histology	Adeno:sqamous:large	38:3:1
Smoking	Never:former:current:unknown	16:12:8:6

Adeno adenocarcinoma, *squamous* squamous cell carcinoma, *large* large-cell carcinoma

19 deletion, 10 patients (45.5%) an L858R point mutation, and 1 case (4.5%) a L861Q point mutation. All mutations detected in this study were gefitinib-sensitive mutations and none had the EGFR-TKI resistant T790M mutation.

Comparison of PCR-invader and direct sequencing methods

The PCR-invader method detected EGFR mutations in 5 patients with a negative result by direct sequencing (Table 3). In contrast, direct sequencing detected a rare but known EGFR mutation (T847I) in one patient (2.4%) with a negative result by use of the PCR-invader method. Direct

Table 3 Comparison of PCR-invader method and direct sequencing for detection of EGFR mutation in 42 patients (a) and 54 samples (b)

	PCR-invader method	
	Mutation (+)	Mutation (−)
(a) Patients, *n* (%)		
Direct sequencing		
Mutation (+)	17 (40%)	1 (2%)
Mutation (−)	5 (12%)	19 (45%)
(b) Samples, *n* (%)		
Direct sequencing		
Mutation (+)	17 (31%)	1 (2%)
Mutation (−)	11 (20%)	25 (46%)

sequencing detected EGFR mutations in 18/42 patients (43%) and 19/54 samples (35%).

Sex, smoking, and EGFR mutation status

EGFR mutations were detected in 19/27 females (70%) and in 4/15 males (27%) (Table 4). EGFR mutations were detected in 13/16 non-smokers (81%) and in 5/20 smokers (25%).

Table 4 Relationship between EGFR mutation and sex (a) and smoking status (b)

Patients, *n* (%)	Mutation (+)	Mutation (−)
(a)		
Female	19 (45%)	8 (19%)
Male	4 (10%)	11 (26%)
(b)		
Never smoker	13 (36%)	3 (8%)
Smoker (current/former)	5 (1/4) (14%)	15 (7/8) (42%)

Mutation positive (+) means positive results from the PCR-invader method or from direct sequencing, or both

EGFR mutation search with different specimens

EGFR mutations detected by use of the PCR-invader method were coincident at different sites and different times in nine out of ten patients (Table 5). In one patient, the primary lung biopsy sample was negative for EGFR mutation, but a pleural effusion sample obtained at the time of relapse was positive for EGFR mutation. More precisely, the PCR-invader method yielded the same results for 9 different samples from 4 EGFR mutation-positive patients, and for 10 samples from 5 EGFR mutation-negative patients. The reproducibility of different times and different sites was verified in most cases by use of the PCR-invader method.

On the other hand, direct sequencing furnished coincident results for different specimens for only one patient out of five EGFR mutation-positive patients.

Treatment with EGFR-TKI

Twenty-four patients were treated with EGFR-TKI (gefitinib) at some point during the treatment courses (from 1st line to 6th line) (Table 6). In the EGFR mutation-positive patients ($n = 17$), RR was 41%, and the disease control

Table 5 EGFR mutations with different specimens

Pt	Sex	Sample	Date	EGFR mutation			
				Mutation	PCR invader result	Sequencing result	K-ras
1	F	Surgery	March 1999	(+)	L858R	(−)	ND
		TBLB	January 2006	(+)	L858R	L858R	ND
		Supra clavicular LN	June 2007	(+)	L858R	(−)	ND
2	F	Pleural effusion	May 2007	(+)	E746-A750del type2	(−)	ND
		TBLB	October 2005	(+)	E746-A750del type2	E746-A750del type2	ND
3	M	Surgery (rt-upper lobe)	March 2007	(+)	L858R	(−)	ND
		Surgery (rt-middle lobe)	March 2007	(+)	L858R	(−)	ND
4	F	TBLB	September 2007	Wild type	(−)	(−)	ND
		Pleural effusion	November 2007	(+)	E746-A750del type1	E746-A750del type1	ND
5	F	TBLB	August 2007	(+)	L747-A750del insP	L747-A750del insP	ND
		Pleural effusion	January 2008	(+)	L747-A750del insP	L747-A750del insP	ND
6	F	Upper lobe (partial resection)	February 2007	Wild type	(−)	(−)	Wild type
		Lower lobe	February 2007	Wild type	(−)	(−)	Wild type
7	M	TBLB	September 2006	Wild type	(−)	(−)	ND
		Neck LN	September 2006	Wild type	(−)	(−)	ND
8	M	Surgery	September 2006	Wild type	(−)	(−)	Mutation (GAT)
		Partial resection	May 2007	Wild type	(−)	(−)	Mutation (GAT)
9	M	TBLB	December 2007	Wild type	(−)	(−)	ND
		Pericardial effusion	January 2008	Wild type	(−)	(−)	ND
10	M	TBLB	December 2007	Wild type	(−)	(−)	ND
		Brain metastasis	January 2008	Wild type	(−)	(−)	ND

Pt patient, *date* the time the samples were taken from patients, *mutation* final decision from both PCR-invader result and sequencing result, (+) mutation positive, (−) EGFR wild type, *ND* not determined

rate (DCR; PR + SD) was 94%. The range of treatment duration was 3 weeks to more than 20 months and the median treatment duration was more than 6 months. In the EGFR mutation-negative patients ($n = 7$), RR was 0% and DCR was 14%. The range of treatment duration was 2 weeks to 2 months and the median treatment duration was 1 month. Patients with EGFR mutation were treated longer and had better disease control with EGFR-TKI.

Assessment of the sensitivity of the PCR-invader method

To show the sensitivity of the PCR-invader method, sensitivity was assessed by use of cell lines with known EGFR mutation status. As shown in Fig. 2, exon 19 deletion was detected in the cell line mixture of 1% (Fig. 2d) to 0.1 % (Fig. 2e) of a mutation-positive cell line (H1650). Also, L858R and T790M were detected in the cell line mixture of 1% (Fig. 2j) to 0.1 % (Fig. 2k) of a mutation-positive cell line (H1975), although with borderline threshold for T790M in the 0.1% mixture (Fig. 2k). For the exon 19 deletion, the results were positive for both DEL1 and DEL2 in Fig. 2b and f, although the fluorescence level is much higher for DEL1. This suggests cross-reaction might be a problem for exon 19 deletion, especially for amounts of mutation-positive cells as high as 50% (Fig. 2b, f). According to the results, the PCR-invader method can detect the known EGFR mutation in lung cancer cells at 100–1000-fold dilution.

Discussion

The PCR-invader method detected EGFR mutations in clinical lung cancer specimens more effectively than direct sequencing. Direct sequencing detected a rare but known mutation in one patient. Most important mutations can be detected by use of the PCR-invader method. By use of the PCR-invader method, EGFR mutations were coincident in samples obtained from the same patients at different sites

Table 6 Comparison between EGFR mutation status and response to EGFR tyrosine kinase inhibitor

Pt	Sex	Histology	EGFR mutation	PCR-invader	Sequencing	EGFR-TKI	Response	Duration[a]
1	F	Ad	(+)	DEL1	DEL1	1st	PR	7M
2	F	Ad	(+)	DEL1	DEL1	2nd	PR	6M
3	M	Ad	(+)	DEL2	DEL2	2nd	PR	10M
4	F	Ad	(+)	L747-T751del[b]	L747-T751del	2nd	PR	7M
5	F	Ad	(+)	L858R	L858R	1st	PR	6M
6	F	Ad	(+)	L858R	L858R	2nd	PR	8M
7	F	Ad	(+)	L858R	L858R	5th	PR	3M
8	M	Ad	(+)	DEL1	(−)	4th	SD	20M
9	F	Ad	(+)	DEL2	(−)	3rd	SD	4M
10	F	Ad	(+)	DEL1	DEL1	1st	SD	7M
11	F	Ad	(+)	DEL1	DEL1	3rd	SD	4M
12	F	Ad	(+)	DEL2	DEL2	2nd	SD	21M
13	F	Ad	(+)	L858R	L858R	1st	SD	1.5M
14	F	Ad	(+)	L858R	L858R	2nd	SD	7M
15	M	Ad	(+)	L858R	L858R	6th	SD	4M
16	F	Ad	(+)	L861Q	L861Q	4th	SD	4M
17	F	Ad	(+)	del insP[b]	del insP	2nd	PD	0.75M
1	M	Ad	(−)			2nd	PD	0.75M
2	F	Ad	(−)			2nd	PD	1M
3	F	Sq	(−)			2nd	PD	1M
4	F	Ad	(−)			3rd	PD	0.5M
5	M	Ad	(−)			3rd	PD	0.5M
6	M	Ad	(−)			3rd	PD	1M
7	M	Sq	(−)			3rd	SD	2M

Ad adenocarcinoma, *Sq* squamous cell carcinoma, *EGFR-TKI* the line of the chemotherapy when the EGFR-TKI was used, *DEL1* E746-A750del (2235-2249del GGAATTAAGAGAAGC), *DEL2* E746-A750del (2236-2250del GAATTAAGAGAAGCA), *del insP* L747-A750del insP

[a] Treatment duration with the EGFR-TKI (months)

[b] Detected on the basis of multiple bands in electrophoresis of PCR product, and after sequencing

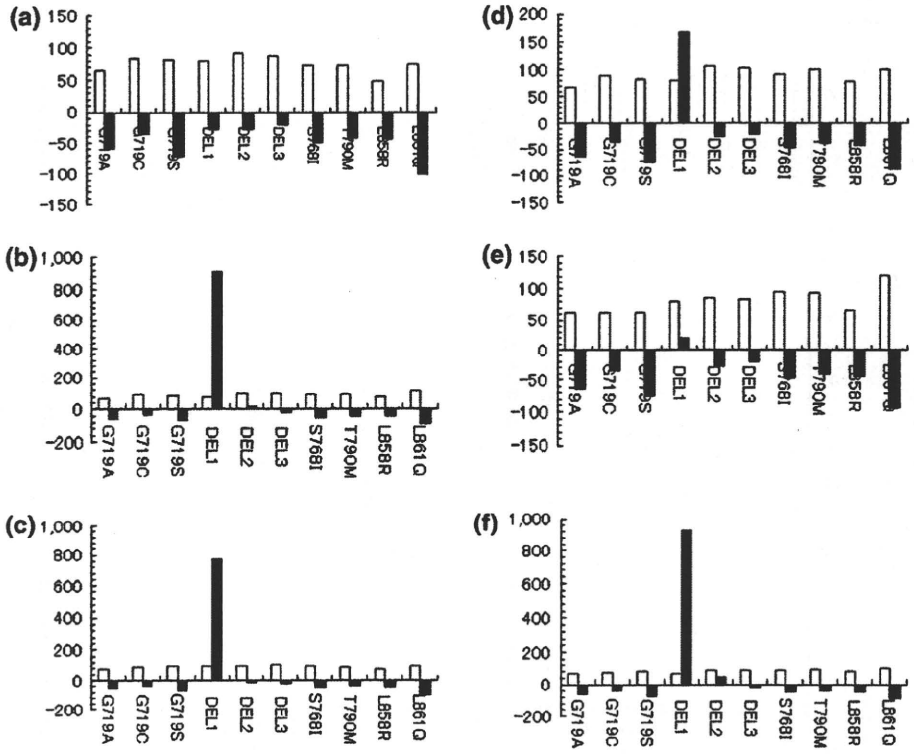

Fig. 2 Sensitivity assay of the PCR-invader method. Sensitivity assay was done using cell lines with known EGFR mutation status, H1650 (exon 19 del: DEL1), H1975 (L858R and T790M), and SK-MES-1 (wild type) (Refs. [8, 19]). Cell lines were mixed in different ratios. For **a** to **f**, H1650 and SK-MES-1 were mixed 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0. For **g** to **l** H1975 and SK-MES-1 were mixed 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0. *White squares* show quality control and a value above zero means PCR quality is good: the value = (fluorescence of the samples with detection probe for wild type sequences) − ([fluorescence of the normal control with the same probe] × 0.8). Values of *solid squares* above zero means positive results with the detection probe for mutation sequences excluding false positives: the value = (fluorescence of the samples with detection probe for mutation sequences) − ([fluorescence of the normal control with the same probe] × 2). To exclude false positivity, this criterion was introduced and used in clinical laboratories. Normal control is the mixture of white blood cell DNA from normal human volunteers. According to the results, PCR-invader methods could detect a known EGFR mutation of lung cancer cell lines diluted 100 to 1000-fold

and different times in nine out of ten patients. Time-independent and site-independent reproducibility were verified. By using the EGFR-invader method, we can only detect known targeted mutations. However, most of the important mutations are covered and other studies such as the IPASS study mainly targeted the major mutations [5].

It is not possible to draw any definite conclusions from the current results regarding the effect with EGFR-TKI, because the study design was not prospective. The RR of our study with mutation-positive patients was relatively low (41%) compared with reported clinical trials (RR 71.2–95%) [20–25]. Several possible reasons for this are the heterogeneity of our patients including treatment line (from 1st line to 6th line), and the fact it was a non-prospective study, which means evaluation to decide a RECIST response may not be sufficient. However, the DCR in this study (94%) was almost the same as that in recent reports (81–96.5%) [5, 20–25]. This higher DCR and longer treatment duration compared with EGFR mutation-negative patients suggested these are meaningful differences and that there are biological differences between these two groups classified according to the presence of EGFR mutation. EGFR mutation detected by the PCR-invader method can be a predictive marker of the effect with EGFR-TKI.

As shown in Table 6, association between results of treatment with EGFR-TKI and EGFR mutation status by the two methods (i.e. PCR-invader/direct sequencing, +/+ vs. +/−) are not evident. There are two EGFR mutation +/− (invader +, direct sequencing −) patients with the treatment effect of SD treated for 4 and 20 months with EGFR-TKI. On the other hand, 15 patients with EGFR mutation +/+ (invader +, direct sequencing +) were treated with EGFR-TKI and resulted in 7 PR, 7 SD, and 1 PD. Because the patients with EGFR mutation +/− had reasonable results (SD) with EGFR-TKI, it seems the EGFR mutation results with PCR-invader method are predictive of treatment.

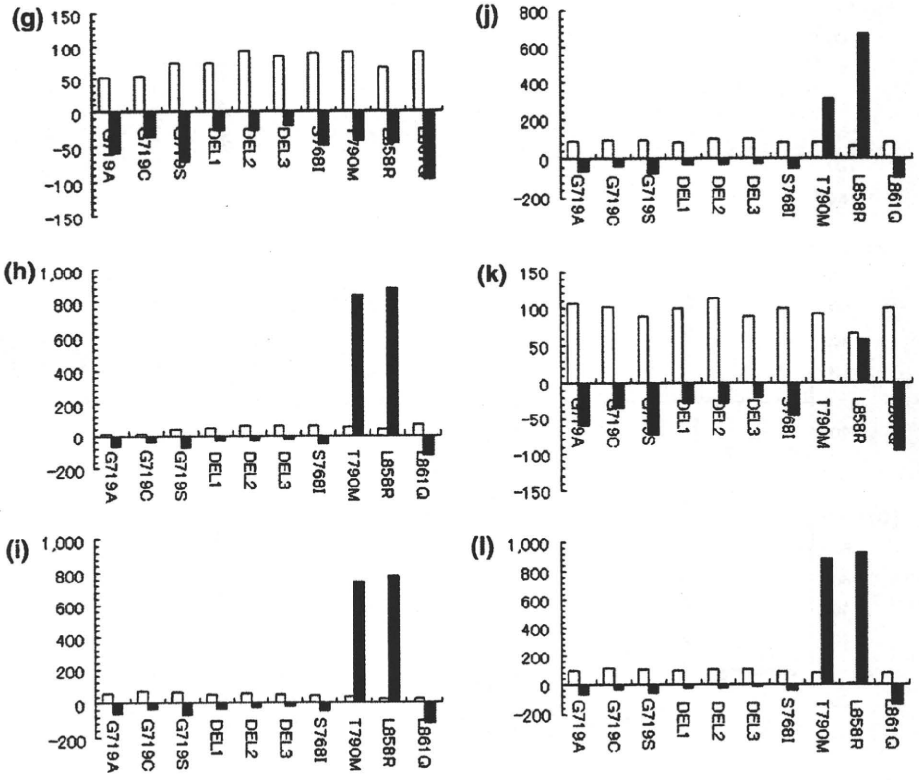

Fig. 2 continued

There are several limitations in this study. First, the work was carried out at a single institution in Japan. Samples were collected for the purpose of general clinical practice and some samples were several years old, so sample quality may be heterogeneous. No one knows the "true" positive rate for EGFR mutation in our samples. Treatments were also heterogeneous and were not prospectively planned. Even though there were sample-quality issues, we were able to detect a fairly reasonable occurrence of mutation in this study. In cases with negative results, the possibility of false negative (because of quality of samples, etc.) should be evaluated, but positive results obtained by this method should be regarded as truly positive.

One of the issues regarding the sensitivity of mutation detection with archived specimens is the quality of the formalin-fixed samples. Previous reports suggested the feasibility of combining conventional DNA extraction and the PCR-invader method using formalin-fixed paraffin wax tissues [26]. In our hands, we could not elucidate the direct effect of formalin fixation on the quality of DNA and the sensitivity of mutation detection method, even though we used the same samples with the PCR-invader method and with direct sequencing. Further study is warranted to clarify the direct effect of formalin fixation on the sensitivity of the PCR-invader method and direct sequencing.

Exon 20 insertion was not detected by the PCR-invader method, because the PCR primers were not constructed to detect those mutations. Exon 20 insertion is thought to be a resistant mutation [27]. In a review paper, the frequency reported is approximately 3% [28]. On the other hand, in the phase III study with 1st line gefitinib treatment, EGFR mutation was checked by the ARMS method, and no exon 20 insertion was detected in 132 EGFR mutation-positive patients (IPASS trial) [5]. In recent phase III studies done in Japan with 1st line gefitinib against EGFR mutation-positive NSCLC [29, 30], the targeted patients were mainly exon 19 deletion and L858R without T790M resistant mutation. Neither study has counted the exon 20 insertion mutation. One study used results from several detection methods including the PCR-invader method [29]. Exon 20 insertion may be important but from those large scale data and clinical trials, in treatment decision making with EGFR-TKI, the inclusion of exon 20 insertion detection does not seem to be mandatory.

For detection of "exon 19 deletion", it is very important that the "exon 19 deletion" are in fact several types as mentioned in "Patients and methods". In the 11 patients with "exon 19 deletion", 8 patients were diagnosed as DEL1 and DEL2 and none had INS-S. One patient was diagnosed as DEL2 by the PCR-invader method, but the sequencing results revealed the exact mutation was

Table 7 Frequency of different types of EGFR exon19 deletion

Repots	Current report	Tanaka (Cancer Sci 2007)	Sequist (JCO 2008)	Gow (Ann Oncol 2008)	Shigematsu (JNCI 2005)	Tamura (Br J Cancer 2008) [28]	Total
Method	PCR-invader	PNA-LNA-PCR clamp	Sequencing	Sequencing	Sequencing	Sequencing	
No. of Pt with del	$n = 11$	$n = 29$	$n = 18$	$n = 15$	$n = 62$	$n = 14$	n=149
Mutation type							
E746-A750del	8 (72.7%)	20 (69.0%)	14 (77.8%)	9 (60.0%)	42 (67.7%)	10 (71.4%)	103 (69.1%)
L747-P753del insS	0 (0%)	3 (10.3%)	1 (5.6%)	1 (6.7%)	3 (4.8%)	1 (7.1%)	9 (6.0%)
L747-T751del	1 (9.1%)	1 (3.4%)	1 (5.6%)	2 (13.3%)	4 (5.6%)	2 (14.3%)	11 (7.4%)
E746-T751del insA	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (7.1%)	2 (1.3%)
L747-A750del insP	1 (9.1%)	2 (6.9%)	1 (5.6%)	0 (0%)	3 (4.8%)	0 (0%)	7 (4.7%)
Others	0 (0%)	3 (10.3%)	1 (5.6%)	3 (20.0%)	10 (16.1%)	0 (0%)	17 (11.4%)

Values given are number of patients (%)

E746-T751del insA, one base different from DEL2. Two other patients had L747-T751del and L747-A750del insP, those were noticed by electrophoresis of the exon 19 PCR product with negative results with the invader assay probes specific to the three "deletion" mutations. Table 7 shows the frequency of different types of exon 19 deletion from several papers. Summation of these reports revealed that the percentage of the same exon 19 deletions detected in this study was 88.6%. Even with other exon 19 deletions which were not seen in our patients, the PCR-invader method with electrophoresis and subsequent sequencing may enable us to check other rare mutations.

It is very important to address false positivity in highly specific techniques. Basically, the PCR-invader method has always been performed with positive and negative controls for quality assurance. Furthermore, detection was conducted with pre-specified thresholds as follows. If the fluorescence of the sample with the mutation detection invader probe was more than twice that of normal control DNA with same probe, the samples were regarded as mutation positive (as shown in Fig. 2). To exclude false positivity, this criterion was introduced and has been used in general practice in Japan. In the sensitivity assay, average of fluorescence values of normal control DNA with mutation detection invader probe were 21–89 according to the probes (data not shown). On the other hand, actual fluorescence values of sample with mutation detection invader probe were approximately 1000–100. For example, in the sensitivity assay with H1975 cell line (Fig. 2g–l) the actual fluorescence values of the sample with L858R probe were 1013–142.5, and that with T790M were 966–79.5 according to the mixture rate with SK-MES-1. In contrast, normal control values were 42.5 for L858R and 39.0 for T790M. So the actual values were always more than twice as high as controls in this situation with up to 1:1000 cell mixture (Fig. 2). Even though we could not eliminate the possibility of contamination, for example sample carry over, the above mentioned threshold seems good enough to eliminate the non-specific false positive results.

Our results with relatively low RR might be because of hidden mutation (for example resistant mutation of exon 19 ins), and/or other clinical factors. We believe that the most likely reasons are the retrospective nature of this study and the patients' characteristics (mainly heavily treated patients). Further validation study in a prospective setting is warranted.

Several different sensitive EGFR mutation-detection methods are available in clinics in Japan. Direct comparisons of these sensitive methods are needed. However, on the basis of the results of this study, which compared the PCR-invader method and direct sequencing, which is the "current standard", the former can be regarded as a standard and reliable sensitive method.

In conclusion, the PCR-invader method detected EGFR mutations in clinical lung cancer specimens more effectively than direct sequencing. Time-independent and site-independent reproducibility was verified.

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Conflict of interest No author has any conflict of interest.

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Phase I Study of Topotecan and Cisplatin in Patients with Small Cell Lung Cancer

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Objective: A single-agent topotecan has an indication for the treatment of small cell lung cancer in Japan. Previous studies demonstrated that topotecan combined with a platinum agent could provide additional antitumor efficacy. This study was to find the recommended dose of topotecan in combination with cisplatin and preferred administration sequence in untreated patients with extensive disease small cell lung cancer for Phase II study.
Methods: Patients received topotecan as a 30 min infusion for 5 days in escalating doses (starting at 0.5 mg/m^2/day), and cisplatin at a fixed dose of 60 mg/m^2, 3 weeks cycle. This study employed the following stages: cisplatin was given before topotecan on day 1 to previously treated patients (Stage 1). After the maximum-tolerated dose level was achieved, the same schedule was applied for untreated patients (Stage 2). Subsequently, cisplatin was given after topotecan on day 5 to untreated patients (Stage 3). The recommended doses of cisplatin on day 1 and 5 schedules were estimated by considering results obtained from Stages 2 and 3, respectively.
Results: A total of 34 patients were enrolled. The maximum-tolerated doses in Stages 1–3 were estimated at 0.65, 0.65, and 1.4 mg/m^2, respectively. The recommended doses of cisplatin on day 1 and 5 schedules in untreated patients were determined at 0.65 and 1.0 mg/m^2, respectively. The major toxicity in this combination was hematological events.
Conclusions: For treatment-naive patients, the combined use of 0.65/60 mg/m^2 topotecan/cisplatin with cisplatin on day 1 schedule or 1.0/60 mg/m^2 topotecan/cisplatin with cisplatin on day 5 schedule is recommended for Phase II study.

Key words: small cell lung cancer – combination chemotherapy – topotecan – cisplatin

INTRODUCTION

Small cell lung cancer (SCLC) is often diagnosed at the extensive disease (ED) stage due to lesion location and rapid disease progression (1). Multiagent chemotherapy is the mainstay of treatment for SCLC, and combination regimens such as cisplatin + etoposide are being used as the standard therapy for ED cases (2). Topotecan, a topoisomerase-I inhibitor, has a favorable toxicity profile compared with most other agents that are active in SCLC. Topotecan has a well-characterized and predictable hematologic toxicity profile that includes neutropenia, which is manageable, short-lived and reversible. The non-hematologic effects of topotecan are generally mild and include manageable gastrointestinal toxicities (3). A single-agent topotecan showed significant activity in SCLC, particularly in patients sensitive to prior chemotherapy; therefore, the incorporation of topotecan in combination chemotherapy regimens for the future treatment of SCLC was warranted (3). Although single-agent topotecan has already an indication for the treatment of SCLC in Japan, previous preclinical and clinical studies have demonstrated that the combination of topotecan with a

platinum agent, such as cisplatin, could provide additional antitumor efficacy (4–9). In addition, one study addressed the impact of cisplatin scheduling and showed that the sequence of cisplatin before topotecan induced significantly worse hematological toxicity than the alternate sequence (10). To improve the therapeutic effect of this combination, the granulocyte colony-stimulating factor (G-CSF) was employed as concomitant therapy in our study.

Thus, we designed the present study to evaluate both administration sequences. The prime objective of the study was to determine the recommended dosage for a subsequent Phase II study from the estimation of maximum-tolerated dose (MTD) of topotecan in combination with 60 mg/m^2 cisplatin on day 1 or 5 in previously untreated patients with SCLC.

METHODS

Eligibility

Written informed consent was obtained from all patients prior to treatment. The protocol and informed consent procedures were reviewed and approved by the Institutional Review Board of each participating institute. Eligibility criteria were as follows: histologically or cytologically proven SCLC; 20–74 years old; previously treated with single-regimen chemo- and/or radiotherapy, or previously untreated patients with ED; no prior treatment with biological response modifiers within 2 weeks; adequate organic function (hemoglobin level $\geq$9.5 g/dl, leukocyte count of 4000–12 000/mm^3, neutrophil cell count $\geq$2000/mm^3, platelet count $\geq$100 000/mm^3, aspartate aminotransferase and alanine aminotransferase levels $<$2.5 times the upper limit of normal, total bilirubin value $<$1.5 mg/dl, serum creatinine below the upper limit of normal, partial pressure of arterial oxygen $\geq$60 mmHg); performance status of 0–1 on the Eastern Cooperative Oncology Group scale; a life expectancy of at least 3 months; and hospitalized patients.

Exclusion criteria included the following: serious infection or other serious concurrent disease; massive pleural effusion or ascites; interstitial pneumonia or pulmonary fibrosis; symptomatic central nervous system metastasis; concomitant malignancies; patients who received bone marrow or peripheral blood stem cell transplantation; a past history of drug allergy; actual or potential pregnancy, breast-feeding status or the intention to become pregnant in the near future; poorly controlled diabetes; previously treated with topotecan; or any other condition that was considered to make the patient ineligible for this study by the investigator.

Treatment Plan and Dose Escalation

This study employed the following three stages: Stage 1, cisplatin administration on day 1 followed by topotecan on days 1–5 to previously treated patients; Stage 2, on the same schedule as Stage 1 to previously untreated patients; and Stage 3, topotecan administration on days 1–5 followed by cisplatin on day 5 to previously untreated patients. The dose level of cisplatin was fixed at 60 mg/m^2 and the dose of topotecan was increased from 0.5 mg/m^2 followed by 0.65, 0.8, 1.0, 1.2 and 1.4 mg/m^2. The stage was moved up to the next after the MTD level was achieved, and the MTD was used as the starting dose in the next stage. Topotecan was administrated by 30 min intravenous infusion for 5 consecutive days. Cisplatin dissolved in 500–1000 ml of physiological saline was infused intravenously over 2 h either on day 1 (prior to topotecan) or day 5 (subsequent to topotecan). Immediately before and after each administration of cisplatin, hydration consisting of 1000–2000 ml of fluid infusion was given intravenously over 4 h. Treatment was repeated every 3 weeks and the next cycle could be expanded to a maximum of 35 days. At all dose levels of all stages, the prophylactic use of G-CSF was initiated from the next day of last administration of topotecan. G-CSF was administered till the recovery of leukocyte or neutrophil after nadir (recovery as either leukocyte count reached 10 000/mm^3 or neutrophil count reached 5000/mm^3).

In the present study, dose-limiting toxicity (DLT) was defined as follows: Grade 4 neutropenia lasting $\geq$4 days; Grade 3 or worse febrile neutropenia and thrombopenia ($<$20 000/mm^3); Grade 4 vasculitis, external auditory canal, fatigue, wound infection, ascites (non-malignant), constipation, central nervous system hemorrhage and hyponatremia; Grade 2 or worse middle ear/hearing, pneumonitis/pulmonary infiltrates and pulmonary fibrosis; and Grade 3 or worse other non-hematological toxicities excepting weight loss, syndrome of inappropriate antidiuretic hormone, anorexia, dyspepsia/heartburn, nausea, vomiting, incontinence and urinary frequency/urgency.

To assess the topotecan dose increase, three patients were enrolled at each dose level and the dose was increased to the next level if none of the patients displayed any DLT. If all the three patients showed DLT, then this dose level was defined as the MTD. If one of the three patients had DLT, an additional three patients were treated at the same level; if none/one of the three additional patients had DLT, the dose was increased to the next level. When two or more of the three additional patients had DLT, this dose level was defined as the MTD. If all the three additional patients showed DLT, the number of additional patients was determined by reference to results of estimation of the dose–response curve with a continual reassessment method. The recommended dose (RD) for Phase II study was determined to reflect the appearance of toxicity and antitumor effect as the drug dose less than the MTD.

Assessment of Treatment

Toxicities were assessed according to the US National Cancer Institute Common Toxicity Criteria (NCI-CTC), version 2.0. The severity of other events not listed in the

NCI-CTC was graded as follows: Grade 1, slight; Grade 2, moderate; Grade 3, severe; and Grade 4, life threatening. During the study, complete blood cell counts and biochemistry tests were repeated at least twice weekly, whereas other investigations were repeated as needed to evaluate marker lesions. Response was evaluated according to the modified World Health Organization (WHO) criteria (11).

Table 1. Patient characteristics

	Stage 1	Stage 2	Stage 3
Cisplatin injection	On day 1	On day 1	On day 5
Prior treatment	(+)[a]	(−)	(−)
No. of patients	9	6	19
Gender			
Male	9	6	16
Female	0	0	3
Age			
Median	59	57	65
Range	23–70	50–74	48–74
Performance status (ECOG)			
0	5	1	5
1	4	5	14
Clinical stage			
I	1	0	0
III	5	0	3
IV	3	6	16

ECOG, Eastern Cooperative Oncology Group.
[a]Cases previously treated with single-regimen chemo- and/or radiotherapy.

RESULTS

Estimation of MTD

Between March 2000 and February 2005, 34 patients were enrolled in this study and all of them received chemotherapy. The characteristics of these patients are shown in Table 1 and the occurrence of DLTs is shown in Table 2.

Stage 1: Cisplatin on Day 1 and Topotecan on Days 1–5, for Previously Treated Patients

No DLT occurred at the first cycle in three patients who received a topotecan dose level of 0.5 mg/m^2, and the dose of topotecan was increased to 0.65 mg/m^2. Since one of the three patients displayed a DLT of thrombocytopenia (<20 000/mm^3), another three patients were treated at the same dose. One of the additional three patients indicated Grade 4 neutropenia lasting 4 days or more as DLTs. No more increase in dose in this stage was, however, decided by Extramural Evaluation Committee (EEC) since in three out of six cases indicated DLTs at the second cycle, although the DLTs were observed in two out of six cases in the first cycle. The MTD of this stage was estimated as 0.65 mg/m^2.

Stage 2: Cisplatin on Day 1 and Topotecan on Days 1–5, for Untreated Patients

The starting dose of topotecan in Stage 2 was 0.65 mg/m^2 based on Stage 1 results. As one of three patients showed Grade 4 neutropenia lasting 4 days or more as DLTs, additional three patients were treated at the same dose. One out of the three cases indicated thrombocytopenia (<20 000/mm^3) and Grade 4 neutropenia lasting 4 days or more as DLTs. Although two out of the six cases indicated DLTs at the first cycle, three of the six cases indicated thrombocytopenia (<20 000/mm^3) only or thrombocytopenia (<20 000/

Table 2. DLTs during the first cycle or other cycles at different dose levels

	Stage 1		Stage 2	Stage 3				
Topotecan (mg/m^2)	0.5[a]	0.65[a]	0.65[b]	0.65[b]	0.8[b]	1[b]	1.2[b]	1.4[b]
No. of assessable patients	3	6	6	4	6	3	3	3
Toxic effects with first cycle/other cycles								
Grade 4 neutropenia ≥4 days	0/0	1/0	1/0	0/0	0/0	0/0	0/0	1/0
Thrombocytopenia (<20 000/mm^3)	0/0	1/0	0/1	0/0	0/0	0/0	0/0	0/0
Grade 4 neutropenia ≥4 days and thrombocytopenia (<20 000/mm^3)	0/0	0/0	1/2	0/0	0/0	0/0	0/0	0/0
Grade3 non-hematological toxicity[c]	0/0	0/0	0/0	0/0	1/0	0/0	0/0	0/0
Gait disturbance	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0
Atrial fibrillation	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0

[a]Prior therapy.
[b]Therapy naive.
[c]Intravenous antibiotic injection for infection.

Table 3. Grade 3/4 toxicities in 85 cycles

	Stage 1		Stage 2	Stage 3					Overall
Topotecan (mg/m^2)	0.5	0.65	0.65	0.65	0.8	1.0	1.2	1.4	
No. of patients	3	6	6	4	6	3	3	3	34
No. of cycles	6	12	23	6	11	9	11	7	85
Mean of cycles	2	2	3.8	1.5	1.8	3	3.7	2.3	
Hematological toxicities									
Anemia	0 (0)[a]	3 (1)	4 (0)	0 (0)	0 (0)	2 (0)	1 (0)	1 (0)	32.4%
Leukopenia	1 (1)	4 (4)	6 (5)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	38.2%
Neutropenia	2 (2)	4 (4)	6 (6)	0 (0)	0 (0)	1 (1)	1 (1)	1 (1)	44.1%
Thrombocytopenia	0 (0)	3 (2)	5 (3)	0 (0)	0 (0)	1 (0)	1 (1)	1 (1)	41.2%
Non-hematological toxicities									
Nausea	0 (0)	1 (1)	3 (2)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	17.6%
Vomiting	0 (0)	1 (1)	2 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	8.8%
Anorexia	0 (0)	1 (1)	3 (2)	0 (0)	1 (0)	0 (0)	0 (0)	1 (1)	17.6%
Interference with daily activity	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)	8.8%
Infection febrile	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2.9%
Increased amylase	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2.9%

Grade by the National Cancer Institute Common Toxicity Criteria (NCI-CTC).
[a]No. of patients in the first cycle are given in parentheses.

mm^3) and Grade 4 neutropenia lasting 4 days or more after the second cycle or later. EEC decided that no more proceed at this stage. Topotecan 0.65 mg/m^2 was estimated as the MTD of Stage 2, same with Stage 1.

Stage 3: Topotecan on Days 1–5 and Cisplatin on Day 5, for Untreated Patients

The first four patients, including one case who was decided as not evaluable from infection due to retaining needle, were treated with 0.65 mg/m^2 of topotecan based on the results obtained in Stage 2, and no DLT appeared. At the next dose level of 0.8 mg/m^2, one of three patients experienced DLTs, Grade 3 of infection as intravenous antibiotic injection. Then, additional three patients were enrolled at the same dose level, and none of the additional three patients had DLT. The dose of topotecan was increased as 1.0, 1.2 and 1.4 mg/m^2 in the protocol sequence. Three patients given 1.0 mg/m^2 and three patients given 1.2 mg/m^2 tolerated their dose level without DLT. Of the three patients given 1.4 mg/m^2, one patient developed Grade 4 neutropenia lasting 4 days. Following the hematological symptom, this patient also experienced Grade 3 gait disturbance. Furthermore, one case indicated an atrial fibrillation on day 3, although the relation between atrial fibrillation and topotecan was not clearly evidenced. Dose escalation was terminated at 1.4 mg/m^2; thus, we estimated the MTD of topotecan in this stage at 1.4 mg/m^2.

Gait disturbance occurred on day 10 of the first cycle following Grade 4 neutropenia. Twenty days later, in this patient, a cerebral infarction around the right lateral ventricle was observed by head magnetic resonance imaging diagnosis. This symptom has taken the medical history of cerebral hemorrhage without aftereffect and complications of hyperlipidemia and hyperuricemia into account. Then, this adverse event was observed for recovery tendency. Atrial fibrillation in a patient, who had a history of slight supraventicular arrhythmia without concomitant medication (not conflicted to exclusion criteria), noted at 1.4 mg/m^2 topotecan dose level, appeared just after completion of topotecan administration on day 3 in the first cycle and disappeared by oral anti-arrhythmic agent on the day 4 of the first cycle.

Toxicities

All 34 patients of 85 cycles were fully assessable for toxicity. Grade 3/4 toxicities during the overall cycles are summarized in Table 3. The most common hematological toxicity was neutropenia, followed by thrombocytopenia, leukopenia and anemia. On total comparison between the first cycle and overall cycles in hematological toxicity, at 0.65 mg/m^2 of Stage 1, 0.65 mg/m^2 of Stage 2 and 1.0 mg/m^2 of Stage 3, the occurrences of anemia and thrombocytopenia were increased. Leukopenia cases in 0.65 mg/m^2 of Stage 2 and anemia cases in over 1.2 mg/m^2 of Stage 3 were also increased. Average administration cycles in each dose

Table 4. Response to treatment

	Stage 1		Stage 2	Stage 3				
Cisplatin injection (fixed at 60 mg/m^2)	On day 1		On day 1	On day 5				
Prior treatment	(+)		(−)	(−)				
Topotecan (mg/m^2)	0.5	0.65	0.65	0.65	0.8	1.0	1.2	1.4
No. of patients	3	6	6	4	6	3	3	3
CR	0	0	0	0	0	0	0	0
PR	0	2	5	1	4	2	3	2
NC	2	3	0	0	0	0	0	0
PD	1	1	1	1	1	0	0	0
NE	0	0	0	2	1	1	0	1

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not evaluable.

level in each stage were less than two cycles in 0.5 and 0.65 mg/m^2 of Stage 1 and 0.65 and 0.8 mg/m^2 of Stage 3, and over two cycles in other doses of Stages 2 and 3.

Principal non-hematological toxicities were observed in six cases of nausea and anorexia, three cases of vomiting and interference with daily activity and one case of infection febrile and increased amylase, excluded as DLT events.

Clinical Response

Clinical response is shown in Table 4. Twelve (63%) of 19 patients in Stage 3 yielded partial response (PR), whereas out of nine patients administered 1 mg/m^2 and more than 1.0 mg/m^2, seven (78%) showed PR.

Recommended Dose

The MTD of topotecan on days 1–5 for therapy-naive patients in combination with cisplatin on day 1 administration was estimated as 0.65 mg/m^2. The RD for Phase II was decided as 0.65 mg/m^2 by considering clinical response and toxicity. The MTD of topotecan in the case of cisplatin on day 5 administration was estimated as 1.4 mg/m^2. The DLTs of this dosage level were atrial fibrillation and gait disturbance in non-hematological toxicity. Since unexpected/serious non-hematological adverse events were observed at topotecan 1.4 mg/m^2 dose level, the RD was tentatively considered at 1.2 mg/m^2, in which dose level, no DLT cases were observed. However, after the first course of 1.0 mg/m^2 dose level, frequencies of Grade 3/4 anemia and thrombocytopenia, as hematological toxicities, indicated increased tendency. Thus, the RD for Phase II was decided at 1.0 mg/m^2, to secure adequate safety.

DISCUSSION

The combination of topotecan and cisplatin has been investigated in several studies. Boabang et al. (5) reported an *in vitro* study result indicating synergistic antitumor activity between topotecan and cisplatin presumably due to the inhibition of DNA repair mechanisms. Since this drug is categorized as an inhibitor against topoisomerase-I, a previous administration of a DNA-injuring drug seems to be useful (12). An *in vitro* combination effect with cisplatin to topotecan was, however, recognized either pre- or post-administration for topotecan. Compared with day 5 administration of cisplatin, day 1 administration indicated an increase in hematotoxicity from the pharmacokinetic mechanism caused by renal dysfunction suspected as subclinical renal tubular damage (10). From this consideration, a Phase I clinical study in therapy-naive SCLC patients aiming at RD finding was planned from the safety and efficacy of cisplatin day 1 and 5 administration schedules, under the G-CSF concomitant use for the prevention of leukopenia/neutropenia.

The MTD of topotecan in this study was 0.65 mg/m^2 for cisplatin day 1 administration schedule and 1.4 mg/m^2 for cisplatin day 5 administration schedule. The DLTs which lead these MTDs were hematological toxicities. At the first course, neutropenia was observed at two of six in the cisplatin day 1 administration group and one of three in the day 5 administration scheduled group. One out of six patients in the cisplatin day 1 group also experienced thrombocytopenia. As for neutropenia, the incidence was similar to the DLT in topotecan monotherapy Phase I (7,13,14). In combination therapy of cisplatin and topotecan, the DLT of thrombocytopenia was reported associated with neutropenia (7,15). In this study, toxicity data on the second and further courses were also evaluated. In this evaluation, no discrepancy was found between the first course and further courses, which established the DLT in this study as neutropenia and thrombocytopenia.

The non-hematological DLTs were gait disturbance and atrial fibrillation, which were observed in the cisplatin day 5 administration group at 1.4 mg/m^2 of topotecan. No non-hematological DLT was observed in the cisplatin day 1 administration group. Grade 3 non-hematological toxicities of nausea, vomiting, anorexia, fatigue and interference with daily activity were observed as similar to other studies such as topotecan monotherapy and cisplatin combination in which Grade 3/4 non-hematological toxicities of nausea, vomiting, anorexia, fatigue and so on were recorded (7,13,15). These DLTs seem not to be this drug specified from clinical observation on occurrence and progress. Furthermore, no occurrence of Grade 3/4 diarrhea was observed, as different from the similar drug of irinotecan (16).

The MTDs of Stages 1 and 2 were estimated by taking not only the DLTs during the first cycle but hematological toxicity after the second cycle or further cycles into account. The RD for cisplatin on day 1 schedule was estimated from

the MTD of 0.65 mg/m^2 derived from consideration on incidence increment of Grade 3/4 hematological toxicities in all cycles, although the DLT observations were seen in two of six cases in the first cycle. On the other hand, cisplatin on day 5 schedule, MTD was estimated as 1.4 mg/m^2 from the observation of DLT in two of three cases at a 1.4 mg/m^2 dose level. As for RD for Phase II, 1.2 to 1.0 mg/m^2 was recommended since hematological toxicity occurrence situations were almost equivalent between 1.2 and 1.0 mg/m^2 dose levels, then for initial dose was recommended as 1.0 mg/m^2 taking safety consideration into account, but increment to 1.2 mg/m^2 was to be allowed from the second cycle based on the occurrence situation of hematological toxicity.

Overseas, the RD for topotecan monotherapy Phase II in therapy-naive SCLC patients was 1.5 mg/m^2 (14). The RDs for cisplatin day 1 administration combination were topotecan 0.75 and cisplatin 75 mg/m^2, respectively (7,9). On the other hand, the RDs were reported as topotecan 1.50 or 1.25 mg/m^2 and cisplatin 50 mg/m^2, respectively, for therapy-naive SCLC in cisplatin day 5 administration schedule (15,17). In Japan, the RD of topotecan in monotherapy was 1.0 mg/m^2. From this study, the RD of topotecan was 65% decreased from monotherapy as 0.65 mg/m^2 in cisplatin day 1 administration schedule and there was no difference from monotherapy in cisplatin day 5 administration schedule. The topotecan RD ratio between cisplatin day 1 administration schedule and day 5 (0.65/1.0) was similar to overseas reports (0.75/1.25–1.50). Therefore, the RD of topotecan was one half of monotherapy in cisplatin day 1 administration schedule and same or 80% in cisplatin day 5 administration schedule, although cisplatin RD still remains uncertain. The reason for RD reduction in cisplatin day 1 schedule is suspected as the increment of hematotoxicity caused by topotecan clearance decrease by subclinical renal tubular damage (10).

The dose of combined cisplatin was employed at 60 mg/m^2 as same dose in the case of the similar drug of irinotecan combination (18). In this study, no DLT occurrence was observed in each three cases of topotecan 1.0 and 1.2 mg/m^2 groups and average administration course numbers were over 3, which indicated the tolerability of this combination. No significant difference in observed toxicities were found compared with monotherapy (19), and previously conducted topotecan and cisplatin combination clinical studies (7,9,15,17). These findings suggested the validity of the cisplatin dose of 60 mg/m^2 in this combination.

Since neutropenia is DLT in topotecan monotherapy (6), the prophylactic use of G-CSF was initiated on day 6 of topotecan administration in this study. As the major DLT of this study was, however, neutropenia, the administration timing of G-CSF in this study might not affect the incidence of DLT. According to Saltz et al. (19), although G-CSF administration did not affect topotecan dose increase, it was suggested that the administration of G-CSF supported prevention against secondary infection or recovery. From safety consideration, the G-CSF concomitant use in this combination seems to be practical.

In conclusion, the RDs of topotecan for 5 consecutive days in combination with 60 mg/m^2 cisplatin in a 3-week cycle were 0.65 mg/m^2 with cisplatin on day 1 with G-CSF and 1.0 mg/m^2, a maximum of 1.2 mg/m^2, with cisplatin on day 5 with G-CSF. Further Phase II study of this combination chemotherapy for advanced/metastasis SCLC as first-line therapy is ongoing.

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Conflict of interest statement

None declared.

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Molecular and Cellular Pathobiology

Cancer Research

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 MMs. *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 oncoprotein YAP, a transcriptional coactivator, via phosphorylation, and inhibited MM cell growth. We also analyzed LATS2 immunohistochemically and found that 13 of 45 MM tumors had low expression of *LATS2*. Because *NF2* is genetically mutated in 40% to 50% of MM, our data indicate that Hippo pathway dysregulation is frequent in MM cells with inactivation of *LATS2* or an upstream regulator of this pathway, Merlin, which is encoded by *NF2*. Thus, our results suggest that the inactivation of LATS2 is one of the key mechanisms for constitutive activation of YAP, which induces deregulation of MM cell proliferation. *Cancer Res; 71(3); 873–83. ©2011 AACR.*

Introduction

Malignant mesothelioma (MM) is an aggressive neoplasm associated with asbestos (1–4). Because MM is usually diagnosed at advanced stages and is largely unresponsive to conventional therapy, the prognosis of patients with MM is very poor (5, 6). MM shows frequent mutation of $p16^{INK4a}$/$p14^{ARF}$ and *NF2* (*neurofibromatosis type 2*) tumor suppressor genes (TSG) 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 MMs (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 MMs 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.

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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% CO_2. 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–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 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).

Oligonucleotide array CGH analysis

All microarrays used were Agilent 244K whole human genome microarrays, with an average distance of 6.4 kb between each probe (array G4411B 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. The scanned TIFF image data were processed with Agilent Feature Extraction software (version 9.5.3.1) by the CGH-v4_95_Feb07 protocol (Agilent Technologies). Extracted data were analyzed with Agilent DNA Analytics 4.0 software (version 4.0.81; Agilent Technologies), and the Aberration Detection Method 2 (ADM-2) algorithm was used to identify contiguous genomic regions that corresponded to chromosomal aberrations. The following parameters were used in this analysis: threshold of ADM-2: 5.0; centralization: ON (threshold: 5.0, bin size: 10); aberration filters: ON (minimum number of probes in region = 2 and minimum absolute average log ratio for region = 1.6 and maximum number of aberrant regions = 10,000 and % penetrance per feature = 0). At a minimum, 2 contiguous suprathreshold probes were required to define a change. To find an obvious homozygous deletion in cell line DNA, aberrant regions with a signal $\log_2$ ratio of less than −1.6 were searched. Genomic positions were based on the UCSC March 2006 human reference sequence (hg18; NCBI build 36.1 reference sequence). The accession number of array CGH analysis data to Gene Expression Omnibus is GSE22237. For tumor tissue DNA, regions of homozygous deletion or one allelic loss of the *LATS2* locus were defined as $\log_2$ ratio < -1.0 or $-1.0 < -0.4$ for at least 3 consecutive probes, respectively.

Mutation analysis

Mutation analysis of all coding exons of the *LATS2* and *SAV1* and *NF2* genes was carried out by direct sequencing after PCR amplification of genomic DNA. The primer sets of *LATS2* are described in Supplementary Materials and Methods. The primer sets of *NF2* were described previously (11, 29), and sequences of the primer sets of *SAV1* are available upon request.

Antibodies and reagents

Rabbit anti-LATS2 antibody (NB200-199) for Western blot analysis was purchased from Novus Biologicals, and mouse anti-YAP (clone 2F12, H00010413-M01) and anti-SAV1 (clone 3B2, H00060485-M02) antibodies were from Abnova. Rabbit anti-LATS2 antibody (ab70565) for immunohistochemistry and rabbit anti-YAP antibody (EP1674Y) were purchased from Abcam, and anti-NF2 (1C4, #9169) and anti-phospho-YAP (S127; #4911) antibodies were from Cell Signaling Technology. Anti-β-actin (clone AC74) and anti-Flag (M2) antibodies were from Sigma, and anti-V5 antibody was from Invitrogen. Rabbit anti-β-catenin (SC-7199) was from Santa Cruz Biotechnology.

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 pLL3.7 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′-GGACCTCACTGCATTAAA-3′). A control shRNA vector for luciferase (Sh-Luc), which contained a target sequence for luciferase (5′-CGTACGCGGAATACTTCGA-3′), was described previously (32).

Cell proliferation assays

A total of 1.0×10^4 and 2.0×10^5 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,

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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-disulfophenyl)-2*H*-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; anti-β-catenin antibody, 1 μg/mL) for overnight at 4°C, followed by incubation with Alexa Fluor 488- or 564-conjugated secondary antibody for 30 minutes at RT. Nuclear staining was carried out with DAPI after incubation with secondary antibodies. The slides were mounted with PermaFluor Mounting Medium (Thermo). Microscopic observation was carried out using an Carl Zeiss LSM510 confocal laser scanning system at 63× magnification.

Additional materials and methods are described in Supplementary Materials and Methods.

Results

Homozygous deletions at 13q12.11 region in MM cells

We carried out array CGH analysis with 14 MM cell lines. As expected, we detected homozygous deletions at 9p21 and 22q12 in multiple cell lines, which harbor $p16^{Ink4a}/p14^{Arf}$ (*CDKN2A/2B*) and *NF2*, respectively (data not shown). Three cell lines showed homozygous deletion at 13q12.11, and the deletion regions in the cell lines Y-MESO-14 and Y-MESO-27 were overlapped and the one in Y-MESO-21 was located 20 to 30 kb away from them (Fig. 1A). FISH analysis also confirmed a deletion in Y-MESO-14 cell line (Fig. 1B). Because a single gene, *LATS2*, was located in this deletion region and homozygous deletions of all 3 cell lines disrupted this gene, we considered *LATS2* to be a strong candidate for a TSG of MM. Meanwhile, we did not detect any deletion at chromosome 6q25.1, which harbors another LATS homologue, *LATS1* (data not shown).

LATS2 mutations in MM cells

We carried out mutational analysis of *LATS2* with the 14 cell lines with other 6 MM cell lines. Genomic PCR analysis confirmed the homozygous deletions in the 3 cell lines, with compatible deletion patterns of the array CGH analysis (Fig. 2A). Because we detected another homozygous deletion in NCI-H2052, we carried out array CGH analysis and found that the homozygous deletion region of NCI-H2052 was different from that of the others (data not shown). We further found short PCR products in 2 cell lines and confirmed that MSTO-211H had a 42-bp deletion in exon 5 and that Y-MESO-30 had a 125-bp deletion, including 14-bp deletion of exon 6. We also found a nonsense mutation, *Y649X*, in Y-MESO-26B. Figure 2B summarizes 7 genetic mutations of *LATS2* in 20 MM cell lines (Fig. 2B). Because the original tumors were available for 2 cell lines with *LATS2* mutation, we analyzed them. In case of Y-MESO-30, we detected the same short fragment of exon 6 in the primary tumor but not in lymphocytes (Fig. 2C). This result indicated that the 125-bp deletion was somatic, which caused an aberrant transcript of *LATS2* (Fig. 2D). We also confirmed the nonsense mutation in the primary tumor of Y-MESO-26B, using a mutation-specific primer set (data not shown).

To validate *LATS2* mutation in primary MM tumors, we analyzed another cohort of 25 primary tumors with array CGH and/or sequencing analyses. We found that 2 tumors had significant loss (their $\log_2$ 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