TABLE 1. Homozygous Deletion and Expression of 17 Genes in Cancerous and Noncancerous Cultured Lung Cells (Extracted from Supplementary Table 1)

No.	Gene[a]	No. of cell lines with HD	Expression[b] T/N ratio	Expression[b] P value by t-test	Region no.	Chr band
3	LRP1B/LRP-DIP	7	1.19	0.30	3	2q22
18	FHIT	4	0.94	0.022	8	3p14
43	KANK1	1	0.81	0.0000067	19	9p24
47	PTPRD	8	1.26	0.0036	20	9p23
58	hsa-mir491*	3			23	9p21
60	PTPLAD2	6	0.72	0.0000000000015		
78	hsa-mir31*	10				
80/81	CDKN2A/p16/p14ARF[d]	21	0.74	0.00046		
82	CDKN2B/p15	19	0.59	0.000032		
131	RB1	1	0.50	0.00030	34	13q14
155	SMAD4	1	0.90	0.0019	41	18q21
157	KEAP1	2	0.97	0.46	42	19p13
167	hsa-mir-99a*	2			44	21q11
168	hsa-let-7c*	2				
169	hsa-mir-125b-2*	2				
175	ADAMTS1	1	0.39	0.000000048	45	21q21

Homozygous deletion / expression[c]: ADC (A427, A549, ABC1, HCC193, ...), SQC, ASC, LCC, SCC, Non-cancerous.

[a]Genes deleted in multiple cases are in RED. MicroRNA genes are marked by *.
[b]Marked in BLUE and RED when T/N ratio was <1 and p value by t-test was <0.05 and 0.00018, respectively.
[c]Homozygous deletion:
Expression examined by Affymetrix chips: Low – High; Presence call; Absence call
[d]p14ARF (Exon 1b) but not p16 is deleted in [illegible].

lines were also obtained from the American Type Culture Collection (Manassas, Virginia), the Japanese Collection of Research Bioresources (Tokyo, Japan), and the RIKEN BioResource Center (Tsukuba, Japan). Genomic DNA and poly A RNA were extracted by standard protocols.

Macro-dissected and micro-dissected cancerous and noncancerous lung cells were obtained from patients who were treated at the National Cancer Center Hospital, Tokyo, Japan. Details of these materials were described previously (Matsumoto et al., 2006; Nakamura et al., 2006). Genomic DNA and total RNA were extracted by standard protocols. This study was performed under the approval of the Institutional Review Board of National Cancer Center.

Detection of Homozygous Deletions by Array CGH Analysis

Copy number changes in genomic DNAs of 27 lung cancer cell lines were assessed using a Human CGH 185-k array covering 181,988 loci and Agilent CGH Analytics Software (Version 3.3) (Agilent Technologies). Genomic DNAs from these cell lines and 10 lymphoblastoid cell lines were analyzed according to the manufacturer's protocol using a human normal genomic DNA mix (Promega) as a reference. First, data for probes that were located in copy number variable regions deposited in the UCSC genome database and those that showed log2 ratios < -2.5 or >2.5 in 10 lymphoblastoid cell line DNAs in the present analyses were removed to mask copy number variable regions. Next, the copy number along the genome of 27 lung cancer cell lines was inferred by the Aberration Detection Method 2 (ADM2) algorithm. Autosomal regions encompassed by ≥ 2 consecutive probes with log2 ratios < -2.5 were defined as candidates for homozygously deleted regions. As the probes were placed at a mean interval of 15-kb, the present homozygous deletion search was undertaken at a 30-kb resolution.

Validation of Homozygous Deletions

One or more set(s) of PCR primers was designed to amplify genomic fragments that encompass an exon of genes deduced to be located in homozygously deleted regions by referring to the information in the UCSC database (http://genome.ucsc.edu/) and was subjected to multiplex PCR using the *IRF1* locus as a reference (Kishimoto et al., 2005). At least one exon was examined for each gene. Primer sequences are listed in Supporting Information Table 2. PCR products were separated by electrophoresis

TABLE 2. 34 Genes Mapped to Regions Other Than 9p21 and Homozygously Deleted in Two or More Lung Cancer Cell Lines

Chromosomal location	Gene	Gene product	No. of cell lines with homozygous deletion	(%)
9p23	PTPRD	Protein tyrosine phosphatase, receptor type D	8	(11)
2q21	LRP1B /LRP-DIP	Low density lipoprotein-related protein 1B	7	(9)
3p14	FHIT	Dinucleosidetriphosphatase	4	(5)
2q24	GRB14	Growth factor receptor-bound protein 14	2	(3)
2q24	COBLL1	COBL-like 1	2	(3)
2q24	SLC38A11	Solute carrier family 38, member 11	2	(3)
2q24	SCN3A	Sodium channel type III, alpha subunit	2	(3)
2q24	SCN2A	Sodium channel type II, alpha subunit	2	(3)
2q24	FAM130A2	TGF-beta induced apoptosis protein 2	2	(3)
2q24	GALNT3	UDP-N-acetyl-alpha-D-galactosamine	2	(3)
5q11	PDE4D	Phosphodiesterase 4D	2	(3)
5q31	CTNNA1	Alpha-catennin	2	(3)
7q35	CNTNAP2	Contactin associated protein-like 2	2	(3)
10p11	PARD3	Par-3 partitioning defective 3 homolog	2	(3)
18q21	ME2	Malate dehydrogenase 2	2	(3)
18q21	ELAC1	elaC homolog 1	2	(3)
19p13	KEAP1	Cytosolic inhibitor of Nrf2	2	(3)
19q13	MZF1	Myeloid zinc finger 1	2	(3)
19q13	MGC2752	Hypothetical LOC65996	2	(3)
21q11	LIPI	Membrane-associated phospholipase A1 beta	2	(3)
21q11	RBM11	RNA binding motif protein 11	2	(3)
21q11	STCH	Stress 70 protein chaperone	2	(3)
21q11	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	2	(3)
21q11	NRIP1	Nuclear receptor interacting protein 1	2	(3)
21q11	USP25	Ubiquitin specific peptidase 25	2	(3)
21q11	C21orf34	Chromosome 21 open reading frame 34	2	(3)
21q11	hsa-mir-99a	miRNA	2	(3)
21q11	hsa-let-7c	miRNA	2	(3)
21q11	hsa-mir-125b-2	miRNA	2	(3)
21q11	CXADR	Coxsackie virus and adenovirus receptor	2	(3)
21q11	BTG3/ANA	BTG/Tob family protein	2	(3)
21q11	C21orf91	Chromosome 21 open reading frame 91	2	(3)
21q11	CHODL	Transmembrane protein MT75	2	(3)
21q11	PRSS7	Enterokinase	2	(3)

on 3% agarose gel and visualized by staining with ethidium bromide. When no PCR product was detected, such an exon was judged as being homozygously deleted.

Expression Analysis of the 176 Genes Homozygously Deleted in Lung Cancer

Information on the expression levels for 23,583 genes of 15 lung cancer cell lines (A549, H157, H322, H1299, H1437, H1648, H2009, H2122, H2126, H2347, HCC95, HCC193, HCC366, and HCC515) was previously obtained by analysis using Affymetrix Gene Chips HG-U133A and HG-U133B (Zhou et al., 2006). Information on those of four other lung cancer cell lines (H223, H209, H841, and H2141) and cultured noncancerous lung epithelial cells (Ramirez et al., 2004) was obtained for the present study. Expression data for 160 of the 176 genes homozygously deleted in lung cancer cell lines were available, and these 160 genes were assessed by 281 probes (Supporting Information Table 1). Differences in expression levels between lung cancer cell lines and noncancerous lung epithelial cells were examined by *t* test, and probes with $P < 0.05$ were judged as significantly different. In addition, probes with $P < 0.00018$ were judged as significantly different after Bonferroni correction for multiple tests (i.e., $0.05/281 = 0.00018$).

Mutation Analysis of the *PTPRD* Gene

All coding exons of the *PTPRD* gene were amplified from 10 ng of DNA from 74 cell lines and 95 surgical specimens of lung cancer by PCR

using 44 sets of primers. PCR products from the cell lines were directly sequenced using a Big Dye Terminator Sequencing kit and an ABI Prism 3700 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). PCR products from the surgical specimens were subjected to WAVE analysis according to the manufacturer's protocol (Transgenomic, Omaha, Nebraska, USA). PCR products with different mobilities in the WAVE analysis were purified and directly sequenced.

Quantitative Real-time Reverse Transcription PCR (QRT-PCR) Analysis

Expression levels of the *PTPRD* gene were evaluated by QRT-PCR using ABI Prism 7900HT (Applied Biosystems). A Taqman probe (5′-AGGATCAATATCAGTTTTCCTA-3′) and a set of primers (5′-TGTTAAGAACACAACGAC CAGCTAT-3′ and 5′- TCAAAGCTGCCCAGG TACTCTAGT-3′) were used as previously described (Sato et al., 2005). PCR was performed in a single tube in duplicate. Results were expressed as the average of these two independent tests.

RESULTS AND DISCUSSION

Identification of Genes Homozygously Deleted in 52 Human Lung Cancer Cell Lines

Homozygously deleted regions in 43 lung cancer cell lines were previously searched for at a 100-kb resolution by a SNP array analysis, and 113 genes were deduced to map to homozygously deleted regions in one or more cell lines (Nagayama et al., 2007). These 113 genes consisted of three genes, *CDKN2A/p16*, *p14ARF* (a gene sharing the same exons with *CDKN2A/p16* but encoding a different protein) (Stone et al., 1995) and *CDKN2B/p15*, which are considered target tumor suppressor genes for homozygous deletions at chromosome band 9p21 (Hamada et al., 2000), and 110 genes located in regions other than 9p21 (Fig. 1).

In this study, homozygous deletions were further searched for by an array CGH analysis at a 30-kb resolution in 27 lung cancer cell lines consisting of 18 cell lines previously analyzed (Nagayama et al., 2007) and 9 cell lines newly prepared (Fig. 1). Among 113 genes found to be deleted in the previous study (Nagayama et al., 2007), 111 were verified in this study. Two genes, *THSD4* and *C20orf133*, considered to be homozygously deleted in the previous study, were not found to be deleted here. Therefore, these two genes were excluded from further analyses. In addition, 83 genes not indicated in the previous study were deduced to be homozygously deleted in the present 30-kb resolution analysis. These 83 genes consisted of 37 genes in the 9p21 region and 46 genes in other regions. Thus, in total, 194 genes (111 + 83) were considered to be homozygously deleted in 52 lung cancer cell lines by using two different DNA array analyses.

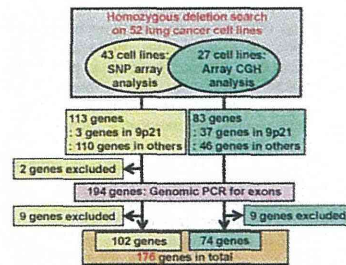

Figure 1. Strategy to identify genes homozygously deleted in lung cancer. A previous search on 43 lung cancer cell lines led to the identification of 113 genes deduced to be homozygously deleted (Nagayama et al., 2007). The present search on 27 lung cancer cell lines (18 cell lines overlaped) led to the identification of an additional 83 genes, and excluded two genes from the 113 genes above. Genomic PCR for exons of 194 genes led to the validation of homozygous deletions in 176 genes.

To confirm the homozygous deletions of these 194 genes, genomic PCR against DNA fragments encompassing an exon located in a homozygously deleted region was performed for all of the 194 genes (primer information in Supporting Information Table 2). One hundred seventy-six (91%) of the 194 genes showed homozygous deletions of at least one exon (Fig. 1). These 176 genes included *CDKN2A/p16* and *CDKN2B/p15*, and the results for deletions of these two genes determined by genomic PCR were consistent with the results determined by Southern blot analysis in our previous study (Okamoto et al., 1995). On the other hand, homozygous deletions of exons were not detected in the remaining 18 genes, probably due to the fact that only intronic or intergenic sequences were deleted or the deletions deduced were spurious ones caused by experimental errors. Therefore, these 18 genes were excluded from the remaining part of this study. Homozygous deletion of one or more genes was detected in 20 (74%) of the 27 cell lines analyzed by the present array CGH analysis, but not in the remaining seven cell lines. Among a total of 52 cell lines subjected to the present and/or previous homozygous deletion scanning, homozygous deletion of one or more genes was detected in 36 (69%) cell lines.

TABLE 3. PTPRD Mutations in Lung Cancer

Sample	Histological type	No. of exon	(nucleotide change)	Predicted effect	mRNA level[a]
Cell line					
Ma29	ADC	11	(C1184T: Homo)	Ala395Val	0.0015
H23	ADC	11	(C1201T: Homo)	Arg401Trp	0.053
Sq-5	SQC	20	(C3299T: Hetero)	Thr1100Met	0.00068
H1155	LCC	14	(C2057T: Hetero)	Thr686Ile	0.035
PC13	LCC	20	(A3164G: Hetero)	Asp1055Gly	0.010
Lu65	LCC	32	(G5258T: Homo)	Gly1753Cys	0.00047
H2171[b]	SCC	5	(G460T: Homo)	Asp154Tyr	0.025
H526	SCC	17	(A2443G: Hetero)	Lys815Glu	0.70
Surgical specimen					
Na68T[b]	SQC	4	(G235T: Hetero)	Gly79STOP	0.10
Na182T[b]	SQC	21	(TG3472-3473AT: Hetero)	Trp1158Met	0.27
S171T[b]	SCC	15	(G2206T: Hetero)	Val1736Leu	Not tested
1662T[b]	SCC	17	(C IVS17+16 T: Hetero)	Unknown	Not tested

[a]Relative expression level to noncancerous lung tissues.
[b]Validated to be somatic mutation.

Characteristics and Genomic Status of 176 Genes Homozygously Deleted in Lung Cancer Cell Lines

The 176 genes with verified homozygous deletions are listed in Table 1 and Supporting Information Table 1. They consisted of 171 protein-encoding genes and five miRNA genes (genes 58, 78, 167–169). These 176 genes were located in 45 regions on 17 chromosomes (Supporting Information Fig. 1). They included known tumor suppressor genes, *CDKN2A/p16, p14ARF, CDKN2B/p15, RB1,* and *SMAD4* (genes 80–82, 131, and 155 in Table 1) (Futreal et al., 2004), as well as candidate tumor suppressor genes shown to be hemizygously or homozygously deleted in several types of human cancers, such as *LRP1B/LRP-DIP, FHIT, PTPRD* and *KEAP1* (genes 3, 18, 47, and 157 in Table 1) (Sozzi et al., 1996; Liu et al., 2000; Sonoda et al., 2004; Sato et al., 2005; Singh et al., 2006; Stallings et al., 2006; Ohta et al., 2008).

Frequencies of homozygous deletion for these 176 genes were examined in 74 lung cancer cell lines, consisting of 52 cell lines used for the array analyses and 22 additional cell lines, by genomic PCR using the same sets of primers as described above (Supporting Information Table 2). One to 75 of these genes were homozygously deleted in 44 of the 74 cell lines analyzed. No gene was deleted in the remaining 30 cell lines (Supporting InformationTable 1). Homozygous deletion of each gene was detected in one (1%) to 20 (27%) of the 74 cell lines. Sixty-four (36%) of the 176 genes were deleted in two or more cell lines (Supporting Information Table 3), while the other 112 (64%) were deleted in a single cell line. The *CDKN2A/p16* and *p14ARF* genes (genes 80–81) in the 9p21 region were most frequently deleted (20/74, 27%). Thirty-four genes deleted in two or more cell lines were located in regions other than 9p21 (Table 2). A candidate tumor suppressor gene, *PTPRD* (gene 47), was most frequently deleted (8/74, 11%) among them. Other known candidate tumor suppressors, *LRP1B* (gene 3), *FHIT* (gene 18), and *KEAP1* (gene 157), were also included in these 34 genes. Therefore, other genes listed in Table 2 will be also strong candidates for lung tumor suppressors.

Expression Status of the 176 Genes Homozygously Deleted in Lung Cancer Cell Lines

Nineteen of the 44 cell lines with homozygous deletion were available for information on expression levels of 23,583 genes obtained by microarray analysis. These cell lines included all four major histological types of lung cancers. Information on expression levels was available for 160 (91%) of the 176 genes. Most of these genes showed nonsignificant signals (i.e., absent call) in cell lines with homozygous deletion of the corresponding genes, and such genes were *LRP1B* (gene 3), *PTPRD* (gene 47), *CDKN2A/p16,* and *p14ARF* (genes 80–81 assessed by the same probes), *CDKN2B* (gene 82), *RB1* (gene 131) and *KEAP1* (gene 157) (Table 1). On the other hand,

some genes with deletions of parts of genes, such as *FHIT* (gene 18), showed significant signals (i.e., present call) in cell lines with homozygous deletions. As for the *FHIT* gene, transcripts lacking exons, which are homozygously deleted, were previously shown to be expressed in lung cancer cells (Sozzi et al., 1996).

Expression data on eight cultured noncancerous lung epithelial cells were also available for the same set of genes as in lung cancer cell lines (Zhou et al., 2006). Therefore, we searched for genes whose expression was significantly lower in lung cancer cells compared to noncancerous lung epithelial cells. In 55 (31%) genes, at least one probe showed a level of expression significantly lower than that in noncancerous lung epithelial cells (T/N ratio <1 and $P < 0.05$ by t test, marked in blue in Table 1 and Supporting Information Table 1). Expression levels in 52 (95%) of these 55 genes remained significantly lower after removing lung cancer cases with homozygous deletion. The differences in expression of 18 genes (10%) remained significant after Bonferroni correction for multiple tests (i.e., $P < 0.00018$, marked in red in Table 1 and Supporting Information Table 1), and that of 13 (72%) genes remained significant after removing cases with homozygous deletion. These genes included the *KANK* and *ADAMTS1* (genes 131 and 175) candidate tumor suppressor genes whose down-regulation by epigenetic alterations rather than genetic alterations in renal and lung cancers, respectively, were reported (Sarkar et al., 2002; Choi et al., 2008). It was noted that homozygous deletions of these three genes were detected only in one cell line, respectively. The results suggest that the present 176 genes include genes preferentially inactivated in lung cancer cells by epigenetic alterations rather than homozygous deletions.

PTPRD Alterations in Human Lung Cancer

Homozygous deletions and mutations in the *PTPRD* gene in human lung cancer and other cancers, as well as the ability of PTPRD protein to inhibit growth and to cause apoptosis have indicated that *PTPRD* is a tumor suppressor gene (Sjoblom et al., 2006; Weir et al., 2007; Ding et al., 2008; Solomon et al., 2008; Veeriah et al., 2009). Thus, we searched for mutations in the *PTPRD* gene in both cell lines and surgical specimens of lung cancer. Sequencing of all coding exons in 74 lung cancer cell lines revealed that eight cell lines (11%) had nonsynonymous (i.e., associated with amino acid change) nucleotide substitutions that were not deposited in the dbSNP database (Table 3). The substitution in H2171 cells was validated to be a somatic mutation, by the absence of this substitution in the corresponding lymphoblastoid cell line (Supporting Information Fig. 2A). The other seven substitutions detected in the remaining seven cell lines were also likely to be somatic mutations because these substitutions were not detected in noncancerous cells of 95 different individuals (see below), and each of them was detected in only one of the 74 lung cancer cell lines and in none of the 95 primary tumors. Among the 95 surgical specimens analyzed for *PTPRD* mutations, four cases (4%) were concluded as having somatic mutations because nucleotide substitutions were detected only in cancer cells and not in the corresponding noncancerous cells (Table 3). One was a nonsense mutation, two were missense mutations, and the remaining one was a mutation in an intronic sequence. By RT-PCR and sequencing, mutant alleles were shown to be expressed in all of the eight cell lines with *PTPRD* mutations and a surgical specimen whose RNA was available for analysis (Supporting Information Fig. 2A).

The *PTPRD* mutations detected in this study were dispersed through the PTPRD protein as previously observed in lung and others cancers (Fig. 2A) (Sjoblom et al., 2006; Weir et al., 2007; Solomon et al., 2008; Veeriah et al., 2009). It was noted that the same mutations were not present among the mutations detected in human cancers up to the present, and hot spots for mutations were not obvious (Supporting Information Fig. 2B and 2C). A recent study indicated that several mutant *PTPRD* proteins have lower abilities than the wild-type protein to inhibit growth and to cause apoptosis in cells (Solomon et al., 2008). In addition, a subset of mutations, including Gly79X detected in the present study, were nonsense mutations causing a production of truncated PTPRD proteins lacking the whole or a part of protein tyrosine phosphatase catalytic domains. These results indicate that somatic *PTPRD* mutations are a genetic event causing functional inactivation of the *PTPRD* gene in human cancer cells.

We next examined the expression of the *PTPRD* gene in both cell lines and surgical specimens of lung cancer. We previously reported that the majority (>90%) of lung cancer cell lines, including eight cell lines with homozygous *PTPRD* deletions showed lower expression levels

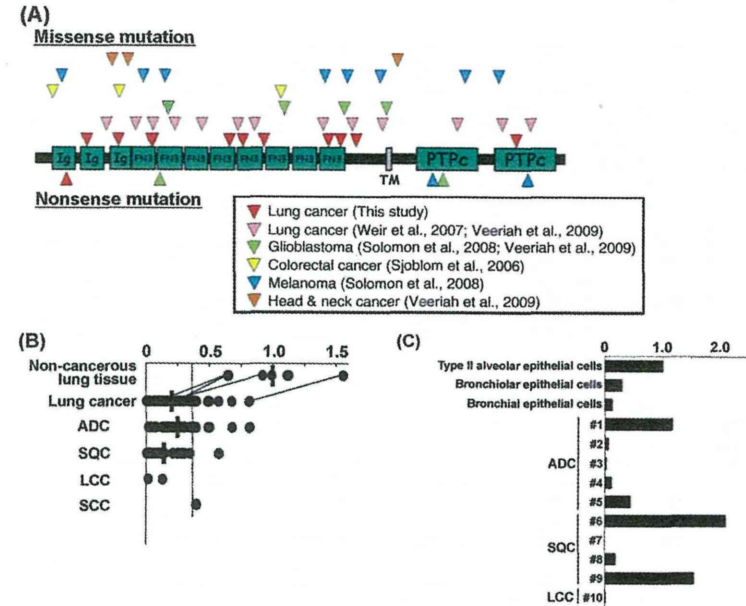

Figure 2. *PTPRD* mutation and expression in lung cancer. (A) Location of missense and nonsense mutations detected in the present and previous studies. Ig, immunoglobulin-like C2-type domain; FN3, fibronectin type III domain; TM, transmembrane domain; PTPc, protein tyrosine phosphatase catalytic domain. (B) Expression in macro-dissected cancerous and noncancerous lung cells. Values for four paired noncancerous and cancerous lung tissues are connected. Expression levels are indicated after adjusting the mean for the levels of expression in seven cases of noncancerous lung tissues to 1. Mean values are indicated by horizontal bars if the group has three or more samples. The threshold level to judge as reduced expression is indicated by a dashed line. (C) Expression in micro-dissected cancerous and noncancerous lung cells. The levels of *PTPRD* expression relative to those of *GAPDH* expression are shown after adjusting the level of *PTPRD* expression in type II alveolar epithelial cells to 1.

than noncancerous lung cells (Sato et al., 2005). In this study, eight cell lines with *PTPRD* mutations were also shown to express lower levels compared to normal lung tissue (Table 3). Sixty surgical specimens of lung tumors were also subjected to QRT-PCR expression analysis. Fifty-one (85%) specimens showed lower levels of *PTPRD* expression than the mean-2SD for seven noncancerous lung tissues, therefore, these samples were judged as having a reduced *PTPRD* expression (Fig. 2B). Two specimens with *PTPRD* mutations for which RNA was available also showed reduced *PTPRD* expression (Table 3). Differences in *PTPRD* levels between noncancerous lung tissues and all lung cancers, ADCs or SQCs were significant ($P < 0.05$ by t test). Two LCCs also showed reduced *PTPRD* expression. A SCC case examined also showed a lower level of *PTPRD*, however, was not judged as having a reduced *PTPRD* expression according to the criteria above.

We also examined *PTPRD* expression in noncancerous lung component cells and cancerous cells which were obtained by micro-dissection of surgical specimens (Nakamura et al., 2006). *PTPRD* expression was detected in noncancerous lung component cells with the highest expression in type II alveolar epithelial cells, candidate precursor cells for lung ADC (Otto, 2002) (Fig. 2C). The levels of expression in five lung cancer

samples were lower than those of any component cells (cases 2, 3, 4, 7, and 10 in Fig. 2C). These results suggested that reduced *PTPRD* expression commonly occurs in lung carcinogenesis. Recently, reduced expression of *PTPRD* was shown to be a frequent event in human glioblastoma, and to be caused by hypermethylation of the promoter region of the *PTPRD* gene (Veeriah et al., 2009). Therefore, it was strongly suggested that *PTPRD* is silenced by a promoter hypermethylation also in lung cancer, although methylation status of the *PTPRD* gene was not examined in this study.

CONCLUSION

We identified 176 genes homozygously deleted in human lung cancer. These genes included known tumor suppressor genes and candidate tumor suppressor genes, whose hemizygous or homozygous deletions as well as intragenic mutations had been reported in several types of human cancers. Furthermore, these 176 genes include genes preferentially inactivated by epigenetic alterations, such as *KANK* and *ADAMTS1*. Indeed, one of these candidates, *PTPRD*, was shown to be genetically and/or epigenetically altered in a considerable fraction of lung cancer. Therefore, this set of genes will be informative to identify novel lung tumor suppressor genes. The Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) suggests that genes with specific functions or involved in specific signaling pathways are not significantly enriched among these 176 genes. Thus, it was suggested that genes involved in a variety of biological processes could function as lung tumor suppressors. Further genetic/epigenetic as well as functional studies of these 176 genes will help understanding of molecular mechanism of lung carcinogenesis. In fact, the present homozygous deletion scanning was performed on a set of 52 lung cancer cell lines including all four major histological types of lung cancer. However, fractions of ADC (30/52, 58%) and SQC (7/52, 13%) were larger and smaller than those in the population of lung cancer patients (Parkin et al., 2004), respectively, therefore, scanning of other sets of lung cancer that are predominant for SQC might provide additional genes.

One to 75 genes were homozygously deleted in 44 lung cancer cell lines analyzed, while no genes was deleted in the remaining 30 cell lines. The result might imply that the intrinsic genomic stability against homozygous deletion is different among lung cancer cases. We recently reported genetic/epigenetic alteration profiles of known oncogenes and tumor suppressor genes in the cell lines used in this study (Medina et al., 2008; Blanco et al., 2009). Therefore, relationships between these alterations and homozygous deletions of those 176 genes were examined. Interestingly, numbers of genes with homozygous deletions are significantly or marginally significantly different according to alterations of tumor suppressor genes, *TP53, CDKN2A/p16, LKB1*, and *PTEN* (Supporting Information Table 4). Multivariate analysis indicated that only the *CDKN2A/p16* alteration among them was independently associated with the number of genes homozygously deleted. The result suggests that *CDKN2A/p16* alteration is involved in genomic instability inducing homozygous deletions as this gene is critical for the maintenance of genome integrity in human cells (McDermott et al., 2006). Interestingly, the cell lines with homozygous *CDKN2A/p16* deletions carried deletions of significantly larger number of genes than those with promoter hypermethylation and mutation of the *CDKN2A/p16* gene; and those without (Supporting Information Fig. 3). Therefore, homozygous *CDKN2A/p16* deletion can be a marker of intrinsic instability for homozygous deletion. More detailed analysis of genetic/epigenetic interactions as well as functional interactions among genes altered in lung cancer cells will further provide insights into the molecular mechanism of lung carcinogenesis.

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Prevalence of human papillomavirus 16/18/33 infection and p53 mutation in lung adenocarcinoma

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Human papillomavirus (HPV) infection is a causative event for the development of uterine cervical carcinoma. Human papillomavirus (HPV) 16, 18, and 33 DNA has been also detected frequently in lung adenocarcinomas (AdCs) in East Asian countries; however, its prevalence in Japan remains unclear. We therefore screened for HPV 16/18/33 DNA in 297 lung AdCs in a Japanese population by multiplex PCR with type-specific primers. As reported previously, HPV 16 DNA was detected in two cervical cancer cell lines, CaSki and SiHa, while HPV 18 DNA was detected in HeLa cells, and 0.1–1.0 copies of HPV-DNA per cell were detectable by this method. However, with this method, none of the 297 lung AdCs showed positive signals for HPV 16/18/33 DNA, indicating that HPV-DNA is not or is very rarely integrated in lung AdC genomes in the Japanese. Furthermore, none of the lung AdCs showed positive signals by nested PCR with HPV 16/18 type-specific primers. Therefore, we further attempted to detect HPV 16/18/33 DNA in 91 lung cancer cell lines, including 40 AdC cell lines. Among them, 30 have been established in Japan and the remaining 61 in the USA. No HPV signals were obtained in any of the 91 cell lines by either multiplex or nested PCR, while the *p53* gene was mutated in 81 of them including 35 of the 40 AdC cell lines. These results indicate that HPV 16/18/33 infection does not play a major role in the development of lung AdC in Japan nor in the USA. (*Cancer Sci* 2010; 101: 1891–1896)

Infection with human papillomavirus (HPV) is a critical event for the development of uterine cervical cancer.[1] E6 protein, encoded by HPV, binds the host cellular tumor suppressor protein p53, and triggers its degradation through the ubiquitin pathway.[2,3] Therefore, the biological significance of continuous p53 degradation by HPV-E6 protein in cervical carcinoma is thought to be equivalent to that of p53 inactivation by genetic alterations in various other types of cancers in human carcinogenesis. The *p53* gene is frequently inactivated in lung adenocarcinoma (AdC) by mutations and/or deletions of both alleles, and the prevalence of p53 mutations in lung AdC is approximately 50% with a higher incidence in smokers.[4,5] However, p53 is not genetically altered in the other half of lung AdCs. Therefore, it is possible that p53 is inactivated by other mechanisms in lung AdC cells without p53 mutations. For this reason, there have been many reports investigating the involvement of HPV in lung AdC development. However, the prevalence of HPV infection in lung AdCs varies drastically among the reports.[5–7] Recently, reasons for a wide variation in the prevalence of HPV infection in lung cancer were investigated by two systematic surveys of a large number of publications.[6,7] A higher prevalence in Asia than in Europe was pointed out by these two investigations,[6,7] and a higher prevalence in studies using HPV type-specific primers than in those using consensus HPV primers was also pointed out in the latter investigation.[7] In East Asia (Supplementary Table S1), a high incidence of HPV infection in lung AdC was reported from Taiwan (92.8%), China (46.9%), and Korea (55.1%).[8–10] In particular, a high prevalence of HPV 16 and 18 infections was reported from Taiwan and China and of HPV 33 infection from Korea. In Japan, the incidence of HPV infection (0–19.4%) has been reported to be not as high as in other East Asian countries, but is still high enough to consider its involvement in lung AdC development.[11–14]

Taiwan, China, and Korea are geographically close to Japan and the people in these countries are ethnically also close to the Japanese. Therefore, in this study, we aimed to elucidate whether or not HPV 16, 18, and 33 are also involved in the development of lung AdC in Japan, as in Taiwan, China, and Korea. We applied a multiplex PCR method as well as a nested PCR method using type-specific primers for detection of HPV 16, 18, and 33 DNA in 275 primary and 22 metastatic lung AdCs in Japanese, and also in 91 lung cancer cell lines established in either Japan or the USA. To validate the specificity and sensitivity of HPV detection methods, three cervical carcinoma cell lines were analyzed by the same methods. In 91 cell lines, the status of p53 mutations was comprehensively analyzed and the results were compared with several p53 databases to evaluate accurately the prevalence of p53 inactivation in lung cancers.

Materials and Methods

Patients and tissues. A total of 275 primary lung AdCs and 22 metastatic lung AdCs to the brain were obtained at surgery from patients treated at the National Cancer Center Hospital, Tokyo, and at Saitama Medical University Hospital. The tumors were pathologically diagnosed according to the tumor-node-metastasis classification of malignant tumors[15] (Table 1). Tumor tissues were stored at –80°C until DNA extraction, and genomic DNA was extracted as previously described.[16] This study was undertaken under the approval of the Institutional Review Board of the National Cancer Center.

Cell line DNA. DNA from 91 lung cancer cell lines[17,18] was screened for HPV-DNA in its genome. These cell lines consisted of 40 AdCs, 11 squamous cell carcinomas (SqCs), two adenosquamous carcinomas (ASCs), nine large-cell carcinomas (LCCs), 27 small-cell lung carcinomas (SCLCs), and two others (one carcinoid tumor and one neuroendocrine tumor), as listed in Table 2. Detailed information will be provided upon request. DNA from three cervical carcinoma cell lines, CaSki, SiHa, and HeLa, and HPV 33 containing plasmid DNA, was used as positive controls for detection of HPV-DNA.

Multiplex PCR with HPV type-specific primers. Sequences for the E1 and L2 regions of HPV 16 and for the E1 region of HPV 18 and 33, together with the aminolevulinate, delta-, synthase 1 (ALAS1) gene segment as an internal positive control, were simultaneously amplified by multiplex PCR in a single tube, as reported.[19] The primer sequences are shown in Supplementary Table S2. Multiplex PCR was performed with Takara Taq (Takara, Shiga, Japan) with a volume of 50 μL containing 1×

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Table 1. Clinicopathologic characteristics of lung adenocarcinomas

	PCR	Primary tumor		Brain metastasis
		Multiplex (%)	Nested (%)	Both (%)
No. of cases	–	275	138	22
Gender	Male	161 (59)	81 (59)	15 (68)
	Female	114 (41)	57 (41)	7 (32)
Age (years)	Mean	60.7	62.0	57.3
	Range	30–84	30–84	48–74
Pathological stage	I	201 (73)	124 (90)	–
	II	27 (10)	6 (4)	–
	III	45 (16)	8 (6)	–
	IV	2 (1)	0 (0)	–
Smoking history	Smoker	71 (55)	69 (58)	15 (68)
	Nonsmoker	57 (45)	51 (43)	7 (32)
	Unknown	147	18	0
p53 mutation	+	34 (32)	34 (33)	16 (73)
	–	72 (68)	70 (67)	6 (27)
	ND	169	34	0

ND, not determined.

PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.025 U Taq polymerase, 3 nM primers, and 10 ng template DNA. Amplifications were performed with the following cycling profiling using a GeneAmp PCR system 9700 apparatus (Applied Biosystems, Foster City, CA, USA): Taq polymerase activation by incubation at 95°C for 1 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 90 s, and elongation at 72°C for 60 s. Five micro liters of the amplicons were analyzed by electrophoresis on 3% agarose gels and ethidium bromide staining.

Nested PCR with HPV type-specific primers. Sequences from the upstream regulatory region (URR) to the E7 region of HPV 16 and HPV 18 were first amplified by PCR with outer primers, and the HPV 16 E6/E7 and HPV 18 E6 regions were secondly amplified by nested PCR with inner primers, as reported previously.[20] The primer sequences are shown in Supplementary Table S2. The first round of PCR was performed under the following conditions: Taq polymerase activation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min. The second round of PCR was performed as follows: 95°C for 1 min, followed by 20 cycles of denaturation for 1 min at 95°C, 1 min of annealing at 60°C, and 1 min of elongation at 72°C. Polymerase chain reaction (PCR) was performed with a Takara Taq with a volume of 20 μL containing 1× PCR buffer, 0.2 mM dNTPs, 0.05 U Taq polymerase, 2 nM of primers, and 10 ng of template DNA for the first round PCR and 1 μL of the first round PCR products for the second round PCR using a GeneAmp PCR system 9700 apparatus (Applied Biosystems).

Mutation analysis of the *p53* gene. A total of 106 of the 275 primary lung AdCs and all of the 22 metastatic lung AdCs were previously examined for mutations in exons 4–8 of the *p53* gene by genomic PCR and direct sequencing.[21,22] All of the 91 lung cancer cell lines were examined for mutations in exons 2–11 covering all the coding sequences of the p53 gene by genomic PCR and direct sequencing as previously described.[18,23] Sequence data for the cell lines obtained in this study were compared with those of the Catalogue of Somatic Mutations in Cancer (COSMIC) (http://www.sanger.ac.uk/cosmic/).[24]

Results

Detection of HPV 16, 18, and 33 DNA by multiplex PCR. Recently, Nishiwaki *et al.* developed a rapid and sensitive multiplex PCR-based HPV genotyping method that allows the simultaneous amplification of 16 different HPV genotypes in a single tube reaction.[19] This method is based on the amplification of multiple HPV-DNA sequences with a set of HPV type-specific primers, and the HPV types are visually distinguished by the size of amplified fragments after separation by gel electrophoresis. Since DNA for HPV 16, 18, and 33 types has been frequently detected in lung AdC cells in East Asia, four primer sets for these three HPV types, in addition to a primer set for a control genome sequence, were used in this study. Two sets of primers were prepared for the amplification of the HPV 16 DNA[19] because of a possible high prevalence of HPV 16 DNA integration in lung AdC genomes.

The sensitivity and specificity of this method was validated using genomic DNA from three cervical cancer cell lines, CaSki, SiHa, and HeLa, and a lung cancer cell line, A549. Human papillomavirus (HPV) 16 has been shown to be integrated into chromosomal DNA in the CaSki and SiHa cell lines, while HPV 18 is integrated in the HeLa cell line.[25–27] A cell line with integration of HPV 33 was not available; therefore, HPV 33 containing plasmid DNA was mixed with A549 cell DNA as a ratio of one copy of HPV 33 DNA per diploid human genome. Specific DNA fragments for HPV 16, 18, and 33 of different sizes from each other were successfully amplified with the control genomic DNA fragment (Genome in Fig. 1) in CaSki, SiHa, and HeLa cells, as well as A549 cells mixed with HPV 33 DNA (Fig. 1). Two bands for HPV 16 DNA (HPV16-U and HPV16-L) were detected in CaSKi and SiHa cell DNA, while a band for HPV 18 DNA was detected in HeLa cell DNA. Human papillomavirus (HPV) 33-specific DNA was amplified from the mixture of plasmid DNA and A549 cell DNA, while no HPV-specific DNA was amplified from A549 cell DNA. Therefore, by this method, three different HPV types were successfully identified and distinguished by the difference in the sizes of amplified DNA. To determine the sensitivity of this method, each sample was serially diluted and mixed with A549 cell DNA to obtain genomic DNA with 0.1–1.0 copies of each HPV-DNA. Approximately 600 copies of HPV 16 DNA are integrated in CaSki cells, one to two copies of HPV 16 DNA are integrated in SiHa cells, and 20–50 copies of HPV 18 DNA are integrated in HeLa cells.[25–27] As shown in Figure 1, 0.1–1.0 copies of the HPV-DNA sequence per cell were detected by this method. Therefore, this method allowed us to detect one copy of HPV 16, 18, and/or 33 DNA integrated in chromosomal DNA of human cells. Further validation of this method was performed using DNA isolated from 18 primary cervical cancers because the presence of the HPV 16/18 DNA in these tumors was previously determined by Southern blot analysis.[28,29] Human papillomavirus (HPV) types detected by multiplex PCR analysis were completely the same as those by Southern blot analysis, and the sensitivity of multiplex PCR analysis for detection of HPV 16 DNA was higher than that of Southern blot analysis. Four cases negative for HPV 16 DNA by Southern blot analysis were positive by multiplex PCR analysis (data not shown). Therefore, we concluded that the sensitivity of the multiplex PCR analysis is higher than that of Southern blot analysis for detection of HPV 16 DNA in cancer cells.

We then applied this method for detection of HPV 16/18/33 DNA in 275 primary lung AdCs and 22 metastatic lung AdCs to the brain (Table 1). However, HPV-specific DNA was not amplified in any of these 297 lung AdCs. Thus, it was strongly suggested that HPV 16/18/33 DNA is not integrated in the chromosomal DNA of these lung AdCs.

Detection of HPV 16 and 18 DNA by nested PCR. It was reported that only a part of HPV-DNA, from the URR to the E6/E7 region, is commonly integrated in chromosomal DNA of cervical cancer cells, and that deletions of other regions occur in the course of viral DNA integration into host cell DNA.[25,26,30]

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Table 2. Status of the *p53* gene in 91 lung cancer cell lines

No.	Cell line	Hist.	Amino acid	Nucleotide
Point mutation				
1	ABC1	AdC	p.P278S	c.832C>T
2	CALU-3	AdC	p.M237I	c.711G>T
3	HCC44	AdC	p.S94X+ p.R175L	c.281C>G+ c.524G>T
4	HCC78	AdC	p.S241F	c.722C>T
5	HCC193	AdC	p.R248Q	c.743G>A
6	HCC515	AdC	p.L194F	c.580C>T
7	Ma10	AdC	p.G245V	c.734G>T
8	Ma17	AdC	p.Y126C	c.377A>G
9	Ma24	AdC	p.R337C	c.1009C>T
10	H23	AdC	p.M246I	c.738G>C
11	H441	AdC	p.R158L	c.473G>T
12	H820	AdC	p.T284P	c.850A>C
13	H1437	AdC	p.R267P	c.800G>C
14	H1975	AdC	p.R273H	c.818G>A
15	H2009	AdC	p.R273L	c.818G>T
16	H2087	AdC	p.V157F	c.469G>T
17	H2122	AdC	p.Q16L+ p.C176F	c.527G>T+ c.47A>T
18	H2126	AdC	p.E62X	c.184G>T
19	PC3	AdC	p.R282W	c.844C>T
20	PC7	AdC	p.H214R	c.641A>G
21	PC9	AdC	p.R248Q	c.743G>A
22	PC14	AdC	p.R248W	c.742C>T
23	RERF-LCMS	AdC	p.R248L	c.743G>T
24	RERF-LC-OK	AdC	p.F113C	c.338T>G
25	VMRC-LCD	AdC	p.R175H	c.524G>A
26	II-18	AdC	p.K164X	c.490A>T
27	H322	AdC	p.R248L	c.743G>T
28	EBC1	SqC	p.E171X	c.511G>T
29	LC1/Sq	SqC	p.M237I	c.711G>T
30	LK2	SqC	p.V272M	c.814G>A
31	HCC15	SqC	p.D259V	c.776A>T
32	H520	SqC	p.W146X	c.438G>A
33	SK-MES-1	SqC	p.E298X	c.892G>T
34	PC10	SqC	p.G245C	c.733G>T
35	HCC366	ASC	p.Y220C	c.659A>G
36	H596	ASC	p.G245C	c.733G>T
37	Lu65	LCC	p.E11Q	c.31G>C
38	Ma2	LCC	p.R175H	c.524G>A
39	Ma25	LCC	p.M237I	c.711G>T
40	H661	LCC	p.R158L+ p.S215I	c.473G>T+ c.644G>T
41	H1155	LCC	p.R273H	c.818G>A
42	PC13	LCC	p.G334V	c.1001G>T
43	HCC33	SCLC	p.C242Y	c.725G>A
44	Lu134	SCLC	p.P278L	c.833C>T
45	Lu135	SCLC	p.G244C	c.730G>T
46	Lu139	SCLC	p.V157F	c.469G>T
47	N417	SCLC	p.E298X	c.892G>T
48	H69	SCLC	p.E171X	c.511G>T
49	H128	SCLC	p.E62X	c.184G>T
50	H345	SCLC	p.Y236C	c.707A>G
51	H446	SCLC	p.Q154V	c.461G>T
52	H841	SCLC	p.C242S	c.724A>T
53	H1184	SCLC	p.G334V	c.1001G>T
54	H1450	SCLC	p.L194R	c.581T>G
55	H1607	SCLC	p.P151H	c.452C>A
56	H1963	SCLC	p.V147D+ p.H214R	c.440T>A+ c.641A>G
57	H2107	SCLC	p.K101X	c.301A>T
58	H2141	SCLC	p.R209X	c.625A>T
59	H2171	SCLC	p.Q144X	c.430C>T
60	H2195	SCLC	p.V157F	c.469G>T
61	H1618	SCLC	p.R248L	c.743G>T
62	H187	SCLC	p.S241C	c.722C>G
63	H510	SCLC	p.R282G	c. 844C>G
64	H1770	Neuroendocrine	p.R248W	c.741-742CC>TT
Small insertion/deletion (≤9 nucleotides)				
1	Ma29	AdC	p.V121fs	c.363delT
2	H522	AdC	p.P191fs	c.572delC
3	H1648	AdC	p.L35fs	c.103-104insT
4	HCC95	SqC	p.G334fs	c.1000(-1003) 1G del
5	H157	SqC	p.L35fs+ p.E298X	c.103-104insT+ c.892G>T
6	H727	Carcinoid	p.Q165-S166 insYKQ	c.496-497ins9
Large deletion				
1	H358	AdC	p?	Large deletion
2	H1299	LCC	p?	Large deletion
Splicing-site mutation				
1	H1703	AdC	p.G262fs	g. Ivs8 +1g>t
2	H1819	AdC	p.A307fs	g. Ivs9 +1g>t
3	H2347	AdC	p.Y126fs	g.375G>A
4	H1650	AdC	p.V225fs	g.Ivs6 -2a>g
5	Sq1	SqC	p.Y126fs	g. Ivs4 +2t>c
6	H82	SCLC	p.Y126fs	g.375G>T
7	H209	SCLC	p.V225fs	g. Ivs6 -2a>t
8	H526	SCLC	p.S33fs	g. Ivs3 -1g>c
9	H1339	SCLC	p.I332fs	g.Ivs9 +1g>t
Wild type				
1	A427	AdC	—	—
2	A549	AdC	—	—
3	Ma12	AdC	—	—
4	Ma26	AdC	—	—
5	H1395	AdC	—	—
6	H226	SqC	—	—
7	Lu99A	LCC	—	—
8	H460	LCC	—	—
9	Lu24	SCLC	—	—
10	Ms18	SCLC	—	—

p, c, and g indicate protein, cDNA, and genomic DNA.
AdC, adenocarcinoma; ASC, adenosquamous carcinoma; LCC, large-cell carcinoma;
SCLC, small-cell lung carcinoma; SqC, squamous cell carcinoma.

The primers for HPV 16, 18, and 33 in the above multiplex PCR analysis were designed to amplify the E1 or L2 region (Supplementary Table S2).[19] Therefore, it was possible that multiplex PCR analysis failed to detect the HPV-DNA sequences because of integration of truncated HPV genomes without the E1 and L2 regions into host cell DNA. To pursue the possible integration of HPV 16 and 18 DNA in lung AdC cells, we performed a nested PCR analysis for the E6 and E7 regions of HPV 16 and 18. The URR to the E7 region of both HPV 16 and 18 genomes was first amplified using outer primers, then, the E6 to E7 region of the HPV 16 DNA and the E6 region of the HPV 18 DNA were amplified using inner primers (Supplementary Table S2), respectively, according to the method previously described.[20] As in the multiplex PCR analysis, HPV 16- and 18-specific DNA fragments were successfully amplified from the CaSki, SiHa, and HeLa cell lines, but not from A549. Next, 138 of the 275 primary AdCs and all of the 22 metastatic AdCs used for

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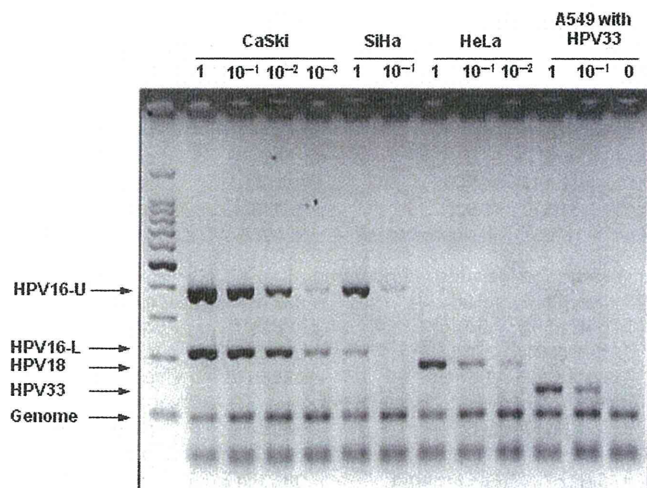

Fig. 1. Detection of human papillomavirus (HPV) 16, 18, and 33 DNA in cervical cancer cell lines by multiplex PCR analysis. Specificity and sensitivity for detection of HPV 16, 18, and 33 DNA. Polymerase chain reaction (PCR) was performed using DNA from CaSki (~600 copies of HPV 16 integrated), SiHa (1–2 copies of HPV 16 integrated), HeLa (20–50 copies of HPV 18 integrated), and A549 cells with/without HPV 33 containing plasmid DNA. Each sample was serially diluted with A549 cell DNA up to the copy number of 0.1–1.0 per cell for HPV-DNA. Five micro liters of the amplicons were analyzed by electrophoresis on 3% agarose gels and ethidium bromide staining. 100 bp DNA Ladder (Takara, Shiga, Japan) was used as a size marker.

multiplex PCR analysis were subjected to nested PCR analysis (Table 1). However, none of them showed positive signals for the E6/E7 regions of the HPV 16 or 18. The results of multiplex PCR analysis as well as those of nested PCR analysis strongly indicated that HPV 16 and 18 are not integrated in lung AdCs developed in Japan, at least in the Tokyo area.

Absence of HPV 16, 18, and 33 DNA sequences in lung cancer cell lines. We next attempted to detect HPV 16, 18, and 33 DNA in a panel of 91 human lung cancer cell lines established in either Japan or the USA. Among the 91 cell lines, 30 originated from Japanese, 42 from Caucasians, and five from African-Americans. Detailed information was not available for the remaining 14 cell lines. Forty cell lines were derived from AdC and the remaining 51 were from other histological types. Both multiplex PCR and nested PCR were performed on all of these cell lines. However, no HPV-specific signals were obtained in any of these cell lines. Therefore, HPV 16, 18, and 33 DNA is not integrated in the 91 lung cancer cell lines established in Japan and the USA. Eleven cell lines were derived from SqC, and 27 cell lines were derived from SCLC; therefore, HPV 16/18/33 integration was not evident in any major histological types of lung cancer.

Status of p53 mutations in lung cancer cell lines. We previously examined for p53 mutations in 106 of the 275 primary tumors and all 22 brain metastases,[21,22] and the mutations were detected in 34 of the 106 primary tumors (32%) and 16 of the 22 brain metastases (73%) (Table 1). We recently reported the status of p53 mutations in 87 of the 91 cell lines analyzed in the present study.[18] In that study, mutation data of several cell lines were obtained not only by direct sequencing of the p53 coding regions but also from the COSMIC database, and the mutations were detected in 70 of the 87 cell lines (80%). However, during this study, we noticed that data for p53 mutations are not the same among three major databases, COSMIC, UMD_TP53 database (http://p53.free.fr), and IARC p53 database (http://www-p53.iarc.fr).[24,31,32] Absence of HPV 16/18/33 integration as well as p53 mutations in 17 lung cancer cell lines prompted us to re-investigate the status of p53 mutations in these cell lines. Therefore, the p53 mutation status in all the 91 cell lines was determined by direct sequencing of all the coding exons, from exon 2 to exon 11, together with exon–intron boundaries of these exons (Table 2). If mutations were detected in the exon–intron boundaries, a possible occurrence of splicing abnormalities due to the mutations was examined by direct sequencing of p53 cDNA products from the corresponding cell lines. Point mutations were detected in 64 of the 91 cell lines, small insertions/deletions in six of them, and large deletions in two of them. Splice-site mutations were detected in nine cell lines, in which shifts of open reading frames due to either exon skipping or intron retention were confirmed. Accordingly, only 10 cell lines were shown to carry the wild-type *p53* gene and express normal p53 protein, including five of the 40 AdC cell lines.

The status of 36 cell lines was not available in COSMIC and thus was defined by our studies (Supplementary Table S3-1) ([18,21–23], this study). The status of 45 cell lines was concordant between our data and COSMIC data (Supplementary Table S3-2), whereas that of the remaining 10 cell lines was discordant (Supplementary Table S3-3). Therefore, although 10 of the 91 lung cancer cell lines carry the wild-type *p53* gene, HPV 16, 18, or 33 are not integrated in these cell lines.

Discussion

To detect HPV-DNA in lung cancer cells, we applied two different PCR methods with HPV type-specific primers, one-step multiplex PCR[19] and nested PCR,[20] because PCR with type-specific primers was reported to be more sensitive than PCR with consensus primers to detect HPV-DNA sequences in human cell DNA.[7] The prevalence and genotype distribution of HPV in cervical cancer precursor lesions defined by one-step multiplex PCR was reported to be compatible with several previous data.[19] In addition, by using these methods, HPV 16 and 18 DNA was distinguishably and efficiently amplified from three cervical cancer cell lines. Therefore, the lack of HPV 16, 18, and 33 DNA in primary lung AdC as well as in lung cancer cell lines would not be due to the low sensitivity of this method for HPV detection. Accordingly, it was concluded from this study that HPV 16, 18, and 33 are not (or are rarely) integrated in lung AdC genomes in the Japanese, particularly those living in the Tokyo area. Lung cancer cell lines analyzed in this study have been established in either Japan or the USA, and consist of all major histological types of lung cancer. Absence of HPV 16/18/33 infection in primary lung AdCs in the US population and lung cancer cell lines established in the USA was previously reported.[33–35] Therefore, the results in the cell lines are consistent with the results in primary AdCs in both Japan and the USA. Indeed, we further attempted to detect HPV-associated DNA sequences in these cell lines by PCR under several low stringent conditions using a set of consensus primers for HPV 16, 18, and 33. However, no HPV-specific signals were detected in any of the 91 lung cancer cell lines examined (data not shown). Therefore, we concluded that no HPV 16/18/33 DNA is integrated in these cell lines. Accordingly, HPV infection seems not to play an important role in the development of lung cancer in Japan nor in the USA, although it is still possible that other HPV types play some role in its development.

A Taiwanese study reported that female never-smokers with lung cancer who were older than 60 years of age had a significantly higher prevalence of HPV 16/18 infections.[8] However, in Korean lung cancer patients, HPV 16/18/33 infections were not associated with gender, smoking status, and histological type.[10] In a study in China, HPV 16/18 infections were not correlated with any clinicopathological parameter, including

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age, gender, smoking status, and histological type, either.[9] In this study, 41% (121/297) and 43% (64/150) of AdC patients were female and non-smokers, respectively (Table 1). Therefore, the etiological role of HPV 16/18 in lung carcinogenesis in non-smokers seems to be restricted to certain geographic areas, and in Japan, HPV 16/18 infection does not play a causative role in the development of lung AdC in female non-smokers.

An inverse correlation of HPV 16/18 E6 protein expression with p53 expression was also reported in Taiwanese lung tumors.[36] However, in a study in China, there was a relationship between the presence of HPV 16/18 DNA and abnormal p53 protein accumulation.[37] Therefore, association of HPV infection with p53 inactivation is still unclear in lung cancer. We previously examined for p53 mutations in 128 of 297 lung AdCs analyzed in this study, and the mutations were detected in 50 cases (39%) (Table 1); therefore, it was possible that HPV is infected in another 78 cases. However, none of the 78 lung AdCs carried HPV 16/18/33 DNA in their genomes. Accordingly, HPV 16/18/33 infections appear to play a limited role in the development of lung AdC in Japan. These results prompted us to analyze comprehensively the status of the *p53* gene in a large panel of lung cancer cell lines. The *p53* gene is inactivated not only by mutations in the coding regions, but also by splicing abnormalities caused by mutations in the exon–intron boundaries and homozygous deletions, and the incidence of p53 genetic alterations in total was 89% (81/91). Therefore, although 10 of the 91 cell lines were shown to carry the wild-type *p53* gene, no HPV 16/18/33 DNA was detected in these cell lines. Since the status of the *p53* gene in these cell lines was not consistent among several databases and reports, the results provided here will be highly informative to diverse scientists using these cell lines for molecular and biological studies.

In Japan, HPV-DNA has been detected in <10% of lung AdC in Chiba and Hokkaido, and ~20% in Okinawa (Supplementary Table S1). Therefore, we cannot totally rule out the involvement of HPV infection in the etiology of lung AdC in Japan. However, the present results strongly indicate that HPV infection plays only a limited role, if any, in the development of lung AdC in Japan.

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Disclosure Statement

The authors have no conflict of interest.

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