

Activation of ERK/IER3/PP2A-B56 γ -positive feedback loop in lung adenocarcinoma by allelic deletion of B56 γ gene

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Abstract. In order to investigate the involvement of the IER3/PP2A-B56 γ /ERK-positive feedback loop, which leads to sustained phosphorylation/activation of ERK in carcinogenesis, we immunohistochemically examined the expression of IER3 and phosphorylated ERK in lung tumor tissues. IER3 was overexpressed in all cases of adenocarcinomas examined, but was not overexpressed in squamous cell carcinomas. Phosphorylated ERK (pERK) was also overexpressed in almost all adenocarcinomas. *EGFR* and *RAS*, whose gene product is located upstream of ERK, were sequenced. Activating mutation of *EGFR*, which is a possible cause of overexpression of IER3 and pERK, was found only in 5 adenocarcinomas (42%). No mutation of *RAS* was found. We further examined the sequences of all exons of B56 γ gene (*PPP2R5C*) and *IER3*, but no mutation was found. Using a single nucleotide insertion in intron 1 of *PPP2R5C*, which was found in the process of sequencing, allelic deletion of *PPP2R5C* was examined. Eight cases were informative (67%), and the deletion was found in 4 of them (50%). Three cases having deletion of *PPP2R5C* did not have *EGFR* mutation. Finally, *PPP2R5C* deletion or *EGFR* mutation that could be responsible for IER3/pERK overexpression was found in at least 8 cases (67% or more). This is the first report of a high incidence of deletion of *PPP2R5C* in human carcinomas.

Introduction

IER3 (immediate early response gene 3) was first identified in fibroblasts from mouse in 1993 (1), and human *IER3*,

formerly referred to as the immediate early gene X-1 (*IEX-1*), was first identified in human squamous cell carcinomas. The human *IER3* gene is located on the short arm of chromosome 6 (6p21.3) and has 2 exons. The gene encodes 156 amino acids (2). IER3 protein is rapidly and transiently induced by a variety of stimuli, including ionizing radiation, inflammatory cytokines, viral infection, anti-cancer drugs and growth factors (2,3), and is associated with apoptosis and cell proliferation (3,4). As it is involved in cell growth, IER3 expression has been examined in several human tumors, including pancreatic carcinoma, ovarian carcinoma, breast cancer, and myelodysplastic syndrome (5-8). However, there has been no study of IER3 expression in human lung carcinoma.

Protein phosphatase 2A (PP2A), which consists of structural subunit A, regulatory subunits B, and catalytic subunit C, has a broad spectrum of functions due to the variety of regulatory subunits, of which there are more than 20 kinds (9). PP2A can act as a tumor suppressor (10,11). B56 regulatory subunits are involved in cell proliferation signaling (11,12). Among them, PP2A-B56 γ 1 dephosphorylates Thr²⁰² of phosphorylated extracellular signal-regulated kinase (pERK), leading to inactivation of ERK (13). Furthermore, Garcia *et al* (14) found that the regulation of cell growth by IER3 is mediated by ERK, which also plays a central role in regulation of a variety of cellular events, including cell proliferation, differentiation, migration, and apoptosis (15,16). IER3 regulates ERK activity through its inhibitory activity on B56-containing PP2A, which dephosphorylates ERK (13). Recently it has been clarified that activated ERK induces IER3 protein synthesis, and then IER3 inhibits B56 γ 1-PP2A, forming the ERK/IER3/B56 γ 1-PP2A positive feedback loop, which eventually leads to sustained activation of ERK (17). The duration of ERK activation is considered to be one of the factors that determine cellular events (18,19). Thus, IER3 and PP2A should contribute to multiple cellular processes, including tumorigenesis, via regulation of ERK.

In this context, mutation of the B56 γ gene (*PPP2R5C*), which is located at 14q32.31, in lung carcinomas as well as other carcinomas has been reported (20). Deletion of 14q has also been reported in lung carcinoma (21). *PPP2R5C* is frequently involved in loss of heterozygosity (LOH) in 14q32

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in a variety of carcinomas (22,23), which suggests an important role of B56 γ in carcinogenesis. Furthermore, BL6 mouse melanoma cells, in which B56 γ is truncated, are highly metastatic (24). In a patient with Sezary syndrome, translocation of t(14;14) (q11;q32) caused rearrangement of *PPP2R5C* (25). All of these findings indicate an important role of B56 γ in carcinogenesis. We recently showed that the ERK/IER3/B56 γ 1 loop is activated by the growth factor-induced RAS/RAF/MEK/ERK cascade (17). After growth factor binds to epidermal growth factor receptor (EGFR), RAS, RAF, MEK and ERK are activated sequentially by phosphorylation or binding (26). The cascade is frequently constitutively activated by mutation or gene amplification in lung adenocarcinomas, including mutation of *EGFR*, amplification of *HER2*, mutation of *RAS*, and mutation of *RAF*. Among these changes, mutations of *EGFR* and *RAS* are most frequent. Activating *EGFR* mutations are found in 5-30% (as high as 50-60% in Asian populations) of non-small cell lung carcinomas, while *K-RAS* mutations are found in 20-12%, and *RAF* mutations in <1% (27,28). Furthermore, it was reported that phosphorylation of ERK was closely associated with mutation of *RAS* or *EGFR* (29).

The aim of this study was to examine whether the IER3/B56 γ 1-PP2A/ERK-positive feedback loop is involved in carcinogenesis of non-small cell lung carcinoma. We also report the high prevalence in these carcinomas of allelic deletion of B56 γ gene, which is a major cause of secondary overexpression of IER3/pERK.

Materials and methods

Tumor samples. Tumor samples, including normal tissues, were obtained from 16 lung cancer patients (12 adenocarcinomas and 4 squamous cell carcinomas) who were operated at Kanazawa University Hospital from September 2013 to December 2014. Tissue specimens were frozen at -80°C.

This work was approved by the ethics committee of Kanazawa University. We obtained informed consent from all patients, and all studies were performed on the basis of the declaration of Helsinki and the ethical guidelines for human genome and gene analysis of the Ministry of Health, Labour and Welfare, Japan.

Immunohistochemistry. Sections (5 μ m) were cut from formalin-fixed, paraffin-embedded (FFPE) tissues. To unmask epitopes, the sections were treated with Target Retrieval Solution (pH 9.0, Dako, Uppsala, Sweden). Endogenous peroxidase activity was blocked with 3% H₂O₂. Sections were reacted with specific rabbit antibodies against human IER3 antibody (Abnova, Taipei, Taiwan) at 1:500 dilution, or specific antibodies against Thr²⁰²/Tyr²⁰⁴-phosphorylated p42/44 ERK (Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution, and then reacted with peroxidase-labeled dextran goat anti-rabbit IgG (Dako). Diaminobenzidine 4HCl (Histofine SAB-PO (M) kit, Nichirei, Tokyo, Japan) was used for visualization. Negative controls were done on serial tissue sections using 0.5% BSA or with antigen-absorbed antibody.

Glutathione-S-transferase (GST) fusion IER3 protein, which was used to absorb antibodies against IER3, was purified with GST beads. Fusion IER3 protein (1 mg) was added

to 20 μ l of antibody solution, and rocked for 60 min at room temperature. Then, the reaction mixture was centrifuged at 14,000 rpm for 10 min. To evaluate possible differences of positivity between non-tumor epithelia and carcinoma cells, the ratio of positive cells were counted in 5 randomly selected areas.

Laser capture microdissection. Frozen sections of lung tumor tissues were fixed with 100% ethanol for 2 min and stained with 0.1% toluidine blue (Merck, Darmstadt, Germany). Approximately 1,000 carcinoma cell sections were cut out using a Laser Microdissection system (LMD 7000, Leica Microsystems, Inc., Bensheim, Germany), and collected in a 0.5 ml RNase-free PCR tube. Extraction of genomic DNA was performed from the frozen sections using a ReliaPrepTM gDNA Tissue Miniprep System (Promega, Madison, WI, USA). Extraction of RNA was performed using an SV Total Isolation system (Promega), and reverse transcription to cDNA was performed using an RT-PCR system.

Direct sequencing. Extraction of genomic DNA from frozen sections was performed using a ReliaPrep gDNA Tissue Miniprep System (Promega). Specific primer sets were used for amplification of human *EGFR*, *K-RAS*, *PPP2R5C* (B56 γ 1 gene) and *IER3*. Exons 18, 19, 20 and 21 in *EGFR* and exon 2 in *K-RAS*, which are hot spots of mutation (30), were evaluated (Table I). All of the exons of *PPP2R5C* (13 exons) and *IER3* (2 exons) were amplified and sequenced. PCR amplification was performed with KOD Fx Neo (Toyobo Co., Ltd., Tokyo, Japan). Cycle sequencing reaction was performed using a Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA). The sequencing reactions were conducted with a capillary sequencer (ABI PRISM 3130x1 Genetic Analyzer, Applied Biosystems).

TA cloning. Fresh PCR products amplified using taq DNA polymerase (Takara Ex Taq[®], Takara, Kusatsu, Japan) were ligated into linearized pCRTM 2.1-TOPO[®] vectors in a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) using T4 DNA ligase. The ligated vectors were transformed into competent TOP10 cells and grown overnight on LB agar plates containing ampicillin and X-gal. Blue-white screening was performed for inserts. The ligated clones were purified and the inserts were sequenced using the M13 (-20) forward primer and a Big Dye Terminator v1.1 Cycle Sequencing kit.

Analysis of LOH in *PPP2R5C*. A single nucleotide insertion in intron 1 in *PPP2R5C*, found in the present study, was used for analyzing LOH. The 3' portions of intron 1 and exon 2 were amplified with the primer sets used to sequence exon 2 (Table I). When the single nucleotide insertion/deletion was found in one allele and not found in the other allele (i.e., duplicated peaks were seen in the electropherogram of the normal lung tissue), the status was considered informative. When the duplicated peaks were not found in the electropherogram of the corresponding tumor, the status was considered LOH.

Statistics. One-way analysis of variance was used for comparing the numbers of immunohistochemically positive carcinoma cells, normal bronchiolar epithelia or alveolar epithelia. When

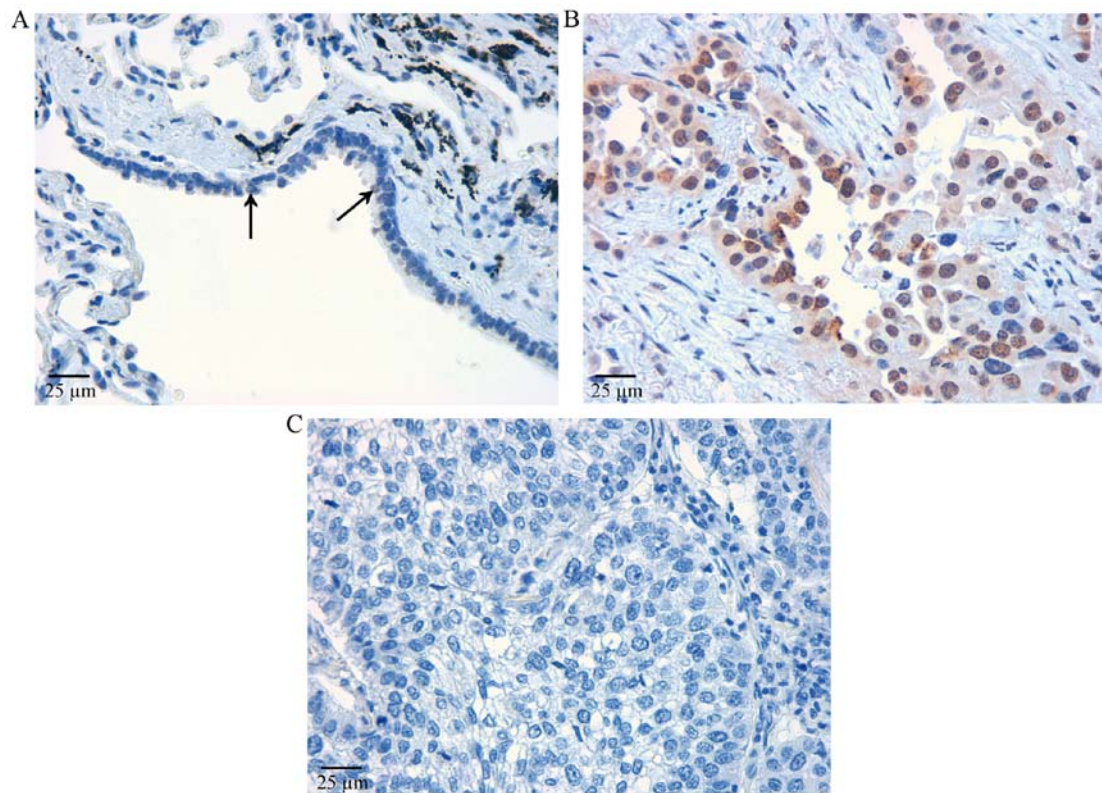


Figure 1. Expression of IER3 evaluated by immunohistochemistry. (A) Respiratory bronchiole. Arrows indicate IER3-positive cells. (B) Adenocarcinoma (case 5). (C) Squamous cell carcinoma (case 15).

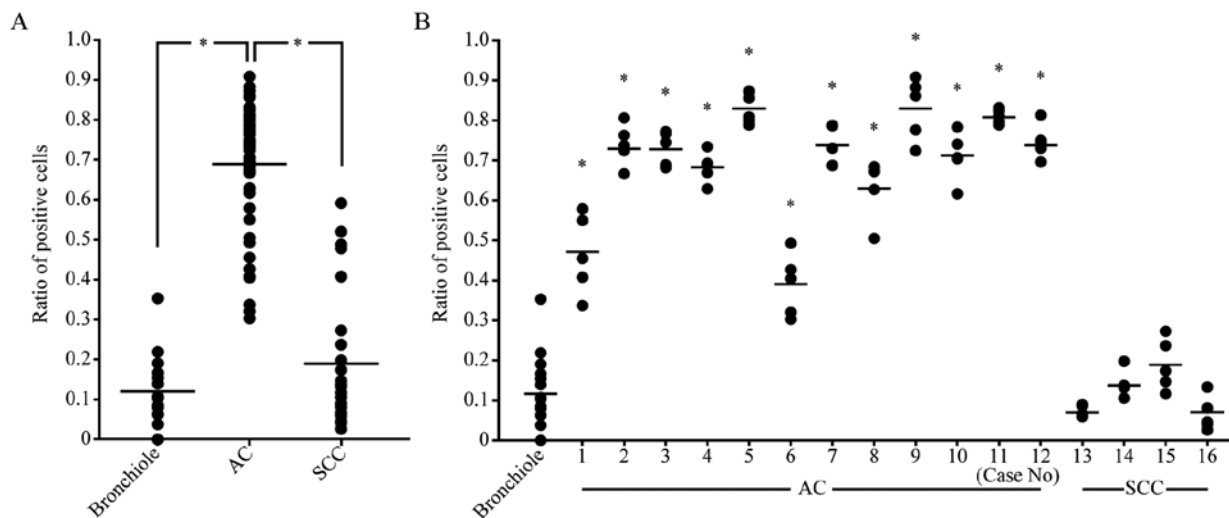


Figure 2. Positive ratio of IER3 determined by immunohistochemistry. (A) Comparisons among bronchiolar epithelia (Bronchiole), adenocarcinomas (AC) and squamous cell carcinomas (SCC). (B) Comparisons between bronchiolar epithelia and each case of carcinoma. The asterisks indicate a statistically significant difference between bronchiolar epithelia and carcinomas ($p < 0.01$).

a significant difference was found, multivariate analysis using Dunnett's multiple comparison test was applied.

Results

Immunohistochemical detection of IER3. A few bronchiolar epithelial cells were positive to anti-IER3 antibodies (Fig. 1A). IER3 was immunolocalized in the nuclei. In adenocarcinoma, IER3 was strongly immunostained in nuclei and faintly in

cytoplasm (Fig. 1B). Granular staining remained positive in cytoplasm when antigen-absorbed antibody was used, suggesting that it was non-specific. Therefore, we considered that only the nuclear staining was positive in the present study. All the adenocarcinomas showed large numbers of positive cells, whereas squamous cell carcinomas showed far fewer positive cells (Fig. 1C).

We counted positive cells in 5 fields of a high-power microscope and calculated the ratio of IER3-positive cells

Table I. The primer sets for direct sequencing.

Genes	1st pair of PCR primers	Nested primers
EGFR	GCTCTGTAGAGAAGGCGAC	GCTCTGTAGAGAAGGCGTAC
Exon 18	TCCCAAACACTCAGTGAAACAAA	TTGGTCTCACAGGACCACTG
EGFR	CCCCAGCAATATCAGCCTTA	GTGCATCGCTGGTAACATCC
Exon 19	GTGGATACCAGCATGGGAGA	TGTGGAGATGAGCAGGGTCT
EGFR	TGAAACTCAAGATCGCATTCA	ATCGCATTATGCGTCTTCA
Exon 20	TGGGACAGGCACTGATTTGT	ATCCCCATGGCAAACCTCTTG
EGFR	AGCCATAAGTCCTCGACGTG	GCTCAGAGCCTGGCATGAA
Exon 21	TGGCTCACACTACCAGGAGA	CATCCTCCCCTGCATGTGT
KRAS	ACGTCTGCAGTCAACTGGAA	GGAGTATTTGATAGTGTATTAACCT
Exon 2	ACCCACTGTATGAGGGTTCC	AGAATGGTCCTGCACC
IER3	CATAAATTACCTCTGCCGGC	CTCACTTGGCCTTACACTCC
Exon 1	TCAAGTTGCCTCGGAAGTCC	AAACAGGAGACAGGTCTAGGT
IER3	CATAAATTACCTCTGCCGGC	ACCTGACCTGTCTCCTGTTT
Exon 2	TCAAGTTGCCTCGGAAGTCC	CGCCTGGTGTCTTCTTTGTGG
PPP2R5C	TGCTGACATCACGAACCAGC	TTCCCGCTGAAGTCTAG
Exon 1	CACCCAACCTCCTCTGGTTA	CCGATTCAGTGAACACAC
PPP2R5C	CTAACAGTGCTCTCAACATGG	ATATGACCAGCGACTAGCTG
Exon 2	TAGGCTCAGAGATCTTGCC	AAATCAGAACTGGGACAC
PPP2R5C	TACGGAGGAGCTAAGTTACC	GCGGCTACTGTTAGAATTACC
Exon 3	ACATCTTCACTGGCTGTTG	CATGCTACTGAGGAGGAGAG
PPP2R5C	TGTAGTCTGTGGGTTTCACC	GTGGCTTTGAGAGGCTAATA
Exon 4-5	TCCTGTCAGAGGAGACGATG	AATGAGCATCACACACAC
PPP2R5C	AGATGCGCTTCTTGTTGCCTG	ATGGCTCCTCCTAGAGCATTG
Exon 6	TCTAAGAGCTCACCAACTCC	CAGCTGGACTCAATGAAATG
PPP2R5C	ACAGGTGCATGGCAACATGC	GCTGGGTTTTGATGGTGA
Exon 7	AGGCCGGATTCACTATCTCG	CTGTGTTCCCTAGAGTCCTG
PPP2R5C	GTTAATGCCCGTTAATCACAC	GGGAAGGTGTTTAACGATG
Exon 8	CCATGAATGAAATGAGCCTG	GCAATTTGGTGAAGCAGATG
PPP2R5C	CACAGACTTCTTACCATGCAG	CATCAGTCACTCCACGTGTC
Exon 9	TGCATGAGAGGCAAAGCATG	GCTACGCATGGAACCTTTTCC
PPP2R5C	GGTTACAGGTTACAGTCTAG	TCTGGTCCAAGGTAGTTTCAT
Exon 10	GAGTCCACAAAGCAACTGAC	ATGGCAGCCAGTTCTTTC
PPP2R5C	CAAGTAACGCGGAATGAGCAG	ACTGTTGGAATATGGAGCAG
Exon 11	GCTTTTCGAGTGAAGAATACCG	GTGGAAGAGGTTTACTTAGG
PPP2R5C	CTCAACATGCCTGTGCCTT	TGTGTTCTTGTGTCTGATGC
Exon 12	CACAGTCTCAGGCTGTATTC	ACGTTAGTGAAATCGAGACC
PPP2R5C	TGTTGCAGGTGTAGGCGAGT	ATGAGAAGCTTGGAGTTCAG
Exon 13	CAGCTTAAACAGAAGGCACTG	TGCAAATCACATCGCCTAC
PPP2R5C	TGGATGCGGCCAACTCCAAT	AACTCCAATGGGCCTTTCC
Exon 1-2	GAGGAAGGTGGTAATGTTTCG	ACGTTGGTTCATCTTCCTCC
PPP2R5C	CGCAACACTGACGTAACCTC	CAGATGTTCTCCTGCTGAT
Intron 1	ATATGACCAGCGACTAGCTG	GGAATAGCCTTTGTTTACC

in each field. The ratios were significantly higher in adenocarcinomas ($p < 0.01$) than in alveolar or bronchiolar epithelia. The average ratio of IER3-positive cells in adenocarcinomas was 4-8 times higher than that in normal epithelia. The ratio in squamous cell carcinomas was not significantly different from

that in bronchiolar epithelia (Fig. 2A). ANOVA followed by Dunnett's multiple comparison showed that the ratios in all the adenocarcinomas were significantly higher ($p < 0.01$) than that in normal epithelia (Fig. 2B). None of the squamous cell carcinomas showed a significant difference from normal epithelia.

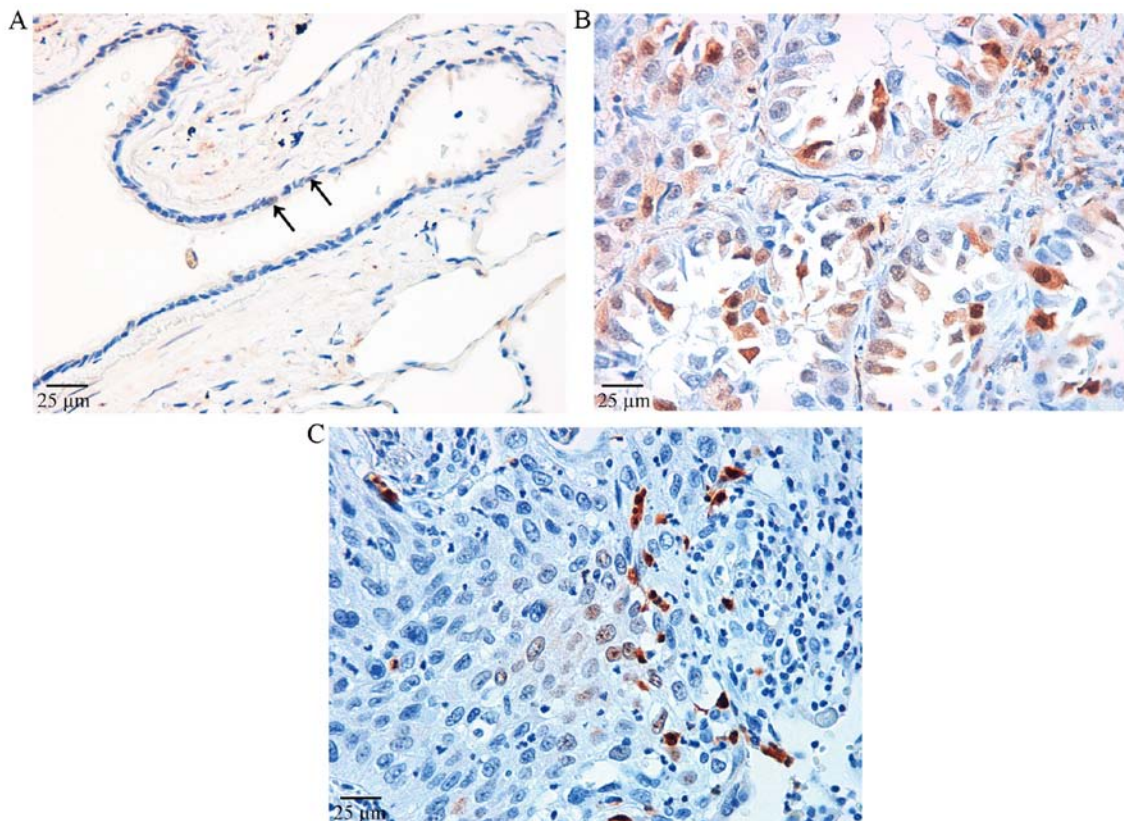


Figure 3. Expression of phosphorylated ERK (pERK) evaluated by immunohistochemistry. (A) Respiratory bronchiolar epithelia. Arrows indicate pERK-positive cells. (B) Adenocarcinoma (case 4). (C) Squamous cell carcinoma (case 15).

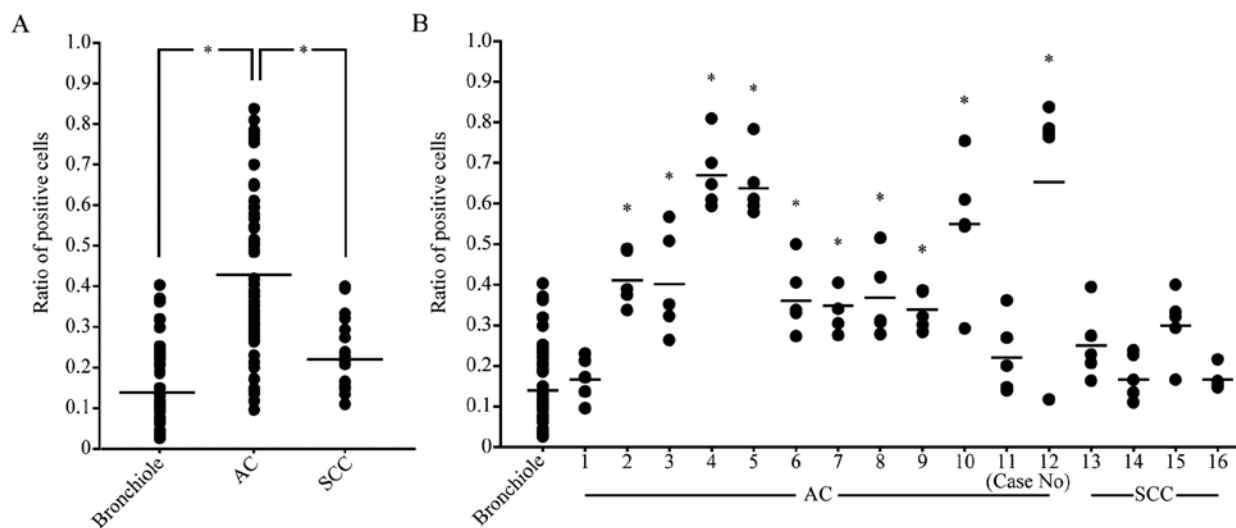


Figure 4. Positive ratio of pERK determined by immunohistochemistry. (A) Comparisons among bronchiolar epithelia (Bronchiole), adenocarcinomas (AC) and squamous cell carcinomas (SCC). (B) Comparisons between bronchiolar epithelia and each case of carcinoma. The asterisks indicate a statistically significant difference between bronchiolar epithelia and carcinomas ($p < 0.01$).

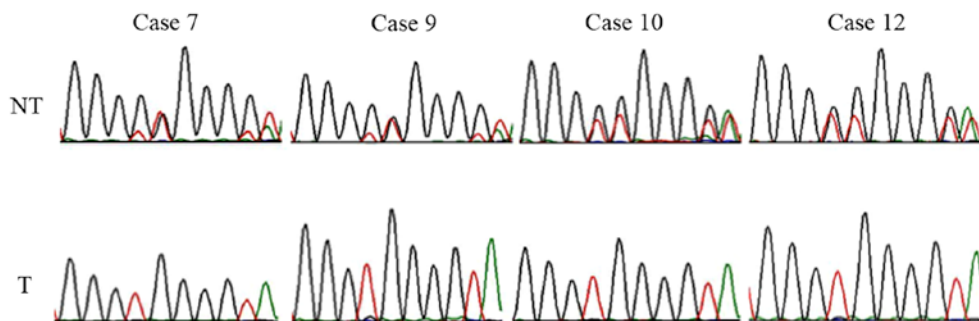
Immunohistochemical detection of phosphorylated ERK. Phosphorylated ERK was immunostained in both nuclei and cytoplasm of bronchiolar epithelia (Fig. 3A). In the present study, only cells immunostained in the nuclei were considered positive. The pattern of distribution of pERK-positive cells was different from that of IER3-positive cells. pERK-positive cells were restricted to the peripheral areas of the tumor in adenocarcinomas, and negative areas were excluded from evaluation

of the cell count. pERK was intensely stained mainly in the nuclei (Fig. 3B). Positive cells were stained in small clusters, which were distributed evenly throughout squamous cell carcinoma (Fig. 3C).

The ratio of pERK expression was significantly higher in almost all adenocarcinomas than in normal epithelia, except for 1 case. None of the squamous cell carcinomas showed a significant difference from normal epithelia (Fig. 4A). When

Table II. Protein overexpression, gene mutations and allelic deletion of PPP2R5C.

Case	Overexpression		Mutation				Deletion
	IER3	pERK	<i>EGFR</i>	<i>KRAS</i>	<i>IER3</i>	<i>PPP2R5C</i>	<i>PPP2R5C</i>
1	+	-	-	-	-	-	-
2	+	+	L858R	-	-	-	-
3	+	+	-	-	-	-	Not informative
4	+	+	L858R	-	-	-	-
5	+	+	L861Q	-	-	-	-
6	+	+	746-753del	-	-	-	Not informative
7	+	+	746-750del	-	-	-	+
8	+	+	-	-	-	-	Not informative
9	+	+	-	-	-	-	+
10	+	+	-	-	-	-	+
11	+	-	-	-	-	-	Not informative
12	+	+	-	-	-	-	+

Figure 5. Electropherograms around the heterozygous point of the single nucleotide (G) insertion at -122 in intron 1 of the PP2A-B56 γ gene. T, tumor; NT, non-tumor.

the ratios in all the cases were compared, multiple comparison showed a significant difference in adenocarcinoma, but not in squamous cell carcinoma (Fig. 4B).

Direct sequencing. To examine the relationship between IER3/pERK overexpression in adenocarcinoma and alteration of the EGFR-ERK cascade or the ERK/IER3/B56 γ 1 feedback loop, we looked for mutations in the sequences of *EGFR*, *K-RAS*, *PPP2R5C* and *IER3*. The results are summarized in Table II.

In 12 cases of adenocarcinoma, *EGFR* mutation was found in 5 cases; deletion of *EGFR* exon 19 (746-750del) was found in 2 cases. In the other 3 cases, point mutation was found in *EGFR* exon 21 (L861Q or L858R). No mutation of *K-RAS* exon 2 was found. No *EGFR* and *K-RAS* mutations were found in squamous cell carcinomas. *IER3* showed no mutation in any of the cases.

In the process of sequencing of *PPP2R5C*, we observed intermittent duplicated waves in the electropherograms from samples of non-tumor lung tissues, indicating the presence of a mixture of different sequences. Insertion or deletion of one nucleotide in the first intron in one allele apparently generated the duplication. We further designed a primer set spanning intron 1, and confirmed the duplication by TA

cloning analysis. The sequence of one allele is the same as the reported sequence (accession no., NC 000014), while the sequence of the other allele showed insertion of a single nucleotide (G) 122 bases upstream of the end of the first intron (IVS1-122insG). The electropherogram using cDNA and the primer sets (Table I) amplifying the boundary of exon 1 and exon 2 showed single waves of the reported sequence (accession no. NM 002719). Finally, it was concluded that there was no mutation in *PPP2R5C* in any of the cases.

Analysis of LOH at PPP2R5C using the single nucleotide insertion. Heterozygous sequences in intron 1 of *PPP2R5C* were found in 8 out of 12 cases (67%) in the non-tumor tissue and in 4 out of 12 cases (33%) in the tumor. Finally, 4 out of 8 informative cases were heterozygous, and 4 were homozygous (50%), indicating LOH (Fig. 5).

Discussion

IER3 inhibits PP2A-B56 γ , resulting in inhibition of ERK dephosphorylation (13,17), so that overexpression of IER3 could result in sustained activation of ERK, leading to cell proliferation. Our immunohistochemical findings that both IER3 and

pERK were overexpressed in adenocarcinomas support this idea. However, it was not clear whether the overexpression was due directly to changes of IER3 or ERK, or to an alteration of upstream signaling protein, such as EGFR. *EGFR* exons 18, 19, 20 and 21 encode an intracellular tyrosine kinase, and mutations in these regions cause ligand-independent EGFR activation (31), leading to overexpression of pERK through the EGFR/RAS/RAF/MEK cascade (26,32). We found EGFR mutation in 42% of lung adenocarcinomas, and all of the EGFR mutation cases showed pERK overexpression. This is consistent with the results of a previous study (33). In addition, overexpression of ERK by IHC is reported to be positive in 89% of lung adenocarcinomas that overexpress EGFR (34). Thus, it is strongly suggested that overexpression of pERK/IER3 is caused by activating mutation of EGFR. However, in the cases with no mutation of EGFR, there is no obvious reason for pERK/IER3 overexpression. Therefore, we considered the possible role of a positive feedback loop of ERK/IER3/PP2A-B56γ1 in the nucleus (17). Both IER3 and pERK were overexpressed in the nucleus in adenocarcinomas examined by us, which is consistent with the existence of an ERK/IER3/PP2A-B56γ1-positive feedback loop in lung adenocarcinoma. This positive feedback loop may be involved in carcinogenesis of lung adenocarcinoma (35).

Recently, Garcia *et al* (36) proposed that pERK enhanced the immediate expression of IER3 in pancreatic cancer, and they found that IER3 is prominently expressed in pancreatic ductal adenocarcinoma. IER3 expression sustains phosphorylation of ERK through PP2A. Invasive breast cancer (7) and multiple myeloma (37) show high IER3 expression, but on the contrary, IER3 expression in ovarian cancer is lower than in benign cystadenomas (6). This might reflect the fact that IER3 has apparently contradictory functions of proapoptosis and cell proliferation (3,4), possibly by inhibiting the activities of other B56 species involved in different signaling pathways from B56γ (13). The positive correlation of IER3 expression and pERK expression may suggest that the interaction of IER3/ERK promotes cell proliferation.

PP2A-B56γ1 dephosphorylates and inactivates ERK, and inhibits cell proliferation and migration (15,17). PP2A functions as a tumor-suppressor gene (38,39). One of the mechanisms of inactivation of tumor-suppressor genes is allelic deletion or loss of heterozygosity (LOH). *PPP2R5C* (PP2A B56γ gene) is located at the tip of the long arm of chromosome 14 (14q32.31). Deletion of 14q has been reported in lung non-small cell carcinoma (21), and may be relevant to the present findings. The reported 14q deletion in lung non-small cell carcinoma occurred only in 4% of cases (21), which is much less frequent than 50% in the present study. However, much more frequent LOH at shorter portions in 14q have been reported (40,41), although LOH of 14q32 has not been examined for lung carcinoma. LOH of 14q32 was found in 32-44% of colorectal cancers (22,42), and is associated with metastatic recurrence (42). LOH was also found in 31% of neuroblastomas (43), 37% of esophageal cancers (23), and 16% of gastric cancers (44). Breast carcinoma with *BRCA2* mutation shows 14q32 LOH in 80% of cases, which may suggest the presence of tumor suppressor genes at 14q32 (45).

Suppressor genes including *RB* and *TP53*, which are recessive genes, are mutated or deleted in one allele in addi-

tion to deletion in the other allele in lung carcinomas (46). In the present study, LOH at *PPP2R5C* was found frequently, but we did not find mutation of *PPP2R5C*. Therefore, some other mechanism of cPP2A-B56γ inactivation must exist if PP2A-B56γ is involved in carcinogenesis. PP2A consists of three subunits, A, B and C. Deletion or inactivating mutation in the subunit A or C gene, in addition to allelic deletion of B56γ, would further reduce function of PP2A-B56γ. Subunit A gene, *PPP1R1B*, is mutated in 15% of lung and colon cancers (47), and expression of Aβ is lost in colon cancer (48). The Aβ gene is located at 11q22-23, and 11q is deleted in 20% of lung cancers (21). Further, 11q22-24 is deleted in 63% of lung adenocarcinomas (49). The subunit C gene, *PPP2CA*, is located at 5q, which is deleted in 20% of lung carcinomas (21). Taken together, these findings suggest it is highly likely that PP2A-B56γ function is frequently lost in lung adenocarcinoma, as well as other carcinomas such as colorectal carcinoma, leading to overexpression of IER3/pERK. We found LOH of *PPP2R5C* in 50% of lung adenocarcinomas, and many of them did not appear to have any other mutation that might cause IER3/pERK overexpression. Thus, deletion of 14q32 could be an important factor in carcinogenesis of the lung.

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References

- Charles CH, Yoon JK, Simske JS and Lau LF: Genomic structure, cDNA sequence, and expression of gly96, a growth factor-inducible immediate-early gene encoding a short-lived glycosylated protein. *Oncogene* 8: 797-801, 1993.
- Kondratyev AD, Chung KN and Jung MO: Identification and characterization of a radiation-inducible glycosylated human early-response gene. *Cancer Res* 56: 1498-1502, 1996.
- Arlt A and Schäfer H: Role of the immediate early response 3 (IER3) gene in cellular stress response, inflammation and tumorigenesis. *Eur J Cell Biol* 90: 545-552, 2011.
- Wu MX: Roles of the stress-induced gene IEX-1 in regulation of cell death and oncogenesis. *Apoptosis* 8: 11-18, 2003.
- Sasada T, Azuma K, Hirai T, Hashida H, Kanai M, Yanagawa T and Takabayashi A: Prognostic significance of the immediate early response gene X-1 (IEX-1) expression in pancreatic cancer. *Ann Surg Oncol* 15: 609-617, 2008.
- Han L, Geng L, Liu X, Shi H, He W and Wu MX: Clinical significance of IEX-1 expression in ovarian carcinoma. *Ultrastruct Pathol* 35: 260-266, 2011.
- Yang C, Trent S, Ionescu-Tiba V, Lan L, Shioda T, Sgroi D and Schmidt EV: Identification of cyclin D1- and estrogen-regulated genes contributing to breast carcinogenesis and progression. *Cancer Res* 66: 11649-11658, 2006.
- Steensma DP, Neiger JD, Porcher JC, Keats JJ, Bergsagel PL, Dennis TR, Knudson RA, Jenkins RB, Santana-Davila R, Kumar R, *et al*: Rearrangements and amplification of IER3 (IEX-1) represent a novel and recurrent molecular abnormality in myelodysplastic syndromes. *Cancer Res* 69: 7518-7523, 2009.
- Janssens V and Goris J: Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J* 353: 417-439, 2001.
- Lee TY, Lai TY, Lin SC, Wu CW, Ni IF, Yang YS, Hung LY, Law BK and Chiang CW: The B56γ3 regulatory subunit of protein phosphatase 2A (PP2A) regulates S phase-specific nuclear accumulation of PP2A and the G1 to S transition. *J Biol Chem* 285: 21567-21580, 2010.
- Eichhorn PJ, Creghton MP and Bernards R: Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* 1795: 1-15, 2009.

12. Westermarck J and Hahn WC: Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 14: 152-160, 2008.
13. Letourneau C, Rocher G and Porteu F: B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J* 25: 727-738, 2006.
14. Garcia J, Ye Y, Arranz V, Letourneau C, Pezeron G and Porteu F: IEX-1: A new ERK substrate involved in both ERK survival activity and ERK activation. *EMBO J* 21: 5151-5163, 2002.
15. Mebratu Y and Tesfagzi Y: How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* 8: 1168-1175, 2009.
16. Wortzel I and Seger R: The ERK cascade: Distinct functions within various subcellular organelles. *Genes Cancer* 2: 195-209, 2011.
17. Kawahara E, Maenaka S, Shimada E, Nishimura Y and Sakurai H: Dynamic regulation of extracellular signal-regulated kinase (ERK) by protein phosphatase 2A regulatory subunit B56 γ 1 in nuclei induces cell migration. *PLoS One* 8: e63729, 2013.
18. Traverse S, Gomez N, Paterson H, Marshall C and Cohen P: Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J* 288: 351-355, 1992.
19. Marshall CJ: Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80: 179-185, 1995.
20. Nobumori Y, Shouse GP, Wu Y, Lee KJ, Shen B and Liu X: Characterization of B56 γ tumor-associated mutations reveals mechanisms for B56 γ -PP2A tumor suppressor activity. *Mol Cancer Res* 11: 995-1003, 2013.
21. Tepeli E, Muslumanoglu MH, Uludag A, Buyukpinarbasili N, Ozdemir M, Oznur M, Aslan H and Artan S: Detection of deletions and/or amplifications of genes related with lung cancer by multiplex ligation-dependent probe amplification (MLPA) technique. *Cancer Biol Ther* 8: 2160-2165, 2009.
22. Bando T, Kato Y, Ihara Y, Yamagishi F, Tsukada K and Isobe M: Loss of heterozygosity of 14q32 in colorectal carcinoma. *Cancer Genet Cytogenet* 111: 161-165, 1999.
23. Ihara Y, Kato Y, Bando T, Yamagishi F, Minamimura T, Sakamoto T, Tsukada K and Isobe M: Allelic imbalance of 14q32 in esophageal carcinoma. *Cancer Genet Cytogenet* 135: 177-181, 2002.
24. Ito A, Kataoka TR, Watanabe M, Nishiyama K, Mazaki Y, Sabe H, Kitamura Y and Nojima H: A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. *EMBO J* 19: 562-571, 2000.
25. Zheng H, Chen Y, Bando T, Niu Y, Yang L, Li B, Lu Y, Geng S, Du X and Li Y: Expression and distribution of PPP2R5C gene in leukemia. *J Hematol Oncol* 4: 21, 2011.
26. Montagut C and Settleman J: Targeting the RAF-MEK-ERK pathway in cancer therapy. *Cancer Lett* 283: 125-134, 2009.
27. Koudelakova V, Kneblova M, Trojanec R, Drabek J and Hajduch M: Non-small cell lung cancer - genetic predictors. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 157: 125-136, 2013.
28. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, Lee J, Jung YJ, Kim JO, Shin JY, *et al*: The transcriptional landscape and mutational profile of lung adenocarcinoma. *Genome Res* 22: 2109-2119, 2012.
29. Hiramatsu M, Ninomiya H, Inamura K, Nomura K, Takeuchi K, Satoh Y, Okumura S, Nakagawa K, Yamori T, Matsuura M, *et al*: Activation status of receptor tyrosine kinase downstream pathways in primary lung adenocarcinoma with reference of KRAS and EGFR mutations. *Lung Cancer* 70: 94-102, 2010.
30. Stella GM, Scabini R, Inghilleri S, Cemmi F, Corso S, Pozzi E, Morbini P, Valentini A, Dore R, Ferrari S, *et al*: EGFR and KRAS mutational profiling in fresh non-small cell lung cancer (NSCLC) cells. *J Cancer Res Clin Oncol* 139: 1327-1335, 2013.
31. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, *et al*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129-2139, 2004.
32. Chung BM, Dimri M, George M, Reddi AL, Chen G, Band V and Band H: The role of cooperativity with Src in oncogenic transformation mediated by non-small cell lung cancer-associated EGF receptor mutants. *Oncogene* 28: 1821-1832, 2009.
33. Rinehart J, Adjei AA, Lorusso PM, Waterhouse D, Hecht JR, Natale RB, Hamid O, Varterasian M, Asbury P, Kaldjian EP, *et al*: Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol* 22: 4456-4462, 2004.
34. Erman M, Grunewald D, Penault-Llorca F, Grenier J, Besse B, Validire P, Morat L, Girard P, Le Chevalier T, Sabatier L, *et al*: Epidermal growth factor receptor, HER-2/neu and related pathways in lung adenocarcinomas with bronchioloalveolar features. *Lung Cancer* 47: 315-323, 2005.
35. Wu MX, Ustyugova IV, Han L and Akilov OE: Immediate early response gene X-1, a potential prognostic biomarker in cancers. *Expert Opin Ther Targets* 17: 593-606, 2013.
36. Garcia MN, Grasso D, Lopez-Millan MB, Hamidi T, Loncle C, Tomasini R, Lomberg G, Porteu F, Urrutia R and Iovanna JL: IER3 supports KRASG12D-dependent pancreatic cancer development by sustaining ERK1/2 phosphorylation. *J Clin Invest* 124: 4709-4722, 2014.
37. Ria R, Todoerti K, Berardi S, Coluccia AM, De Luisi A, Mattioli M, Ronchetti D, Morabito F, Guarini A, Petrucci MT, *et al*: Gene expression profiling of bone marrow endothelial cells in patients with multiple myeloma. *Clin Cancer Res* 15: 5369-5378, 2009.
38. Shi Y: Serine/threonine phosphatases: Mechanism through structure. *Cell* 139: 468-484, 2009.
39. Janssens V, Goris J and Van Hoof C: PP2A: The expected tumor suppressor. *Curr Opin Genet Dev* 15: 34-41, 2005.
40. Abujiang P, Mori TJ, Takahashi T, Tanaka F, Kasyu I, Hitomi S and Hiai H: Loss of heterozygosity (LOH) at 17q and 14q in human lung cancers. *Oncogene* 17: 3029-3033, 1998.
41. Kwong FM, Wong PS and Lung ML: Genetic alterations detected on chromosomes 13 and 14 in Chinese non-small cell lung carcinomas. *Cancer Lett* 192: 189-198, 2003.
42. Al-Mulla F, AlFadhli S, Al-Hakim AH, Going JJ and Bitar MS: Metastatic recurrence of early-stage colorectal cancer is linked to loss of heterozygosity on chromosomes 4 and 14q. *J Clin Pathol* 59: 624-630, 2006.
43. Hoshi M, Shiwaku HO, Hayashi Y, Kaneko Y and Horii A: Deletion mapping of 14q32 in human neuroblastoma defines an 1,100-kb region of common allelic loss. *Med Pediatr Oncol* 35: 522-525, 2000.
44. Dai YC, Ho CL, Tsai YC, Hsu YH, Chang YC, Liu HS, Chen HH and Chow NH: Allelic loss of 14q32 in the pathogenesis of gastrointestinal and ampullary malignancies: Mapping of the target region to a 17 cM interval. *J Cancer Res Clin Oncol* 131: 94-100, 2005.
45. Pécuchet N, Popova T, Manié E, Lucchesi C, Battistella A, Vincent-Salomon A, Caux-Moncoutier V, Bollet M, Sigal-Zafrani B, Sastre-Garau X, *et al*: Loss of heterozygosity at 13q13 and 14q32 predicts BRCA2 inactivation in luminal breast carcinomas. *Int J Cancer* 133: 2834-2842, 2013.
46. Sekido Y, Fong KM and Minna JD: Progress in understanding the molecular pathogenesis of human lung cancer. *Biochim Biophys Acta* 1378: F21-F59, 1998.
47. Wang SS, Esplin ED, Li JL, Huang L, Gazdar A, Minna J and Evans GA: Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* 282: 284-287, 1998.
48. Carmen Figueroa-Aldariz M, Castañeda-Patlán MC, Santoyo-Ramos P, Zentella A and Robles-Flores M: Protein phosphatase 2A is essential to maintain active Wnt signaling and its β tumor suppressor subunit is not expressed in colon cancer cells. *Mol Carcinog* 54: 1430-1441, 2015.
49. Rasio D, Negrini M, Manenti G, Dragani TA and Croce CM: Loss of heterozygosity at chromosome 11q in lung adenocarcinoma: Identification of three independent regions. *Cancer Res* 55: 3988-3991, 1995.