Activation of ERK/IER3/PP2A-B56γ-positive feedback loop in lung adenocarcinoma by allelic deletion of B56γ gene

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Abstract. In order to investigate the involvement of the IER3/PP2A-B56γ/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. TP53, whose gene product is located upstream of ERK, was sequenced. Activating mutation of TP53, which is a possible cause of overexpression of TP53 and pERK, was found only in 5 adenocarcinomas (42%). No mutation of TP53 was found. We further examined the sequences of all exons of B56γ gene (PPP2R5C) and IER3, but no mutation was found. Using a single nucleotide insertion in intron 1 of PPP2R5C, which is located at 14q32.31, in lung carcinomas as well as other carcinomas has been reported (20). Deletion of 14q was also 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, PPP2R5C, whose gene product 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.

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.

In this context, mutation of the B56γ 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

Introduction

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in a variety of carcinomas (22,23), which suggests an important role of B56γ in carcinogenesis. Furthermore, BL6 mouse melanoma cells, in which B56γ is truncated, are highly metastatic (24). In a patient with Sezary syndrome, translocation of t(14;14) (q11.3;q32) caused rearrangement of PPP2R5C (25). All of these findings indicate an important role of B56γ in carcinogenesis. We recently showed that the ERK/IER3/B56γ 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/B56γ-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 B56γ 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 μm) 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 μl 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 ReliaPrep™ 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 (B56γ 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 pCR™ 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.

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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.
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.
Immunohistochemical detection of phosphorylated ERK. Phosphorylated ERK was immunostained in both nuclei and cytoplasm of bronchial 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
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/B56γ 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 (L858R 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-B56γ, 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

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

<table>
<thead>
<tr>
<th>Case</th>
<th>IER3</th>
<th>pERK</th>
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<th>KRAS</th>
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<td>3</td>
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<td>L858R</td>
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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). In addition, 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, García 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 diphosphorylates 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 addition 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 cPPP2A-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 1q22-23, and 1q 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


