AKT1 and AKT2 mutations in lung cancer in a Japanese population

HIDEFUMI SASAKI, KATSUHIRO OKUDA, OSAMU KAWANO, HARUHIRO YUKIUE, MOTOKI YANO and YOSHITAKA FUJI

Department of Surgery II, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

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Abstract. Mutations of the AKT1 (v-akt murine thymoma viral oncogene homologue 1) gene at amino acid 17 (E17K) and in the kinase domain of the AKT2 gene have been reported in non-small cell lung cancer (NSCLC). We investigated AKT1 and AKT2 mutations (n=146 and n=124, respectively) in surgically-treated NSCLC cases. The presence or absence of AKT1 (E17K) and AKT2 kinase domain mutations was analyzed by direct sequencing. Although there were no AKT1 mutations at the N-terminal, an AKT2 mutation was detected in 1 of 124 lung cancer patients (0.8%). The sample was also analyzed for EGFR, K-ras, PIK3CA and BRAF mutations. In addition to the AKT2 missense mutation (R371H), this lung adenocarcinoma was shown to harbor an EGFR (L861Q) and a PIK3CA (E542Q) mutation. This study demonstrated that mutations in the kinase domain of AKT2 occur in a small percentage of Japanese patients with lung cancer, while E17K AKT1 mutations do not occur at all.

Introduction

Lung cancer is a major cause of death from malignant disease due to its high incidence, malignant behavior and the lack of major advancements in treatment strategy (1). It has been proposed that the activation of the intracellular pro-survival signal transduction protein AKT (v-akt murine thymoma viral oncogene) is a central signaling event in carcinogenesis (2), and has been shown in experimental models to confer chemoresistance in lung cancer (3). In addition, expression of the activated form of the cell survival protein AKT (phospho AKT) in primary non-small cell lung cancer (NSCLC) is an independent indicator of poor prognosis (4). A previous report, which investigated AKT1, 2 and 3 mutations in common cancers (5), indicated that AKT2 mutations were present in the kinase domain in 2.5% of lung cancers, while no AKT1 or AKT3 mutations were found (5). Recently, a novel somatic AKT1 mutation resulting in a glutamic acid to lysine substitution at amino acid 17 (E17K) in the lipid-binding pocket of AKT was identified in several cancers (6). Lys 17 alters the electrostatic interactions of the pocket and forms new hydrogen bonds with a phosphoinositide ligand (6). Malanga et al demonstrated that E17K mutations are predominantly found in squamous cell histotypes of lung cancer, and did not identify mutations in lung adenocarcinoma cases (7).

Because we have previously found mutations of the other kinase gene, epidermal growth factor receptor (EGFR) (8-11), to be predominant in a Japanese population, we now chose to investigate AKT1 and AKT2 gene status by direct sequencing. Our findings were compared to the clinicopathologic features of lung cancer.

Patients and Methods

Patients. The study group included lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School. Lung tumors were classified according to the general rules for the clinical and pathological recording of lung cancer in Japan (12). All tumor samples were immediately frozen and stored at -80°C until use for assay. For the AKT1 study, because Malanga et al had previously demonstrated that E17K mutations were predominantly found in squamous cell histotypes of lung cancer and had found no mutations in adenocarcinomas (7), we focused on squamous cell carcinomas. The clinical and pathological characteristics of the 146 NSCLC patients used for AKT1 gene analysis were as follows: 134 patients (91.8%) were male and 12 were female; 138 (94.5%) were smokers and 8 were non-smokers. Ninety cases (61.6%) were diagnosed as squamous cell carcinoma, 48 as adenocarcinoma and 7 as adenosquamous cell carcinoma. For the AKT2 study, since Soung et al had demonstrated that the AKT2 missense mutation was found in adenocarcinomas (5), we too focused on adenocarcinomas. The clinical and pathological characteristics of the 124 NSCLC patients used for AKT2 gene analysis were as follows: 58 patients (46.7%) were male and 66 were female; 45 (36.3%) were smokers and 79 were non-smokers. Thirteen cases (10.5%) were diagnosed as squamous cell carcinoma, 107 as adenocarcinoma and 4 as adeno-squamous cell carcinoma.

Correspondence to: Dr Hidefumi Sasaki, Department of Surgery II, Nagoya City University Medical School, 1 Kawasaki, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
E-mail: hisasaki@med.nagoya-cu.ac.jp

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PCR assay for AKT1 and AKT2 mutations. Total-RNA was extracted from lung cancer tissues using the Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer and adjusted to a concentration of 200 ng/ml. Approximately 10 cases were excluded from each assay as the tumor cells were not sufficient for the extraction of tumor RNA. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco-BRL, Gaithersburg, MD) with 0.5 μg oligo (dT)12-16 (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 μl of each cDNA sample for PCR analysis. PCR reactions were performed using the LA Taq kit (Takara Bio Inc., Shiga, Japan) in a 25-μl reaction volume. The primer sequences for the AKT1 gene N-terminal domain were as follows: forward, 5'-ATGAGCGACGTGGCTATTGT-3'; reverse, 5'-TCCTCCTCCCTTCCTGCTTCTT-3' (350 bp, exons 1-3). Cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 45 sec, 60°C for 45 sec and 72°C for 45 sec. The primer sequences for the AKT2 gene kinase domain were as follows: forward, 5'-GACAAAGATGGCCACATCAA-3'; reverse, 5'-CTGGGCGGTAAATTCATCAT-3' (489 bp, exons 9-12). Cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 45 sec, 58°C for 45 sec and 72°C for 45 sec. The products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). Samples were sequenced using the ABI PRISM 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review. The results of EGFR mutation in the kinase domain have previously been reported (8-11).

Statistical analysis. Statistical analysis was conducted using the Mann-Whitney U test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and the $\chi^2$ test. All analyses were performed using a StatView software package (Abacus Concepts Inc., Berkeley, CA). A p-value <0.05 was considered significant.

Results

AKT1 gene mutation status in Japanese lung cancer patients. Sequencing was performed for the N-terminal of the AKT1 gene in 146 NSCLC samples. Among the 146 patients, no mutations were detected by direct sequencing with cDNA samples.

AKT2 gene mutation status in Japanese lung cancer patients. Sequencing was conducted for the kinase domain of the AKT2 gene in 124 NSCLC samples. Among the 124 patients, only one mutation at exon 11 (G1112A, R371H) was detected by direct sequencing with cDNA samples (Fig. 1). The patient was a female non-smoker with well-differentiated adenocarcinoma with a pathological stage of T1N2 (stage IIIa). The patient also had a L861Q mutation at exon 21 (Fig. 2) and a PIK3CA mutation (E542Q). In this sample, sequencing was additionally performed for K-ras, N-ras and BRAF. However, neither the sequencing nor genotyping analysis revealed any mutations. Comparison of protein sequences indicated that R371 was highly conserved with the other AKT family proteins, AKT1 and AKT3.

Discussion

We found AKT2 mutations in the kinase domain in 0.8% of Japanese lung cancer patients, and no cases of AKT1 mutation in Japanese NSCLC patients. Although the incidence of AKT2 mutations was low, the location of the mutation at an important functional site (the kinase domain) suggests that AKT2...
mutations contribute to the pathogenesis of lung cancer.

AKT has been found to play a role in the survival of cancer cells (13-16). It has also been shown to confer chemoresistance in NSCLC cell lines (3,17). AKT activation is driven by membrane localization initiated by the binding of the pleckstrin homology domain (PHD) to phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3) or phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P2) (2). The importance of AKT in human cancer is largely inferred based on common recurring mutations in enzymes that produce or degrade short-lived second messenger phospholipids and consequently indirectly activate AKT through membrane recruitment. We and others have identified the activating somatic mutations in PIK3CA, the catalytic subunit of phosphatidylinositol-3-0H kinase (PI(3)K)-enzyme, which catalyzes the production of D3-phosphorylated phosphoinositides in lung carcinomas (18-20).

The PHD plays a critical regulatory role in AKT function, and its disruption has an impact on biology and disease. Cellular survival in the presence of high levels of PtdIns(3,4,5)P3 is only possible if the Drosophila AKT PHD is inactivated (21). The introduction of a homologous mutation into the PHD of AKT1 (R25C) results in a kinase that does not efficiently bind phosphoisoitides, fails to localize to the membrane and is not activated (22). More recently, an E17K AKT1 mutation was shown to activate AKT1 by means of pathological localization to the plasma membrane, the stimulation of downstream signaling and the transformation of cells, inducing leukemia in mice (5). Malanga et al demonstrated that E17K mutations were present in 5.5% of squamous cell carcinomas, but in no adenocarcinomas (7). However, in our analysis we did not find any mutations in the N-terminal of AKT1. This may be due to racial differences between the studies conducted on mutant E17K.

AKT2, a member of the AKT family, is associated with the development of human cancer. Overexpression of AKT2 has been observed in 20% of pancreatic adenocarcinomas (23). Transfection of antisense AKT2 RNA into pancreatic adenocarcinoma cell lines inhibits tumorigenesis (24). Additionally, it has been demonstrated that AKT2 expression is correlated with prognosis in human hepatocellular carcinoma (25). Moreover, a previous report revealed that 1 in 79 lung cancer cases had a missense AKT2 mutation. Proteins along the AKT signaling pathway, including K-ras, EGFR, erbB2 and BRF, are frequently mutated in lung cancers (8-11,26,27) and act as oncogenic proteins. Of these genes, we found an AKT2 mutation in a lung adenocarcinoma, suggesting that alterations in AKT signaling by both AKT2 and EGFR mutation can occur. These together could contribute to the pathogenesis of lung adenocarcinomas.

In summary, AKT1 mutations in Japanese lung cancer patients would appear to be extremely rare; however, AKT2 mutations may play a role in function.

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