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PIK3CA mutation status in Japanese lung cancer patients

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Summary Somatic mutations of the PIK3CA (phosphatidylinostitol 3-kinase catalytic subunit) gene have been found in human cancer patients. Previous reports suggested that about 4% of lung cancers harbored PIK3CA gene mutations. However, the clinico-pathological background for PIK3CA gene mutations has not yet been investigated in lung cancer. We have genotyped the PIK3CA gene in Japanese lung cancer patients. The study included 235 lung cancer cases surgically removed in Nagoya City University Hospital. The two PIK3CA mutation hot spots (exon 9 and exon 20) were analyzed by real time polymerase chain reaction (PCR)-based assay. The data were confirmed by direct sequencing. In exon 9, somatic mutation was found in eight patients (3.4%). The mutation included three E542K (G1624A), three E545K (G1633A), one E542Q (G1624C), and one Q546K (C1636A). However, in exon 20, there was no mutation in our lung cancer patients. PIK3CA mutations were not correlated with gender (women versus men, $p = 0.4162$), age ($\leq$60 versus >60, $p = 0.8027$), or smoking status of the lung cancers (never versus smoker, $p = 0.5666$). PIK3CA mutation incidence was significantly lower in adenocarcinoma (2/135, 1.5%) than in squamous cell carcinoma (5/77, 6.5%, $p = 0.0495$). Among eight patients with a PIK3CA mutation, three patients also harbored an EGFR somatic mutation. PIK3CA gene mutations were rare in lung cancer; rarer in adenocarcinoma. Further functional analyses of the PIK3CA mutations are warranted to study if they could be the target of therapy for the lung cancer.

1. Introduction

It is now well established that cancer is a genetic disease and that somatic mutation of the oncogene and tumor suppressor genes are the initiator of the carcinogenic process [1]. The phosphatidylinositol 3-kinase signaling pathway has recently

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been suggested to play a pivotal role in the oncogenesis of human cancers [1].

Recently, high frequencies of somatic mutation in the PIK3CA gene have been reported in several cancer types, including colon, brain, stomach, breast, and ovary [2—7]. More than 75% of these mutations are clustered in the helical (exon 9) and kinase domains (exon 20) of the PIK3CA gene [2]. Mutations in the three mutation hotspots in PIK3CA (i.e., E542K, E545K, and H1047R) have been shown to elevate its lipid kinase activity and lead to the activation of the downstream Akt-signaling pathway [2,8]. The PIK3CA mutation was identified in 4% of lung cancer cases, however, this particular study included only one case with the mutation [2].

For known mutations, real time polymerase chain reaction followed by melting curve analysis, using hybridization probes, is highly sensitive, rapid, and an efficient approach to mutation detection [9—11]. To determine the PIK3CA mutation status in Japanese lung carcinoma for screening and diagnostic purpose, we wanted to develop a faster and easy method to detect mutations. In this report, we investigated PIK3CA mutation status by the real time PCR-based assay using LightCycler [12] wild type specific sensor an anchor probes. The PIK3CA gene was then sequenced to confirm the PCR study. The findings were analyzed in reference to the clinico-pathologic features of the lung cancer.

2. Materials and methods

2.1. Patients

The study groups included 235 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2003. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan [13]. All tumor samples were immediately frozen and stored at −80 °C until assayed.

The clinical and pathological characteristics of the 235 lung cancer patients are as follows; 116 cases at stage I, 47 at stage II, and 72 at stages III—IV. The mean age was 65.153 years (range, 38—85). Among the lung cancer patients, 139 (57.4%) were diagnosed as adenocarcinoma, 77 (32.8%) were as squamous cell carcinoma, and 11 (8.8%) were as adenosquamous cell carcinoma. EGFR mutation status of 235 lung cancer patients was already reported elsewhere [14—17].

2.2. PCR assays for EGFR

Total RNA was extracted from lung cancer tissues and adjacent non-malignant lung tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About 10 cases were excluded because tumor cells were too few to sufficiently extract 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 DNA for LightCycler analyses. The genotyping PCR reactions were performed using LightCycler DNA Master Hybridization probes kit (Roche Molecular Biochemicals, Mannheim, Germany) in a 20-μl reaction volume. The primer sequences for PIK3CA gene in exon 9 were as follows: the forward primer, 5-GAATTGGTCTGTATCCCGAG-3 and the reverse primer, 5-CGGGGATAGTTACACAATAGT-3 (200 bp). For the exon 9 genotyping, sensor (LC Red 640-AGGATCTCGTGTAGAAATTGCTTTGAGCTGTTCTT-phoshate) and anchor (TTTCTCCTGCTCAGTGATTTCAGAGA-Fluorescein) probes were used. The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 8 s. The primer sequences for PIK3CA gene in exon 20 were as follows: the forward primer, 5-CTCTGGAATGCCAGAACTAC-3 and the reverse primer, 5-ATGCTGTTTAATTGTGTGGAAG-3 (175 bp). For the exon 20 genotyping, sensor (ACCCT-AGCCTTAGATAAAACTGAGCAAGAGGCTTT-Fluorescein) and anchor (LC Red 640 GAGTATTTCATGAAACAAATGAATGCA-CATC) probes were used. The cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 7 s. The cDNA from HCT-116 colon cancer cell line was used as a positive control. This cell line has H1047R (exon 20) mutation [18]. The primers for PIK3CA gene in exon 9 were also used for direct sequencing.

2.3. Direct sequencing

Samples with positive results with the PCR-based study were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed using ABI prism Seq Scape Version 2.1.1.

2.4. Statistical methods

Statistical analyses were done 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 χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan-Meier methods, and differences were examined by the Log-rank test and Breslow-Gehan-Wilcoxon test. All analysis was done using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA), and was considered significant when the *p*-value was less than 0.05.

3. Results

3.1. Genotyping of PIK3CA at exon 9 and exon 20 in lung cancer tissues

For the PCR-based genotyping of the exon 9 of PIK3CA, the anchor probe was matched for the wild type sequence. As shown in Fig. 1a, wild type PCR product showed a single peak at 64 °C, whereas the heterozygous products (mutant) showed an additional peak at 57 °C. Among the 235 lung