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Abstracts of the 2009th Alumni Association Medical Research Fund Prize Memorial Lecture (1)

Development of New Molecular Markers for Cancer: A Highly Sensitive Assay for Detecting Mutations of Phosphatidylinositol 3-kinase and p53

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Introduction

Cancers are caused by a series of mutations of several somatic genes, such as p-53, APC, and K-ras. The mutation of the p53 gene is a frequent genetic change seen in cancer cells. Inactivation of the p53 tumor-suppressor gene is a critical and early event in carcinogenesis.

Phosphatidylinositol 3-kinases (PI3Ks) are heterodimeric lipid kinases composed of catalytic and adaptor/regulatory subunit variants encoded by separate genes and alternative splicing. They have recently received a great deal of attention from regulating signaling pathways important for neoplasia, including cell proliferation, adhesion, survival, and motility. Somatic mutations in many different human cancers were discovered in the gene encoding for the PI3K catalytic subunit (PIK3CA). These somatic missense mutations have been suggested to increase the kinase activity of PIK3CA contributing to cellular transformation.

The early detection of these mutations can provide clues to the etiology and pathogenesis of human cancer, and be applicable to cancer prevention and clinical treatment for patients.

Methods

The mutation load assay, a highly sensitive assay to estimate the p53 mutation load in the DNA of nontumorous tissue, was developed for specific mutations[1]. In brief, the mutation load assay procedure involves enrichment of mutated DNA fragments by means of specific restriction-enzyme digestion, followed by 2 consecutive polymerase chain reaction (PCR) amplifications, enzyme digestions, and a quantification of p53 mutated fragments with use of an internal control and slot blot hybridization. The products from the second

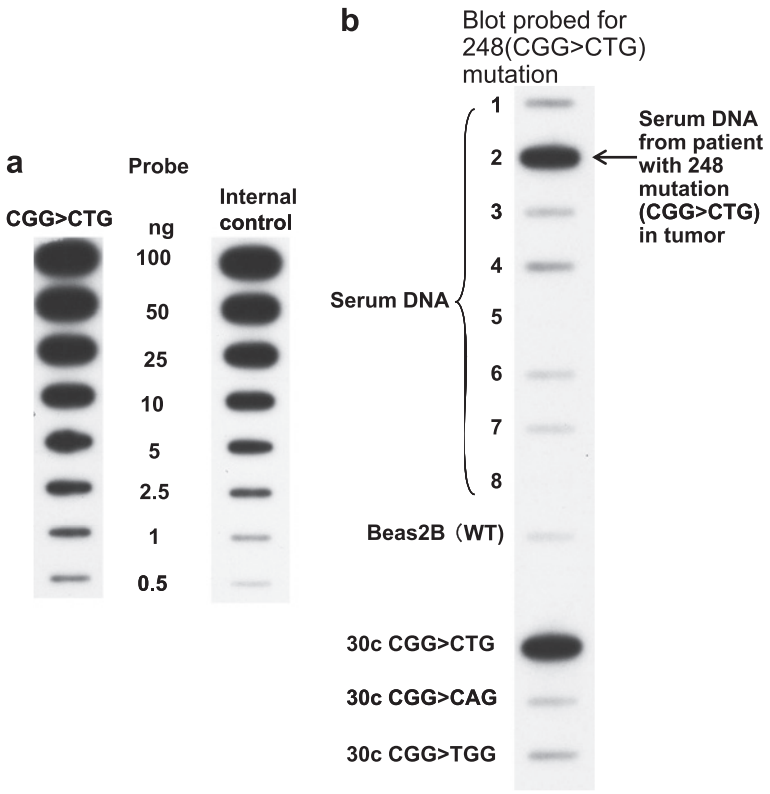

Fig. 1 p53 mutation load assay

a: Quantification control ladder for known amount of mutant (CGG>CTG at codon 248) DNA and internal control DNA showed linear intensities for the specific probes.

b: An identical codon 248 mutation was detected in amplified serum DNA from patient with a 248 (CGG>CTG) mutation in tumor.

PCR amplification were purified with a PCR purification kit and prepared for application to slot blot hybridization. Slot blot membranes were hybridized with specific labeled oligonucleotides which identify mutations under optimal conditions. Signal intensities were obtained with a phosphorimager and quantified. To determine the number of p53 mutant copies in blood DNA samples, negative control intensities from Beas2B (wild-type p53 cell line) were subtracted from the measured intensity for each blood sample. The amounts of internal control and each p53 mutant were determined by generating standard curves using known nanogram amounts applied to each membrane.

Mutation analysis of PIK3CA was performed for tissue DNA of the esophagus[2]. Genomic DNAs extracted from tumor and nontumor tissues of the esophagus were amplified with designed primers. Exons 1, 9, and 20 of PIK3CA were sequenced directly with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an automated capillary sequencer (ABI 3730, Applied Biosystems).

Results and Discussion

Optimal conditions of DNA extraction, PCR amplification, digestion, specific hybridization and washing were determined for the modified p53 mutation load assay. Under the specific conditions, quantification control ladders for known amounts of mutant and internal control DNA showed linear intensities for each probe within the ranges examined (**Fig. 1a**).

The p53 mutation load assay was applied for blood circulation DNA. An identical codon 248 mutation was detected in amplified serum DNA from a patient with a 248 (CGG>CTG) mutation in tumor under double-blind

testing **(Fig. 1b)**.

The original p53 mutation load assay had a higher sensitivity for mutation detection of 1 : 10^7 due in part to the larger amount of DNA available for assay application[3]. Based on our modified experiment, the estimated lower limit of determination was 1 : 6,400 because of the limitation in the amount of applicable DNA for the assay. However, the estimated limit of determination of our assay for p53 mutations in blood DNA was still higher than previous reports using restriction fragment length polymorphism (RFLP) (1 : 250) and similar to the combination of RFLP and short oligonucleotide mass analysis. In addition to its high sensitivity, another advantage of this assay is that is quantitative and can be used clinically to assess changes over time.

All detected PIK3CA mutations in our experiment were identical alterations of A1634C in exon 9. This finding is consistent with previous reports, in which somatic missense mutations of PIK3CA were predominantly found in the kinase and helical domains of the PIK3CA subunit. Because mutational hotspot regions are suitable targets, the mutation load assay is readily applicable to the detection of PIK3CA mutations.

Future prospects of our research are the development and improvement of highly sensitive methods for detecting PIK3CA and p53 mutations, their application to various limited specimens, and the establishment of less-invasive examinations for patients, which may lead to the early detection of cancers, more accurate diagnoses, and the assessment of treatment effects.

References

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