Sensitive Methods for the Detection of ras Mutations in Lung Cancer: Some Answers, More Questions

The ras family consists of three evolutionarily conserved genes, H-ras, N-ras, and K-ras. The ras genes code for 21-kDa proteins that mediate signal transduction between cell surface receptors and intracellular regulatory molecules. The proteins attach to the inner surface of the cytoplasmic membrane via a posttranslationally added farnesyl group. This lipid modification is essential for mediating growth response, and there is a dynamic interconversion between the two forms. Point mutations of ras are the most frequent dominant oncogene mutation found in human tumors and usually target only three codons: 12, 13, and 61. Most activating mutations are defective in GTPase activity and thus are locked into the GTP-bound form, resulting in continuous growth stimulation. Detection of ras mutations in tumors enables us to understand cancer biology and pathogenesis and may be of clinical importance by providing information useful for early diagnosis and prognosis and the development of novel therapeutic approaches (1, 2). As discussed below, ras mutations may be detected by a large variety of methodologies, both conventional and, as reported in this issue of Clinical Chemistry, supersensitive (3).

Although about 20–25% of human tumors have ras mutations, especially involving the K-ras gene, the distribution demonstrates considerable tumor type specificity (4, 5). Thus, although K-ras mutations are found in nearly 90% of pancreatic carcinomas and about 50% of colorectal carcinomas, they are rare in breast cancers. Lung cancers are an example of the heterogeneity of mutation frequency found in different histologic forms by conventional detection techniques. Of the three major forms of lung cancer, K-ras mutations are present in about 30% of adenocarcinomas, a small percentage of squamous cell carcinomas, and <1% of small cell carcinomas (5–7). ras mutations in lung cancers are almost exclusively found in smokers, and it is reasonable to assume that carcinogens in tobacco smoke directly cause the mutation (6, 8). The specific activating mutations in lung cancers are mostly G-to-T transversions, presumably from exposure to polycyclic aromatic hydrocarbons in cigarette smoke. ras mutations are also present in radon-associated tumors (9). ras mutations are associated with prolonged survival with all stages of lung cancer. (10–13). In particular, substitution of a polar or charged amino acid for the wild-type glycine residue may be a negative prognostic indicator (14). Why ras mutations in lung cancers are a negative prognostic factor is unknown, because they are not associated with a higher metastatic rate or resistance to chemotherapy (15, 16). Mutations may also be present in the putative precursor lesions of peripheral adenocarcinomas, namely foci of atypical adenomatous hyperplasia (17). In our experience, they are very rare in the nonneoplastic bronchial epithelium of lung cancer patients (18), although at least one report describes contrary findings (19). Spontaneously arising or nitrosamine-induced pulmonary tumors in mice and hamsters are almost exclusively adenocarcinomas and frequently contain activating ras mutations (20, 21), but rats may also develop ras-associated squamous carcinomas (22). Transgenic mice bearing an albumin enhancer/promoter linked to a mutated human H-ras gene develop multiple peripherally located pulmonary adenocarcinomas that are eventually lethal (23). Human bronchial epithelial cells transformed by v-K-ras form poorly differentiated adenocarcinomas in athymic nude mice (24). Despite the above cited evidence demonstrating the strong association between ras mutations and pulmonary adenocarcinomas, some reports indicate that there may be geographic differences and that in some parts of the world ras mutations may occur in squamous carcinomas as frequently as in adenocarcinomas (25).

Techniques for the routine detection of activating mutations of oncogenes in cancer cells are often laborious and time-consuming (26). With recent advances in molecular and cell biology, one can now broadly classify mutation detection into two categories. The first category of methodologies are for screening of genes such as p53 or BRCA1, where the mutations may be present at numerous sites scattered over a large portion of the gene. Methods in the second category are designed for the detection of specific mutations in the nucleotide sequence at one or a few known sites. Such methods are useful for the detection of activating ras mutations, which usually are limited to three codons of the gene, or for detection of a mutation previously identified by some other technique—such as screening family members for a specific germline mutation. Because of the relative ease of screening for a very restricted number of activating mutations, multiple methods have been developed for the detection of ras mutations. These methodologies include allele-specific hybridization (27), allele-specific amplification (28), ligase chain reaction (29), and designed restriction fragment length polymorphism (7).

The lower limits of detection of the conventional methods are in the range of one mutant form of ras in 10–20 wild-type genes or cells (7). During the last few years several groups have developed supersensitive methods that can detect mutations even at dilutions of 1 in 105 to 106 (30–33). Although the approaches of these groups vary, they involve some form of selective enrichment of the mutant allele relative to the wild-type form, followed by detection of the mutant form. Somers et al. (34) have developed a simplified, highly sensitive approach (named Point-EXACCT) for the detection of rare mutant alleles.
After amplification of the region of interest (the critical codon 12, the site of most of the ras mutations), they prepare single-stranded DNA by a novel method. After hybridization to mutant-specific and wild-type biotinylated probes, the products are incubated with T<sub>4</sub> DNA ligase. If complementarity is perfect, the ligase covalently joins the 5′ biotinylated probe and the 3′ digoxigenin-labeled detection probe. If the probes and target are mismatched, ligation does not occur. Ligated products are detected by a colorimetric method. A possible shortcoming of their method is its requirement of relatively large amounts of DNA (1–4 μg) for PCR amplifications. In our opinion, their method may be further improved by the use of small peptide nucleic acid probes (PNAs) during PCR, which suppress amplification of wild-type alleles and consequently increase the relative ratio of mutant to wild-type sequences (35). In addition, the use of a chemiluminescent assay or fluorescent dyes may give stronger signals. The entire process, including the suggested improvements, can be easily automated and adapted for use in a routine clinical laboratory.

Utilizing the Point-EXACCT method, Somers et al. (3) in this issue report the incidence of ras mutations in lung carcinomas and compare the results with more conventional methods for mutation detection by sequencing. In an analysis of 89 non-small cell lung carcinomas, the Point-EXACCT method detected a total of 21 mutations, at least 11 of which were not detected by single-strand sequencing. However, the Point-EXACCT method detected ras mutations in 8 (15%) of 52 squamous cell carcinomas, a figure much higher than would be expected using methods for high fraction mutations. The investigators did not determine whether intratumor heterogeneity existed, nor did they determine the relative ratio of wild-type to mutant forms.

What lessons can we learn from the new generation of very sensitive assays for detection of activated oncogenes such as ras? Clearly, such assays may be useful for the detection of mutant cells when they occur at very low frequencies. Thus, sensitive methodologies may be used for early cancer detection in exfoliated cells present in spuia, bronchioloalveolar lavage fluids, and other samples (30, 36, 37). However, because ras mutations occur in preneoplastic lesions, can we distinguish between mutations present in subjects at increased risk vs those with invasive cancers? Are such screening methods clinically useful and cost effective? Another application is the detection of mutations occurring in cancers that were not found by conventional methods. Although varying percentages of stromal cells are always present in tumor tissues and may mask the presence of mutations, even conventional methods may detect mutant cells present as 5–10% of the cell population (15). Thus, the sensitive detection of ras mutations in tumors not found by conventional methodologies almost certainly indicates the presence of tumor heterogeneity. Of interest, intratumor heterogeneity for ras mutations was not detected in lung carcinomas by conventional methodology (38). Patients with tumors having a low fraction of ras mutations are younger and have a better prognosis than those with high-fraction tumors (32). These results “suggest that using highly sensitive methods of K-ras mutant detection in tumor DNA could obscure differences between patients in whom the mutation is found throughout the tumor, those in whom the mutation is only present in a small subpopulation and those who have no mutation” (32). Clearly, whenever a new, highly sensitive assay is introduced into clinical practice, a new database has to be generated before its true usefulness can be evaluated.

References
1. Qian Y, Sebti SM, Hamilton AD. Farnesyltransferase as a target for antican-


