Symposium article

Molecular genetic tumor markers in the early diagnosis and screening of non-small-cell lung cancer

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Summary

Background: Little progress has been made in decreasing lung cancer mortality by applying conventional methods to early diagnosis and screening. Recent advances in molecular oncology, however, have provided tools which may be of use in this area. Many genes involved in controlling cell growth and differentiation are abnormal in lung cancer cells. Such genes include K-ras, p53, rb, myc, her2/neu, and probably one or more tumor suppressor genes on chromosome 3p. The involvement of these genes in lung cancer is reviewed. The K-ras oncogene contains a mutation in codon 12 in many cases of non-small-cell lung cancer, particularly adenocarcinoma, and is thus a potentially useful lung cancer tumor marker.

Design: We have developed a highly sensitive, simple assay for ras mutations, and applied it to bronchoalveolar lavage fluid obtained from patients undergoing evaluation for suspected lung cancer.

Results: In many cases, the ras assay was more sensitive than routine cytology and histopathology, demonstrating that this is a potentially clinically useful assay.

Conclusion: Molecular genetic tumor markers, including mutations in ras and other genes, and/or immunohistochemical tumor markers, may provide tools which can be applied to bronchoalveolar lavage fluid or sputum, for use in diagnostic tests and in screening programs. The use of such markers may lead to decreased lung cancer mortality.

Key words: biomarker, early diagnosis, lung cancer, ras, oncogene, screening

Rationale for the clinical use of molecular tumor markers

Clinically useful tests for cancer include both screening tests, used in an asymptomatic population, and diagnostic tests, used in patients clinically suspected of having cancer. In regard to lung cancer, a screening test must be able to identify patients at an early enough stage of disease such that local resection will be potentially curative. In contrast, a diagnostic test applied to a patient suspected of having lung cancer may be of use if it facilitates the diagnosis of cancer, reducing the need for invasive diagnostic procedures, and expediting the institution of therapy.

Lung cancer screening is important because most cases are unresectable at the time of diagnosis, and thus are no longer curable [1]. It has long been hoped that earlier lung cancer detection would decrease lung cancer mortality, but in 1995, despite many efforts, this goal remains unattained. Many studies of lung cancer screening have found that periodic chest X-rays and/or sputum cytology has no impact on lung cancer mortality; thus, such screening is generally not recommended [2]. These negative results may have resulted not from any problem with the concept of screening, but rather from the relative insensitivity of sputum cytologic examination. At present, no test for cancer other than cytologic examination is routinely performed on BAL fluid, but molecular tumor markers, applied to both sputum and BAL fluid, might improve cancer detection. Eventually, the application of multiple molecular markers to clinical specimens may lead to reduced lung cancer mortality. Furthermore, even in cases where cancer is advanced at the time of diagnosis, sensitive molecular markers may facilitate definitive diagnosis, in some cases eliminating the need for additional invasive diagnostic procedures.

Most malignancies, including non-small-cell lung cancer (NSCLC), develop because of an accumulation of changes (point mutations, rearrangements, amplifications, deletions) in genes affecting cell growth and differentiation. Individual genetic changes rarely, if ever, are sufficient to cause malignancy, but several changes together may lead to cancer. Tumor suppressor genes normally function to suppress unrestrained cellular growth; typically, mutation or deletion of both alleles must occur before a cell becomes malignant. Such genes, for example p53 and rb, often contain deletions and/or point mutations in tumor cells. In contrast, cellular proto-oncogenes are usually changed to...
active oncogenes by point mutations (as for ras) or by translocations, and an abnormality in one of the two alleles is typically sufficient for tumor development. Both ras activation and the inactivation of several tumor-suppressor genes are common in human lung tumors. The genes which presently appear to be most suitable as clinical tumor markers in lung cancer are ras and p53, both because of their prevalence in NSCLC and because the nature of ras and p53 mutations makes these the easiest to detect, at present, in clinical samples.

The ras gene family

The ras oncogene family consists of three highly conserved functional genes, N-ras, K-ras, and H-ras, which encode related proteins of 189 amino acids, together termed p21. Point mutations confer transforming properties on the ras genes; nearly all oncogenic ras mutations have occurred in codons 12, 13, and 61 [3]. The ras proteins possess GTPase activity and function in signal transduction. Oncogenic mutations yield p21 proteins with altered GTP binding, leading to constitutive activation [4].

Many cases of NSCLC contain ras mutations within a single codon, K-ras codon 12; thus, an assay for mutations in this codon should be of use as a tumor marker. In the largest published series of ras mutations in human lung cancer, a K-ras codon 12 mutation was found, by an assay using the polymerase chain reaction (PCR) followed by allele-specific oligonucleotide hybridization (ASO-h), in 45/258 (17%) NSCLC samples, including 43/181 adenocarcinomas (24%) [5]. Several other series reported similar mutation frequencies in adenocarcinomas, and mutations in other K-and N-ras codons were less often present [6–15]. We have detected K-ras codon 12 mutations in BAL fluid obtained from patients undergoing evaluation for suspected lung cancer, indicating that this is a marker of potential clinical utility (see below).

The p53 gene

The wild-type p53 protein plays a role in regulating gene expression and suppresses transformation. Germline p53 mutations cause an inherited cancer susceptibility syndrome, the Li-Fraumeni syndrome [16], and somatic mutations of the p53 gene are the most common genetic abnormality described in human cancers. Many mutant p53 proteins bind to and downregulate the activity of concomitantly present wild-type protein. Such mutants are not, technically, recessives; they act, rather, in a ‘dominant negative’ manner. Cells with one wild-type p53 allele and a missense mutation in the other allele thus may have a growth advantage over cells with two wild-type alleles, and with subsequent loss of the wild type allele (or a second mutation), such cells may become more malignant.

Along with ras, p53 has been the most widely studied gene in lung cancer. p53 mutations are found in over 50% of all lung cancers [4, 17]. Most p53 mutations in NSCLC are missense point mutations in exons 4–9; nonsense mutations, deletions, and mutations outside of exons 4–9 also occur. Within exons 4–9, certain codons (‘hotspots’) are mutated in large numbers of cases [17, 18]. The most common mutations in smokers are G→T transversions on the coding strand, whereas nonsmokers and patients with exposure to other carcinogens such as radon exhibit different mutational patterns [19, 20]. p53 mutations occur not only in fully-malignant lung cancer cells, but also in premalignant (dysplastic) cells [21–23], suggesting that such mutations could be particularly useful clinically for early cancer detection. A small number of patients have had p53 mutations detectable in sputum preceding the diagnosis of p53 mutation-positive lung cancer [24], further demonstrating the potential of p53 mutations as clinically useful tumor markers.

Various assays have been used to detect p53 mutations in DNA extracted from lung tumors; most common has been PCR of segments of the p53 gene followed by single strand conformation polymorphism (SSCP) analysis. Missense mutations in exons 4–9 generally lead to proteins with an increased half-life over the wild-type protein; therefore, immunohistochemical p53 assays have been used by many investigators as surrogate assays for mutations; an increased level of immunohistochemically-measurable p53 protein suggests that the protein contains a missense mutation [19, 21, 25–28]. The difficulty with using immunohistochemistry as the only marker of a p53 mutation, however, is that frameshift and nonsense mutations do not lead to increased p53 protein levels, and thus are not detected by this method [19].

Other genetic changes

The retinoblastoma (rb) gene

The rb gene, on chromosome 13q14, encodes a nuclear protein which plays a role in cell cycle regulation. rb, first found to be abnormal in retinoblastoma cells, was the first isolated tumor suppressor gene. Nearly all small-cell lung cancers (SCLC) and about 25% of NSCLC exhibit a deletion, mutation, or translocation involving the rb gene, and expression of the rb protein product is often decreased or nondetectable [18, 29–31]. At present, rb gene analysis does not appear to offer immediate clinical applicability as a lung cancer marker, because the size of the gene (27 exons over 190 kb of DNA) and the variety of abnormalities, including large deletions, make PCR analysis difficult.
Overexpressed oncogenes: myc and her2/neu

The myc genes (c-, L-, and N-myc) encode nuclear proteins which appear to regulate gene expression. These protooncogenes are activated not by point mutations, but by overexpression, either via gene amplification or deregulated transcription, in nearly all SCLC and occasional NSCLC cases [4, 32]. Similarly, the her2/neu protooncogene, which has characteristics of a tyrosine kinase growth factor receptor, is overexpressed in about 30% of NSCLC, primarily adenocarcinomas [31, 33]. As the overexpression of both myc and her2/neu is quantitative rather than qualitative, these would appear problematic as tools for lung cancer diagnosis and screening, although it has been suggested that high-titer antibodies against her2/neu, used in single-cell immunohistochemistry on sputum, could find clinical application [33].

Chromosome 3p and other frequently-deleted chromosomal regions

Many NSCLC and SCLC cases have a deletion of part of the short arm of chromosome 3, suggesting that one or more tumor suppressor genes with a role in lung cancer reside on 3p [31, 34, 35]. Although a number of genes involved in cell growth and differentiation reside in this region, the specific critical tumor suppressor genes(s) deleted has not yet been established. Once such a gene is identified, it may provide another tool for early disease detection. Similarly, recurrent deletions on other chromosomes have been described, including a region of chromosome 9p containing the interferon gene complex [4, 31, 34]. Any of these regions may include tumor suppressor genes which may eventually provide clinically useful molecular cancer markers.

Immunocytochemical markers

In addition to genetic tumor markers, immunocytochemical tumor markers may be of use for lung cancer screening and diagnosis. In one study, two murine monoclonal antibodies reactive with antigens on lung cancer cells were utilized on sputum from patients with dysplastic pulmonary changes, and had sensitivity and specificity of about 90% for predicting subsequent lung cancer development [36]. One of the antibodies used in this study recognizes an antigen ordinarily expressed during fetal development, and the expression of fetal markers on epithelial cells is recognized as a potential target for identifying malignant cells [36]. These antibodies and other potential tumor markers are now being evaluated in a prospective study of patients at very high risk for the development of NSCLC [36, 37].

Application of K-ras mutation detection to lung cancer diagnosis

Any cancer-specific genetic change can potentially be utilized for diagnostic purposes. Body fluids may contain cells or cell debris bearing the same oncogene mutations characterizing their parent tumors, as has been shown, in small numbers of patients, for p53 mutations in urine from patients with bladder cancer [38], for ras mutations in stool from patients with colorectal tumors [39], and for both p53 and ras mutations in sputum from patients with lung cancer [24].

In order to utilize ras mutations clinically, a simple, highly sensitive assay is required, because even when tumor cells are present in sputum or BAL fluid, these fluids always also contain many genetically normal non-malignant cells; thus, any genetic tumor marker specific for DNA derived from tumor cells will be diluted in DNA derived from non-malignant cells. ‘Standard’ methods of identifying ras mutations include PCR followed by either ASO-h, direct sequencing, SSCP analysis [9, 15, 40], or denaturing gradient gel electrophoresis (DGGE) [11, 41]. Detection via such methods require that a mutation must be present in at least 10% of the total number of alleles in the specimen [7, 41, 42], and are therefore not sensitive enough for practical use on sputum or BAL fluid.

To overcome this difficulty, we have developed a more sensitive assay for ras mutations [42, 43]. In this assay, a mismatched PCR primer is used to introduce a restriction site into PCR products derived from normal, but not mutant alleles. Restriction enzyme digestion leaves intact only PCR products derived from mutant alleles, after which further PCR permits identification of any mutation present in the original sample (Fig. 1). Our original protocol was able to detect 1 mutant allele per 10^6 normal alleles [42]. We now use a simplified protocol, readily applicable to clinical samples, which detects 1 mutant allele per 10^3–10^4 normal alleles [43].

We have applied this assay to identify K-ras codon 12 mutations in BAL fluid obtained during bronchoscopies performed on patients clinically suspected of having lung cancer. In several cases, a mutation was present in BAL fluid obtained during an otherwise non-diagnostic bronchoscopy, i.e., mutation analysis has been more sensitive than routine BAL fluid cytology and histologic examination of bronchoscopic biopsies [44]. The clinical utility of this method is illustrated by the case histories of two patients.

Case 1

An asymptomatic 62-year-old woman had a subtle density seen on routine chest X-ray. Chest CT scan suggested a small mass. Bronchoscopy with transbronchial biopsy (TBBx) and BAL were performed. The biopsy specimen and cytology of the BAL fluid were negative. An aliquot of BAL fluid was frozen for study. The
A 62-year-old man presented with a persistent cough. Chest X-ray and CT scan revealed lung masses. Bronchoscopy with BAL and TBBx were nondiagnostic. A second procedure, transthoracic needle aspiration (TTNA), was also negative. The third procedure performed was mediastinoscopy, which suggested squamous cell carcinoma. Finally, a resection was performed, and revealed separate areas of squamous cell carcinoma and adenocarcinoma. BAL fluid from the first bronchoscopy contained a K-ras codon 12 mutation GTT. The first nondiagnostic bronchoscopy with BAL and TBBx were nondiagnostic. A BAL fluid aliquot was obtained at both of her bronchoscopies contained the same mutation as in the BAL fluid, and DNA from the squamous cell carcinoma was mutation-negative.

In these patients and others we have studied [44], the BAL fluid obtained at an otherwise nondiagnostic bronchoscopy contained a mutation, indicating that molecular tumor markers may facilitate lung cancer diagnosis. The clinical utility of any tumor marker depends upon the specificity of the marker for cancer, and it appears that ras mutations fulfill this criterion; we are unaware of a ras mutation ever having been reported in non-malignant pulmonary tissue, and none of the BAL fluid samples we have obtained from patients without lung cancer has contained a mutation [44]. One obvious limitation to the use of K-ras mutations in lung cancer diagnosis is that not all cases contain a mutation. This marker may be more useful, however, than would be expected based upon the published K-ras codon 12 mutation frequency of under 25%. Using our sensitive assay, we have found that K-ras mutations are actually present in about 50% of lung adenocarcinomas, and some of these mutations are not detectable by PCR/ASO-h [43].

Sensitive assays for ras mutations would appear also to have potential applicability to lung cancer screening programs, if applied to sputum samples. In one study, patients provided sputum specimens as part of a randomized trial of lung cancer screening. Eight patients were later identified who developed lung cancer containing a K-ras codon 12 mutation. In 7/8 patients, the identical mutation was detected in a sputum sample obtained at least one month prior to the diagnosis of lung cancer [24]. In this study, after DNA was isolated from sputum, PCR of K-ras segments was performed, the PCR products were cloned into a bacteriophage vector, transferred to nylon filters, and the filters were assayed for mutant clones by ASO-H. This method is very sensitive, but also too labor-intensive and expensive for routine clinical application. These data suggest, however, that a simpler, sensitive assay applied to sputum samples could be clinically useful in screening programs.

**Conclusions and future directions**

Tremendous progress has been made in understanding lung cancer at a molecular level, and this understanding is now beginning to be applied to the clinical arena. The application of molecular tumor markers may finally lead to improved ability to diagnose lung cancer, with, eventually, decreased lung cancer mortality. Since only a fraction of lung cancers appear to harbor any particular genetic or immunocytochemical marker, each marker will be informative in only a fraction of cases. The most important consideration for any tumor marker, however, is not its ability to detect all cases of
cancer, but its specificity for cancer. In this regard, ras and p53 mutations appear to be ideal tumor markers. Eventually, the use of a panel of cancer-specific markers, including perhaps ras mutations, abnormalities in other oncogenes and/or tumor suppressor genes, and immunocytochemical markers, should increase the probability that any individual tumor will be positive for at least one tumor marker, and thus amenable to molecular detection.

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