Alu profiling of primary and metastatic nonsmall cell lung cancer

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Abstract

The metastatic potential of nonsmall cell carcinoma of lung (NSCLC) is currently recognized post factum, when lymph nodes or distant organs are already involved. Our ability to determine which tumors have acquired metastatic potential could help direct therapy to be more aggressive or less aggressive based on this information. Evaluation of microsatellite instability via detection of LOH at specific loci may be useful in identifying specific markers and/or genes associated with this process. We examined Alu insertional elements as a potential marker of genetic changes associated with the metastatic potential of NSCLC. We analyzed archived, paraffin embedded tissue from 20 proven cases of NSCLC. DNA was extracted from 10 micron paraffin sections and amplified using an Alu PCR protocol. This technique does not examine specific loci but rather results in a banding profile of cellular genomic DNA. Informative allelic banding patterns, noted as differences between primary and metastatic lesions from the same patient, were observed in five of six cases (83%) with intrapulmonary metastases and in only nine of 14 (64%) cases with extrapulmonary metastases. Multiple genomic changes were detected in metastatic tumor cells as compared to normal lung tissue or primary lung tumor tissue. It appears that Alu profiling may be useful in the detection of metastatic vs primary lesions, and this technique may offer a method for identifying novel genes responsible for tumor progression and metastases. © 2003 Elsevier Science (USA). All rights reserved.

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Introduction

Evaluation, management, and prognosis of patients with nonsmall cell lung carcinoma (NSCLC) depend on tumor staging (1–4). Staging is based on the TNM system, including tumor size, local or distant lymph node involvement, and presence of metastases to other organs. Increased tumor size and more distant metastases are associated with a higher stage/more aggressive tumor requiring more complex treatment and thus, a less favorable prognosis (5).

The metastatic potential of lung tumors is currently recognized post factum when lymph nodes or distant organs are already involved. Early detection of tumors with metastatic potential may provide an opportunity for prevention of metastases by directing treatment in primordial stages of diagnosis to a more aggressive and complex type. Consequently, to reduce potential side effects concomitant with anticancer therapy, the tumors negative for metastatic ability, may be treated with more conservative protocols.

Transformation of normal cells to their malignant counterparts typically involves loss of tumor suppressor gene expression, activation of protooncogenes, and/or defects of mismatch repair mechanisms (MMR). Microsatellite instability, an indicator of faulty MMR, has been identified in several structural genes that participate in various aspects of normal cellular homeostasis in different tumor types. Detection of microsatellite instability involves PCR analysis of multiple loci that do not appear to have a significant amount of specificity for any given tumor type (6–45). A more ideal marker would not necessarily be specific for a single tumor type, but rather would be universal in its ability to detect genome-wide genetic alterations as a profile. Currently,
many studies are being performed using gene expression profiling via microarray technologies to associate recognizable patterns with various tumor types. The Alu insertional elements may be a useful tool in the analysis of genomic DNA for the variety of genetic alterations that can occur. The universal presence of Alu repeats throughout the genome may allow for the nonspecific detection of insertions/deletions of many genetic sequences located between two Alu repeats (46–51). These gains or losses of genomic material could represent large or small gene sequences associated with LOH and/or gene amplification. Alu-PCR is a very sensitive method with the added advantage of being able to detect novel genomic alterations without knowledge of these sequences (52). We refer to this analysis of unknown banding patterns as Alu profiling.

In this study, we present Alu profiling data for the examination of primary and metastatic NSCLC.

Materials and methods

Patients

Our specimens for this study consisted of paraffin-embedded tissues from 20 patients with NSCLC obtained after pneumonectomy, lobectomy, or open lung biopsy. All patients underwent surgery at Hartford Hospital (Hartford, CT) between 1996 and 2001. Of the 20 cases, 12 were male and eight were female. The age ranged from 43 to 87 years (66.6 ± 21.9). All tumors were confirmed as primary pulmonary carcinomas by compatible histology, absence of other nonpulmonary primary tumors, and immunohistochemical studies including CK7, CK20, and TTF-1. Of the 20 cases, 11 were adenocarcinomas of similar histology, six were squamous cell carcinomas, and three were adeno/squamous carcinomas. All adenocarcinomas were CK7 and TTF-1 positive and negative for CK20.

Patients were categorized by pathological diagnosis as either local intrapulmonary (N1) or distant extrapulmonary (N2, N3, M1) metastatic NSCLC. Of the 20 patients, six cases had intrapulmonary metastases only (N1) and 14 cases had extrapulmonary (N2, N3, M1) metastases. The extrapulmonary metastases group was further divided into three subgroups: four cases with extrapulmonary only metastases (N2, N3, N1), six with combined intra- and extrapulmonary metastases (N, N2, N3, M1), and four that represent a distinctive group of extrapulmonary metastases only to the brain (M1) (Fig. 1).

DNA extraction

DNA was extracted from four 10 μM serial unstained sections of formalin-fixed paraffin-embedded tissue. Tissue blocks were chosen for which primary or metastatic tumor represented the predominant histologic cell type to eliminate cellular heterogeneity. Xylene was used to deparaaffinize tissue sections. After washing in 70% ethanol, tissue
was lysed overnight with cell lysis solution (Gentra Systems, Inc., Minneapolis, MN) and proteinase K. Proteins were then precipitated and the supernatant was placed in 100% isopropanol containing glycogen for DNA precipitation. DNA pellets were washed with 70% ethanol and hydrated overnight at room temperature with DNA hydration solution and then stored at 2–8°C.

PCR analysis

Alu PCR was performed using a single Alu primer: 5’GGC AGA CTC CAT CTC AAA 3’. Genomic DNA (0.5–1.0 μg), isolated as described above, was incubated in a total reaction volume of 50 μL containing 40 ng of primer, 2.0 units Hot Start Taq (Qiagen, Inc., Santa Clarita, CA), 200 μM each deoxynucleotide triphosphate, and Qiagen PCR buffer (1.5 mM MgCl₂, 200 mM ammonium sulfate, 750 mM Tris-HCl, pH 9.0, 0.1% w/v Tween). The cycling parameters for Alu PCR were as follows: initial denaturation at 94°C for 12 min followed by 40 cycles consisting of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The final extension was at 72°C for 10 min.

Electrophoretic gel analysis

The amplification products of the Alu PCR were separated by polyacrylamide gel electrophoresis and visualized using ethidium bromide staining and UV light. Loss of allelic sequences was evaluated by direct visualization. Genomic instability was evaluated by the presence or absence of additional bands in PCR products from tumor compared to normal tissue.

Results

As a result of Alu profiling, we obtained discernable amplified DNA fragments in the range of 126 to 460 bp in size. In four of six (67%) patients with intrapulmonary metastases, the absence or presence of bands compared to normal tissue was noted (Fig. 2). The genetic changes were most prominent in two electrophoretic regions related to DNA size of 126–135 bp and 160–175 bp. In other cases containing simultaneous intra- and extrapulmonary metastases, genetic alterations as determined by Alu profiling were seen in five of six (83%) cases. Interestingly, these changes in banding profiles were detected in the same (126–135 bp and 160–175 bp) two size regions (Fig. 3), indicating gain or loss of similar sequences during these metastatic events.

In general, there were two types of observed electrophoretic changes that could be associated with genomic instability and tumor progression. Qualitative changes were observed as differences in banding patterns or intensity of bands within a given pattern. Quantitative changes were also observed that resulted in different numbers of bands within a profile.

Of the four cases with metastatic tumor to the brain, three (75%) showed a single discrete band (126 bp) in normal tissue that was absent in primary and metastatic tumor. There were also several other changes in banding patterns, as well as, band intensity differences (Fig. 4).

In cases with extrapulmonary metastases only, three of four (75%) showed changes that were present in the same two electrophoretic regions (126–135 and 179–250 bp). Overall, nine of 14 (64%) cases with extrapulmonary metastases showed informative allelic changes (Table 1).

![Fig. 2. S01-30 and S01-39 absence of band in primary and metastatic tumor compared to normal tissue. S01-50 presence of abnormal band in cancer compared to normal tissue.](image1)

![Fig. 3. There are two types of electrophoretic changes revealing allelic nonidentity, qualitative changes expressing as a changeable pattern or intensity of electrophoretic bands (S01-22 and S01-02) and quantitative changes in number of bands (S01-44).](image2)
Discussion

Cancer represents the acquisition of many genetic and epigenetic changes, some of which remain to be defined. In order to further our understanding of these changes, gene expression profiling of various forms has become critical. We have begun profiling of another sort that could help establish changes in genomic DNA patterns using a modified PCR protocol. In this study, Alu PCR was used to profile genetic changes that may occur during carcinogenesis and metastasis in NSCLC. We identified 20 patients with metastatic NSCLC for which archived paraffin-embedded specimens were available. The presence of metastatic disease was established preoperatively by radiographic examination and verified during surgical procedures. Alu profiling in patients with intrapulmonary metastases only, combined intra- and extrapulmonary metastases, extrapulmonary metastases only, or distinctive extrapulmonary metastases to the brain demonstrated differences in allelic banding patterns.

While traditional microsatellite instability assays can be used to analyze tumor tissues, these protocols involve multiple primer sets and may not be specific for a given tumor type. Alu profiling utilizes one primer set and can be applied to all tumor types due to the nature of the profiling process. The high sensitivity and low specificity of microsatellite PCR assays would not make this suitable as a routine screening method in clinical laboratories. Microsatellite markers are designed to amplify one particular DNA sequence located in proximity to a known tumor suppressor gene, protooncogene, etc. Noninformative or variable results obtained by different authors with microsatellite PCR are probably not because there were no allelic changes, but more likely because of the limited numbers of markers used.

Discovered and described in the mid-1980s, Alu insertion elements are the most numerous short interspersed repeats in the human genome, accounting for 3% to 6% of the total DNA. These sequences consist of directly repeating 300 bp monomer units, with an average distance between copies of approximately 4 kb. Because of a variety of Alu functions and the dispersion of these elements throughout the human genome, many structural alterations may be detected by Alu PCR as changes to intervening sequences between these repeats. The presence or absence of DNA sequences amplified by Alu PCR may represent the insertion or deletion of sequences, respectively. This in turn could represent amplified oncogenes or loss of tumor suppressor genes.

Alu PCR is a very sensitive method and can be accomplished on samples containing low concentrations of template such as with paraffin embedded tissue. Because Alu

![Fig. 4. Metastatic tumors to the brain: There is a single discreet band (126 bp) in normal tissue, which disappears in primary and metastatic tumor and there is a changeable pattern and intensity of bands in 222 and 350 bp region.](image-url)
profiling highlights multiple changes in a neoplastic cell genome compared to normal tissue, interpretation of Alu amplification results is based more on banding pattern comparisons rather than on single band analysis as with conventional microsatellite PCR. Our data suggest that there are significant genomic changes between normal, primary tumor, and metastatic tumor cells in NSCLC that can be detected by Alu profiling. In addition, these studies provide proof of principle for application of this technique to other tumor types followed by DNA sequencing for allelic identification.

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References