

High-throughput screening of bronchial lavage and blood samples for early detection of lung cancer

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Genetic instability is a characteristic feature of cancer and has previously been detected in the bronchial lavage of lung cancer patients (1). Here we used an improved screening technology and increased throughput to further development of an assay for the early detection of lung cancer.

Lung cancer causes more deaths than any other cancer, accounting for 17.8% of all cancer deaths worldwide — more than from breast, prostate, and colorectal cancer combined (2, 3). Lung cancer is estimated to develop over a period of 15 to 20 years. Much of this time is spent in carcinogenesis, during which inhaled carcinogens bind to DNA, leading to genomic instability and mutation and, possibly, to mutation of a proto-oncogene. Altered oncogene proteins may result in changes in tissue structure, which can be identified by light microscopy. Genomic instability can be passed on in daughter cells which continue to mutate and proliferate as independent clones, eventually becoming malignant and invading local structures (3).

The use of early detection methods has meant that many of the most common cancers, such as breast and colon tumors, are diagnosed at earlier stages when chances for successful treatment are greater. However in the case of lung cancer, a successful screening method is not yet available. Symptoms often first present at an advanced stage resulting in late diagnosis of the disease. Consequently, five-year survival rates are extremely poor, being as low as five percent in the Merseyside region of England (1).

Screening for lung cancer markers among asymptomatic individuals is therefore an approach for early detection, diagnosis, and successful treatment of lung cancer. Recent advances in tumor biology have led to the development of various techniques for detecting

cells undergoing carcinogenesis. However, until such techniques are refined and validated for mass screening, detection of lung cancer using radiographic and cytologic methods is the only alternative. Tools to read and interpret the molecular features of cancer and identify markers are therefore crucial, and their diagnostic and prognostic value must be assessed and integrated into clinical diagnostics (4).

Materials and methods

Bronchial lavage and corresponding blood samples were taken from 80 individuals with suspected lung cancer who were referred to the Cardiothoracic Center in Liverpool. Patients were selected according to the availability of an adequate cytology preparations and blood samples. Bronchial lavage specimens were obtained from all patients. The choice of site was based on bronchoscopic findings within the large airways, where approximately 50 ml of saline was introduced via the bronchoscope and then aspirated.

Smoking data were available for 69 of the patients, of whom 51 were current smokers, 13 former smokers (stopped >5 years prior to presentation) and 5 nonsmokers. The total smoking exposure was calculated in pack-years = [(age at presentation) – (age started) – (years stopped)] × [cigarettes/day].

A differential cell count was undertaken for all bronchial lavage samples reported as having “no malignant cells seen” and for the lung cancer patients with genomic instability. The epithelial cells present varied by 20–90%. ►

References

1. Field, J.K., et al. (1999) Genetic alterations in bronchial lavage as a potential marker for individuals with a high risk of developing lung cancer. *Cancer Research*, **59**, 2690.
2. Hoffman, P.C., Mauer, A.M., and Vokes, E.E. (2000). Lung cancer. *Lancet*, **355**, 479.
3. Merck & Co., Inc. (2000) Chapter 50: Lung cancer. In: *The Merck Manual of Geriatrics*. New Jersey: Whitehouse Station (http://www.merck.com/pubs/mm_geriatrics/50x.htm)
4. National Institutes of Health, National Cancer Institute (1997) <http://rex.nci.nih.gov/massmedia/backgrounders/cancerinbrief.html>
5. Liloglou, T., et al. (2000) Sensitivity and limitations of high throughput fluorescent microsatellite analysis for the detection of allelic imbalance. Application in Lung Tumours. *Int. J. Oncol* **16**, 5.

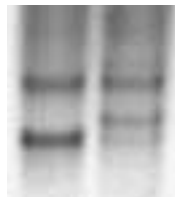
Detection of Genomic Instability in Bronchial Lavage DNA of Patients with Lung Cancer

A Loss of heterozygosity



Blood BL

B Microsatellite instability



Blood BL

Figure 2 DNA was purified from blood using the QIAamp 96 DNA Blood Kit and from bronchial lavage (BL) using the DNeasy 96 Tissue Kit. PCR products were analysed on 10% native polyacrylamide gels (silver staining). Genomic instability in bronchial lavage DNA was determined as **A** loss of heterozygosity and **B** microsatellite instability.

Detection of Genomic Instability in DNA from Bronchial Lavage using Fluorescent-labeled Microsatellite Markers

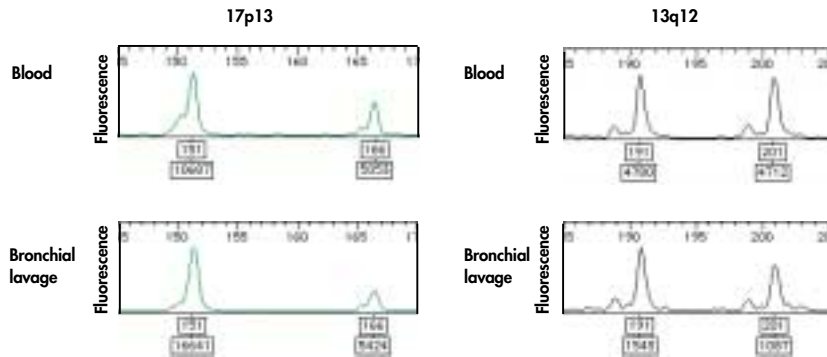


Figure 1 GENOTYPER analysis of PCR products from blood and bronchial lavage DNA isolated using QIAamp 96 DNA Blood and DNeasy 96 Tissue Kits respectively. The PCR product was mixed with GENESCAN-350 molecular weight standard (Applied Biosystems) and analyzed on an ABI PRISM 377 DNA Sequencer. The figure shows a typical scan from a patient with lung cancer where DNA from bronchial lavage shows allelic imbalance in comparison to the patients' germline blood DNA.

DNA isolation and PCR

DNA from 200 µl blood was isolated using the QIAamp® 96 DNA Blood Kit, following the QIAamp DNA Blood protocol. Bronchial lavage (approximately 1 ml) was centrifuged for 5 minutes at 2000 x g, and the resulting pellet, containing a variable amount of mucus, was processed using the DNeasy® 96 Tissue Kit.

Primers for the microsatellite loci were selected from the LMS High Density Panel Set (Applied Biosystems). The 10 µl multiplex PCR mixture contained 1x PCR buffer, 2.5 mM MgCl₂, 500 mM dNTPs, and 0.75–1.0 mM each primer pair, 0.75 units hot-start DNA polymerase, and 2–3 µl of the purified DNA. The

thermal cycler program included a hot start at 95°C for 12 minutes, followed by 30 cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. A 30-minute final extension step was included at the end in order to maximize addition of an extra adenosine residue at the 3' terminus of the amplicon, thus eliminating split peaks. Two microliters of the PCR product was mixed with 3 µl of loading buffer (5:1:1 formamide: dextran blue/EDTA:ROX 350 size standard). The mixture was denatured at 95°C for 5 minutes, chilled on ice, and loaded on a 6% denaturing polyacrylamide gel on an ABI PRISM® 377 automatic sequencer (Figure 1). The gel image was analyzed using GENESCAN® and GENOTYPER® software (Applied Biosystems, Inc.).

Results and discussion

Bronchial lavage samples for the 80 patients with suspected lung cancer were analyzed for genomic instability. Genomic instability was determined by loss of heterozygosity (LOH) and microsatellite instability (MSI) in bronchial lavage DNA as compared to normal germline DNA isolated from the corresponding blood samples (Figure 2). The diagnoses of all patients were determined and used to validate the specificity of the markers used (Table 1).

Table 1. Resulting patient diagnoses

Diagnosis	Number of patients
Squamous cell carcinomas of the lung (SqCCL)	22
Adenocarcinoma	13
Small cell lung carcinoma (SCLC)	10
Chronic obstructive pulmonary disease (COPD)	9
Chest infection	13
Asthma	10

The markers used have previously been shown to carry genetic alterations (LOH, MSI) in 97% of lung tumors (5). Samples were scored as positive when they had at least one marker with LOH or MSI. Using this criterion 39 of the 45 patients with lung cancer and 27 of the 35 patients with a nonmalignant disease were found to have genomic instability. This provided 22.8% specificity (Fisher's test; $p=0.2$), which is unacceptable as a potential diagnostic tool. (Note that all positive results were confirmed by repeating a separate PCR at least twice.)

By analyzing positive results per chromosomal location, four markers were found that scored positive specifically in lung cancer: 3p14, $p=0.001$; 3p21, $p=0.026$; 13p12, $p=0.005$; and 17p13, $p=0.01$; while markers on 5q15, 9p21, 9p23, and 13q14 were nonspecific (Table 2). By using only the specific markers, 32 from 45 lung cancer cases and 9 from 35 non-malignant disease cases scored positive (sensitivity = 71.1%, specificity = 74.3 %; Fisher's $p=5.6 \times 10^{-5}$).

In comparison, cytological examination (currently typical in clinical practice) scored 23 from 45 lung cancer cases positive in comparison to the 32 from 45 that the DNA assay reported. Combining these two detection methods, the lung cancer cases that scored positive increased to 37 out of the 45 patients with lung cancer, providing an overall sensitivity of 82.2%.

We suggest that, given the fact that only five nonsmokers were included in the study, the nonspecific markers reflect smoking-related DNA damage or DNA alterations related to inflammatory reactions that do not make cells commit to malignant transformation whereas the cancer-specific markers reflect genetic changes related to a tumorigenic determination of the cell.

Conclusions

- ◆ Genomic instability, as determined by loss of heterozygosity (LOH) and microsatellite instability (MSI), is a potential marker for the early detection of lung cancer prior to clinical manifestation of the disease.
- ◆ The development and validation of molecular diagnostic techniques provides an alternative to detection of lung cancer at an advanced stage of the disease and a positive outlook for development of a DNA assay for early detection of the disease.
- ◆ The QIAamp 96 DNA Blood and DNeasy 96 Tissue Kits offer efficient and reliable high-throughput DNA purification from blood and bronchial lavage, respectively, and are ideal for diagnostic screening purposes.
- ◆ Further investigations are required to expand the current set of cancer-specific markers.

Table 2. Marker specificity to lung cancer in bronchial lavage samples

Chromosomal loci	LOH/MSI detection in:		LOH/MSI incidence vs clinical diagnosis: Fisher's p-value
	Lung cancer cases (%)	Nonmalignant lung conditions (%)	
3p21	46.4	19.2	$p=0.026^*$
3p14	40.0	6.4	$p=0.001^*$
13q12	45.2	12.5	$p=0.005^*$
17p13	50.0	15.8	$p=0.01^*$
13q14	50.0	48.3	$p=0.44$
5q15	67.9	42.9	$p=0.008$
9p23	41.7	21.7	$p=0.16$
9p21	32.3	11.5	$p=0.20$

* Four chromosomal loci showed a significant level of LOH/MSI specificity in lung cancer cases.

Further reading

Hosler, G.A., Bash, R.O., Bai, X., Jain V., and Scheuermann, R.H. (1999) Development and validation of a quantitative polymerase chain reaction assay to evaluate minimal residual disease for T-cell acute lymphoblastic leukemia and follicular lymphoma. *Am. J. Pathol.* **154**, 1023.

A quantitative polymerase chain reaction (PCR) assay targeting tumor-specific chromosomal rearrangements in pediatric T-ALL and in follicular lymphoma was developed and validated. This quantitative PCR assay based on DNA isolated using the QIAamp DNA Blood Mini Kit utilizes a synthetic internal calibration standard that contains priming sequences identical to those found flanking the chromosomal rearrangement breakpoints. The limits of detection were 5 tumor cells at ratios of 1 tumor cell in 10⁵ normal cells and a linear range up to 100% tumor cells.

Basso, K., Frascella, E., Zanesco, L., and Rosolen, A. (1999) Improved long-distance polymerase chain reaction for the detection of t(8;14)(q24;q32) in Burkitt's lymphomas. *Am. J. Pathol.* **155**, 1479.

A method based on DNA isolated from cultured cells and frozen tissue and on simplified and efficient long distance PCR for the detection of t(8;14)(q24;q32) was established. PCR product ranged in size from 2.0 to 9.5 kb.

Khanna, M., Park, P., Zirvi, M., Cao, W., Picon, A., Day, J., Paty, P., and Barany, J.F. (1999) Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors. *Oncogene* **18**, 27.

A multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) method based on DNA isolated from paraffin-embedded archival colon carcinomas was developed. The PCR/LDR identifies all 19 possible single-base mutations in K-ras codons 12, 13, and 61, with a sensitivity of 1 in 500 wild-type sequences.

Zhong, X.Y., Lin, Y.S., Kaul, S., and Bastert, G. (2000) Sensitive and specific detection of carcinoembryonic antigen cDNA using the hot start polymerase chain reaction technique. *Clin. Lab.* **46**, 7.

Total RNA was prepared from tenfold dilutions of breast carcinoma cultured cells in peripheral blood mononuclear cells using the RNeasy[®] Mini Kit. Hot start PCR was performed to enable increased specificity and sensitivity when detecting CEA cDNA. Using the assay described 12 out of 12 samples were positive for CEA cDNA from ten CEA-mRNA-positive tumor cells in 10⁷ normal cells.

Lamberti, C., Kruse, R., Ruelfs, C., Caspari, R., Wang, Y., Jungck, M., Mathiak, M., Malayeri, H.R., Friedl, W., Sauerbruch, T., and Propping, P. (1999) Microsatellite instability — a useful diagnostic tool to select patients at high risk for hereditary non-polyposis colorectal cancer: a study in different groups of patients with colorectal cancer. *Gut* **44**, 839.

Microsatellite instability was investigated as a tool to increase the likelihood for uncovering a mismatch-repair germline mutation in patients with colorectal cancer and to identify a genotype-phenotype relation in families with verified mutations. For this purpose, DNA was isolated from paraffin-embedded tumors and short tandem repeats analyzed by PCR.

Peck, K., Sher, Y.P., Shih, J.-Y., Roffler, S. R., Wu, C.-W., and Yang, P.-C. (1998) Detection and quantification of circulating cancer cells in the peripheral blood of lung cancer patients. *Cancer Research* **58**, 2761.

Nested RT-PCR of cytokeratin 19 mRNA was used as a means of quantifying the number of circulating cancer cells in the peripheral blood. This method appeared to be reliable for estimating the cancer cell number in the peripheral blood of lung cancer patients. A detection limit of one cancer cell in 10⁷ peripheral blood mononuclear cells was determined. The QIAamp RNA Blood Mini Kit was used to isolate total cellular RNA from peripheral blood samples for RT-PCR.