Abstract. Background: CLCA2, HMGB3, L587S and ASH1 were identified in lung cancer tissues using genetic subtraction, microarray and quantitative PCR, and found to be specific and complementary for detection of non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Materials and Methods: A real-time RT-PCR assay, simultaneously detecting four genes, was developed and tested on lung cancer specimens. Results: Twenty-two out of 24 adenocarcinomas, 18/18 squamous, 4/5 large cell, 2/2 small cell and 2/2 bronchoalveolar/ neuroendocrine cancer tissue samples tested positive. Specificity was demonstrated by evaluation of 194 other tumor and corresponding normal tissues. Circulating tumor cells in the peripheral blood of 49/108 lung cancer patient samples tested positive, and general correlations of multigene expression signals to disease status were observed. Changes in multigene expression during treatment and disease recurrence in individual patients could be detected. Conclusion: These data indicate the diagnostic and prognostic utility of a multigene real-time RT-PCR assay to detect tumor cells in the peripheral blood of lung cancer patients.

Lung cancer carries the highest mortality rate of all cancers, leading to an estimated 1 million deaths annually worldwide (1, 2). The diagnosis of lung cancer is complicated by the presence of several pathologically distinct tumor types, segregated by treatment approach into two groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The NSCLC group includes adenocarcinoma, large cell carcinoma and squamous cell carcinoma. NSCLC comprises ~75% of all lung tumors, with adenocarcinoma and squamous cell carcinoma being the most prevalent forms.

In most cases, the early diagnosis of NSCLC is elusive and most tumors have already metastasized at detection. Only 16.7% are localized on initial diagnosis (1, 2). If tumors can be detected while still anatomically confined, the combination of chemotherapy and radiation is reasonably successful, but overall, the 5-year survival rate is only 10-15% (1, 2). The prognosis for patients with SCLC is even bleaker; only 6% are localized at initial diagnosis and the 5-year survival rates are ~6% (1, 2).

Current diagnostic methods include physical examination of the patient followed by X-ray and computer tomography, histological examination of the tumor or biopsy, and evaluation of mediastinal and sentinel lymph nodes (3-8). The use of gross tissue collection strategies is severely limited by the specificity and detection limits of commercially available diagnostic tests, and by invasive collection methods. In addition, the macro-diagnostic approach lacks sensitivity for detecting small foci, such as micrometastases in samples such as blood or sputum. The development of immunohistochemistry (IHC) or molecular tests, that can facilitate the detection of micrometastases and identify their source of origin as the lung, would greatly improve and contribute to the earlier diagnosis of lung tumors and monitoring of experimental therapies (9, 10).

Molecular assays using PCR can greatly improve the diagnostic sensitivity to detect malignant cells. Several groups have been investigating hypermethylation in the CpG islands of tumor suppressor genes that are known to be modified in lung cancer (11-14). Oligonucleotide and tissue array analyses led to the identification of the cancer antigens,
MAGE A sub family members and NY-ESO-1, which demonstrated little prognostic utility (15, 16). CEA mRNA has been described as being able to detect circulating tumor cells in NSCLC and other tumors in patients with or without evidence of distant metastases (17-21). MUC-1 and Cytokeratin 19 have also been used to detect circulating lung tumor cells, but lack specificity (22-25). In addition, Neureomedin B receptor (a bombesin-like receptor) PCR has been shown to detect occult SCLC tumor cells in peripheral blood, but was only present in approximately 30% of the cases (26).

These studies point to the presence of circulating tumor cells and the need for improved sensitivity and specificity, and correlation to the disease state. The presence of circulating tumor cells post-treatment is a strong indicator of treatment failure or recurrence of disease and has been shown, in many cancers, to be an independent prognostic indicator of poor clinical outcome or the need for treatment changes (10, 27). Thus, new genetic markers need to be identified for the molecular diagnosis and monitoring of lung cancer.

In the present study, a multigene real-time RT-PCR assay was developed for the detection of tumor cells in the peripheral blood of NSCLC and SCLC lung cancer patients. By using a combination of genetic and electronic subtraction techniques with microarray analyses, our group identified several over-expressed lung cancer targets and demonstrated their tumor specificity by real-time RT-PCR, Northern blot and immunohistochemistry (28-32). Four of the candidates, calcium-activated chloride channel 2 (CLCA2), human high mobility group box 3 protein (HMGB3), L587S (novel), and Aschaetescut homologous protein (ASH1), were selected for use in a real-time RT-PCR diagnostic assay due to their complementation in detecting both NSCLC and SCLC tumors. In order to demonstrate the potential utility of this test in molecular diagnosis and monitoring of lung cancer, blood specimens from lung cancer patients at various stages of disease were evaluated for the presence of circulating tumor cells.

Materials and Methods

Tissue sources, RNA extraction and cDNA synthesis. Primary cancers and healthy tissues were obtained from the Cooperative Human Tissue Network (CHTN), National Disease Research Interchange (NDRI), and other clinical sources. SCLC tumor cell lines were obtained from the American Type Culture Collection (ATCC). Total RNA was isolated from homogenized tissue samples using Trizol total-RNA isolation kit (#15596-018). DNase treatment was performed using DNA-freeTM (Ambion #1906), according to the manufacturer’s protocol. mRNA was reverse-transcribed into cDNA using oligo(dT) primer and Superscript Reverse Transcriptase (Invitrogen #Y01212 and 18064-014) for 1 h at 42°C and diluted to a final volume of 500 µl. cDNA was diluted 1:10 and 2 µl was used in each subsequent real-time PCR reaction.

Blood sources, RNA extraction and cDNA synthesis. Patient consent and IRB approval were obtained prior to sample collection. Peripheral blood (typically 5-8 ml) was drawn into 10 ml EDTA containing vacutainers (BD) from cancer patients at the Swedish Medical Oncology Clinic, Seattle, WA and through Proteogenex, Manhattan Beach, CA, USA. The specimens were processed within 3 h using RosetteSep™ tumor cell enrichment antibody cocktail (StemCell Technologies Inc. #15122). The enrichment cocktail contains tetrameric antibodies that cross-link normal hematopoietic cells (CD45), granulocytes (CD66b) and monocytes (CD36) to red blood cells (glycophorin A). The depleted tumor mononuclear cells were collected using Accuspin™ System Histopaque®-1077 (Sigma-Aldrich #A-6929). mRNA was isolated using the Roche mRNA Isolation Kit (#1741985). DNase treatment was performed using DNA-freeTM (Ambion #1906), according to the manufacturer’s protocol. mRNA was reverse-transcribed into cDNA using oligo(dT) primer and Superscript Reverse Transcriptase (Invitrogen #Y01212 and 18064-014) for 1 h at 42°C in a final volume of 120 µl. Two µl cDNA was used in each subsequent real-time PCR reaction.

Primers, probes and real-time PCR. Specific primers and 6-carboxy-fluorescein (FAM)-labelled Taqman® probes were used in combination to detect mRNA expression of different cancer-specific genes. The primers were designed to be intron spanning (exon specific) to eliminate reactivity with genomic DNA for all the genes except actin. In addition, the multigene PCR test was engineered such that unique amplicon sizes resulted from the amplification of individual target genes, which could be used to indicate the tumor type. Primer pairs were screened individually for tumor specificity, using a 300 nM concentration. Subsequently, individual primer concentrations were titrated to non-competing levels (lower delta R, no change in Ct) to obtain approximately equal efficiencies during multiplex (MPLX) amplification. For the MPLX amplification, all 4 gene-specific probes and primer sets were combined in a single assay where cumulative FAM signal was detected. Typical 25-μl PCR reaction mixtures included 1X TaqMan Buffer (TaqMan Core Reagents Kit #4304439; Applied Biosystems, Foster City, CA, USA), 5 mM MgCl₂, 0.2 mM dCTP, 0.2 mM dATP, 0.2 mM dUTP, 0.2 mM dGTP, 0.01 U/ml AmpEraseUNG, 0.0375 U/ml Amplitaq Gold Polymerase, 8% v/v glycerol, 0.05% v/v Gelatin, 0.01% v/v Tween20, 300 nM CLCA2-2ISF 5'-ATGGCAAGAGGCTGACAAGCTC-3' and CLCA2-2ISR 5'-TTCAACCACCCTCAATTCTTTCTTA-3' primers, 200 nM LS87-2ISF 5'-CCTCAACGTGGTTTAAAGGATC-3' and LS87-2ISR 5'-GTAAACGGGATTCTCATGTACG-3' primers, 50 nM each of HMG2B-2ISF 5'-GGCTTTGATTGGATTTCGG-3' and HMG2B-2ISR 5'-CCGGCGCGGCGCACCATCGCTA-3' primers. Each reaction also contained 3 pmol of each gene-specific probe: CLCA2 5'-6FAM-TCAATCAATCTTCATTCTTCGGA-3' and CLCA2-2ISR 5'-TTCAACCACCCTCAATTCTTTCTTA-3'; ASH1-1R 5'-CCTCAACGTGGTTTAAAGGATC-3' and ASH1-1ISR 5'-TTCAACCACCCTCAATTCTTTCTTA-3'; CEA 5'-6FAM-TCAATCAGAGGCCCAAGGAGGACTC-3' and CEA-2ISR 5'-CCTCAACGTGGTTTAAAGGATC-3'.

The combined gene expression levels were measured by quantitative real-time PCR using the ABI 7700 Prism® sequence detection system (Applied Biosystems) using the cycle 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 15 sec, 60°C for 1 min and 68°C for 1 min. MPLX copy numbers and Actin messag concentration were calculated by...
constructing standard curves using the TaqMan® SDS analysis software from serial dilutions of 4 combined purified PCR amplicons containing target gene cDNA sequences, and of human genomic DNA (Clontech #6650-1) for Actin analysis, respectively. Standard curves were assayed on each PCR plate. In addition, a lung tumor cell line and a lung tumor tissue pool were included as positive controls on each PCR plate and used in conjunction with the standard curve to eliminate plate-to-plate variation. Final MPLX copy numbers were determined as means of triplicate reactions for blood samples and of duplicate reactions for tissue specimens. Actin expression was measured in separate reactions as a quality control for the blood and tissue cDNA samples using reactions with 300 nM ActinF 5'-ACTGGAACGGTGAAGGTGACA and ActinR 5'-CGGCCACATTGTGAACTTTG primers, and 3 pmol Actin-probe 5'-6FAM-CAGTCGGTTGGAGCGAGCATCCC-3'-TAMRA. The expression levels for tissue specimens are reported as MPLX copies normalized per ng of actin. Blood specimens with Actin expression <50 pg were excluded from the analysis.

Electrophoresis. PCR products were analyzed by agarose gel electrophoresis to determine the differential expression of the 4-gene multiplex including CLCA2 (249 bp), HMGB3 (204 bp), L587S (169 bp), and ASH1 (157 bp) as indicated by the ladder (lane 1) and labelled arrows. Lane 3 shows the no template, negative control.

Results

A panel of 51 lung tumors and 13 normal lung tissues was assayed by the 4-gene multiplex RT-PCR to determine the specificity of the test for lung cancer. Initially, each gene was assayed individually to determine the tumor-type specificity and potential for complementation with other genes (Figure 1A). In our hands, ASH1 and HMGB3 detected adenocarcinomas, with additional complementation from L587S, CLCA2 and HMGB3 detected squamous carcinomas, CLCA2, ASH1, HMGB3 detected large cell carcinomas, while ASH1, HMGB3 and L587S detected small cell, bronchoalveolar and neuroendocrine carcinomas (Figure 1A). The single-tube multiplex assay, using optimized primer concentrations and cumulative FAM detection, detected all lung tumor types above the levels of individual gene expression (Figure 1B). Actin gene expression was used to normalize the individual and MPLX data. The mean actin values for tumor and
normal tissues were not significantly different, \( p = 0.38 \) (tumor mean actin=6614 ng/PCR reaction, normal lung mean=7095 ng/PCR reaction). The MPLX PCR products were assayed by agarose gel electrophoresis to determine which genes were expressed in individual tumors (Figure 2). Amplicon detection by gel electrophoresis was in agreement with the single gene PCR results, with all 4 lung markers being amplified in several samples.

Based on a cut-off value of 3 standard deviations above the mean MPLX copies of 13 normal lung tissues, we were able to establish MPLX sensitivity for lung cancers of greater than 94% (Figure 3). A high MPLX expression signal was detected in 2/2 neuroendocrine/bronchoalveolar, 2/2 SCLC primary tumors, 2/2 SCLC cell lines, 4/5 large cell carcinomas, 22/24 adenocarcinomas and 18/18 squamous cell carcinomas in comparison to normal lung tissues (Figure 3). The data from the SCLC cell lines was excluded from the total sensitivity calculations since cell line expression may not correlate with tumor expression.

In order to examine the specificity of our assay relative to normal tissues, a panel of 160 human tissues and cell lines was assayed by the 4-gene multigene RT-PCR (Figure 3). Low reactivity was found in a panel of 85 normal tissues, which included peripheral whole blood (not epithelial cell-enriched), bone marrow, spinal cord, small intestine, colon, breast, prostate, ovary and other organ specimens. An elevated MPLX signal (mean±standard deviation) was detected in a sub-group of normal specimens including from the esophagus (23.86±18.15), skin (115.45±31.63),

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bronchus (44.74) and trachea (98.59±40.29) (Figure 3). Additionally, the multigene assay was tested on a panel of 100 tumor tissues representing 12 tumor types and elevated MPLX expression was found in 2/6 pancreatic cancers (397.01 and 110.66, with a mean pancreatic cancer MPLX signal of 91.73), 1/6 kidney tumors (85.17, with a mean kidney cancer MPLX signal of 17.62), 1/6 bladder tumors (66.86, with a mean bladder cancer MPLX signal of 22.38) and 2/6 breast tumors (137.21 and 118.01, with a mean breast cancer MPLX signal of 61.49), with minimal expression in the remaining 76 tumors (data not shown).

Given the low background detection of the multiplex targets in peripheral blood (mean expression 3.33±3.86 MPLX copies), tumor-enriched blood samples from lung cancer patients at various stages of disease and undergoing different treatment regimens were evaluated. Each of the 4 major tumor types was represented (1-14 patients per diagnosis, Table I) and a total of 108 blood specimens were obtained. Repeat draws were obtained from 20 of the patients to monitor disease progression during therapy and potential relapse. The mean age of the patients was 61.8 years, including 24 male, 21 female and 4 donors who did not provide gender information (Table I). Twenty-five additional blood samples were collected from normal, healthy donors, subjected to the tumor-enrichment procedure, and used to establish cut-off values for positive multiplex gene expression. The blood assay specific cut-off was established at 2 standard deviations above the mean of the normal donor MPLX signal (Figure 4). Patients with no current evidence of disease (NED) were separated into groups: those receiving treatment and untreated patients. Of the 15 patients receiving treatment, 7 showed MPLX copies above the cut-off value while 5/12 of the untreated patients tested positive or at cut-off (Figure 4). Twenty-seven out of 64 patients with active disease and undergoing treatment had a significant MPLX signal. Ten out of 17 patients with active disease not receiving treatment also had a significant MPLX signal (Figure 4). Multigene signals for the positive patients ranged from 28 MPLX copies to 991.74 MPLX copies.

Repeat draws from individual patients were monitored for changes in gene expression related to the treatment regimens, relapse, or changes in disease state. One patient, with large cell carcinoma in a progressive disease state, was treated with a new course of chemotherapy (Figure 5A). Over a 7-month treatment session, the MPLX copy number fell below the cut-off, suggesting a reduction in circulating tumor cells in the patient’s blood. These results were reflected in a second patient, with small cell carcinoma, who received a new chemotherapy treatment at the onset of progressive disease and experienced a pronounced drop in MPLX copies within a 6-month period (Figure 5B). An increased signal was found on 4/3/02, prior to diagnosis of progressive disease on 5/29/02 and the next diagnostic blood-draw on 7/31/04 (Figure 5B). In a squamous cell carcinoma patient, elevated MPLX copies in 2 blood samples, taken on 1/31/03 and 3/14/03, were detected 6 months prior to a clinical diagnosis of recurrent cancer in the lung with lymph node involvement on 9/5/03 (Figure 5C).

**Discussion**

The current methods used to diagnose lung cancer include physical examination of the patient followed by X-ray and computer tomography (CAT scan, MRI or bone scans) of
the chest and abdomen, with primary diagnosis relying on histological examination of the tumor or biopsy. Sputum cytology is a potential screening method in high-risk individuals, but it is partially effective and often does not yield the tumor type. Bronchoscopy is effective in obtaining tissue samples, brushings, or washes, but is best used when the tumor is visible and localized. Evaluation of mediastinal lymph nodes using mediastinoscopy or mediastinotomy has been used to evaluate spread to the lymph nodes (3-8). In particular, sentinel lymph node analysis is useful in staging disease (4). Additionally, a variety of diagnostic markers have been evaluated for use in the diagnosis or monitoring of lung cancers and treatments with varying effectiveness based on the type of tumor and treatment regimes. The development of tests that can facilitate the detection of micrometastases and identify their tissue source of origin would greatly improve and contribute to the earlier diagnosis of lung tumors and monitoring of experimental therapies.

Recently, new focus has been placed on designing diagnostic applications for easily obtained clinical samples such as peripheral blood, specifically nucleic acid evaluation assays such as DNA methylation and real-time RT-PCR. Micrometastases are effectively detected in patient serum using DNA hypermethylation assays for genes such as p16/CDKN2, DAP Kinase, GSTP1 and MGMT (33). In that particular study, the detection of abnormal promoter hypermethylation in serum DNA correlated to the presence of DNA alteration in the primary tumor. This is in contrast with alternative studies where DNA methylation was detected in serum or lavage fluid, but was absent in the primary specimens (33, 34). These data could indicate the persistence of certain genomic DNA in the serum in the absence of a tumor, making prognostic and diagnostic evaluations difficult post surgical resection. However, the detection of circulating tumor cells by gene expression (mRNA) is advantageous over DNA screening, since it targets active disease and can be applied to monitor treatment efficacy.

Several groups have reported detection of circulating lung tumor cells in the blood using a variety of gene-expression assays, including a combination of Muc1, Luxn, KSI/4, CK19, CEA and PSE (35, 36). Although the expression of the single marker, Luxn, was found in 42% of the samples analyzed, the assay is limited in the detection of tumor cells from stage II-IV NSCLC patients (36). CEA mRNA, detected in circulating tumor cells in NSCLC pre- and post-treatment, was analyzed and CEA mRNA detection post-operatively appears to be an independent indicator of poor prognosis (17-21). Recently, RT-PCR was used to detect the expression of the Pre-Pro-gastrin-releasing peptide (Pre-ProGRP) in peripheral blood, bone marrow, pleural effusions and sputum samples from SCLC patients (37, 38). KSI/4 and luxn have also been detected by real-time RT-PCR in mediastinal lymph nodes of NSCLC patients (39). Typically, the aforementioned genes are assayed individually and diagnostic potential is hampered by the relative paucity of lung cancer-specific markers.

Assay sensitivity and tumor type coverage for the detection of circulating tumor cells can be improved by evaluating multiple markers. For example, Simi et al. simultaneously detected MMP9 and TIMP1 in NSCLC by multigene real-time PCR, but the tumor coverage was limited to adenocarcinoma and squamous cell carcinoma types (40). Furthermore, matrix metalloproteinases and their inhibitors are subject to regulation by a variety of diseases including pulmonary fibrosis, cigarette smoke-linked NSCLC and asthma and may not be specific markers of cancer, but of general lung disease (41-43). A new study identified a panel of 4 markers, KRT19, Ubiquitin thiolesterase, HSFIIB1-like gene for fibronectin, and TRIM28, in silico used to detect the circulating tumor cells in peripheral blood samples limited to NSCLC patients (44). In these studies, the detection of lung cancer was limited to NSCLC, which accounts for approximately 75% of lung cancer tumors. The ideal multiplex or panel assay would include markers for both NSCLC and SCLC to target complete lung cancer detection in all stages of disease. In order to determine the comparative clinical diagnostic efficacy of all these molecular diagnostic tests, side by side comparison studies should be performed in the future.

In this proof-of-concept study, we developed a quantitative real-time RT-PCR assay specific to lung cancer by simultaneously detecting 4 gene products with complementary expression profiles in NSCLC and SCLC tumor types from patients with all stages of disease. The combination of genes used in the multiplex assay presented here are specifically advantageous in the diagnosis of lung cancer, because molecular profiling of tumors can provide information about clinical status, tumor progression, treatment monitoring and patient prognosis.

Aschaetescute homologous protein ASH1 (HASH1, ASCL1) is known to be highly expressed in SCLC tumors (31). In normal lung tissue, the gene expression is restricted to epithelial bronchial neuroendocrine cells. However, ASH1 is up-regulated in lung cancers sharing neuroendocrine features and gene expression changes are easily monitored by RT-PCR (45).

CLCA2 belongs to the CLCA family of calcium-activated chloride channels, initially being identified in human lung, trachea and mammary gland as hCLCA2 and since reported to be overexpressed in squamous cell lung cancer (29, 46). Loss of CLCA2 expression, in contrast, has been associated with tumorigenicity in breast cancer. It is likely that the overexpression of CLCA2 is associated specifically with lung cancer and squamous cell tissues.
HMGB3 is a family member of the high mobility group architectural genes, initially classified as HMG2a, restricted to embryogenesis, and normally diminished or silent in adult tissues. In addition, HMG2 gene rearrangements and truncated proteins have been detected in benign mesenchymal tumors and lipomas and natural killer lymphomas. Recently, reports have demonstrated the role of HMG2a in the activation of the endogenous cyclin A gene, a factor in cell cycle regulation and tumor growth inhibition through HMG2a silencing (47, 48). HMG2a-related tumorogenesis may be related to the loss of exons 4 and/or 5, rather than the presence of sequences encoding functional domains (49). Since our sequence was discovered as a splice variant, it is possible that our sequence preferentially detects HMGB3 in cancer over that of normal gene expression.

L587S is a novel gene located on chromosome 18. It was identified through subtraction libraries to be specific to lung cancer (31, 32).

We demonstrated the application of this multigene assay in examining patient blood specimens for the evidence of circulating tumor cells. In addition to peripheral blood analysis, the assay could potentially be applied to detect tumor cells in various tissues including lymph node, bone marrow, sputum and pleural effusion samples. Our findings also suggest that the 4-gene multigene assay may be useful as a prognostic indicator and for prediction of disease relapse or progression. In 2 patients, a reduction in multigene gene expression was detected once chemotherapy had been initiated (Figure 5, patients A, B). In patient B, elevated levels of multigene gene expression were detected in the blood sample from 4/3/02, prior to the progressive disease diagnosis on 5/29/02 and subsequent blood draw on 7/31/02 (Figure 4, Patient B). In a third patient, disease recurrence was indicated by elevated multigene copies as early as 7 months prior to clinical diagnosis (Figure 5, Patient C).

These findings demonstrate the potential application of circulating tumor cell detection to evaluate the treatment response in NSCLC and SCLC patients. This application is supported by studies demonstrating that cytokeratin-positive minimal residual disease in bone marrow is indicative of relapse in breast cancer patients (27). Similarly, in NSCLC patients the presence of micrometastatic tumor cells in the lymph nodes correlates with poor prognosis (50). Moreover, the detection and molecular analysis of metastatic cells could provide an important tool to assess the status of residual disease for individualized antibody and vaccine therapy in the future.

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Received January 5, 2006
Accepted January 16, 2006