ORIGINAL ARTICLE

Gene Expression Profilingfor Cancer Targeted Therapy

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ABSTRACT

Background: Lung cancer is still the leading cause of cancer-related deaths and most of them are normally diagnosed in late stages, So there is an urgent need to establish reliable tools for the identification at early stages of the disease. In this study, we use an eleven gene signature test to examine lung biopsy specimens for diagnostic, prognostic & therapeutic purposes.

Methods: Bronchoscopy was performed to collect 102 fresh frozen specimens from 51 patients. 32 lung tumors& their adjacent normal tissues were examined along with biopsy specimens. In all specimens, total RNAs were isolated & after generating cDNA, were used for qRT-PCR analysis.

Results: The results indicate that the expressions of 3 oncogenes Bcl-2, k-ras & h-ras were significantly high ($p<0.05$) in surgically removed tumors as well as biopsy specimens compare to adjacent normal tissues. The differential expressions of these 3 genes were strikingly higher in cigarette using patients than nonsmokers.

Conclusion: Considering the overall results of this study, it can be concluded that, lung tissue abnormalities other than cancer could cause the elevation of some oncogene expression. This study also verifies that smoking can definitely cause oncogenic mutation, activation &/or amplification; which is now a globally proven fact.

Keywords: Lung biopsy, specimen, Gene expressionprofiling

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Introduction

Lung cancer is still the leading cause of cancer-related death in both men and women.[1] Prognosis is poor with 2-year survival rate of approximately 15% because most lung cancers are normally diagnosed in late stages.[2] Therefore the majority of patients are not cured by the therapeutic regimens that are available today.[3] There is an urgent need to establish reliable tools for the identification of lung cancers at early stages of the disease, prior to the development of clinical symptoms. The use of tissue-based biomarkers for lung cancers might circumvent the pitfall of imaging technologies diagnostics.[4, 5] Biomarkers screening can be utilized to direct imaging approaches to those individuals identified as potential lung cancer patients.

All cancers are considered genetic diseases caused by alterations in cancer-associated genes.[6] The identification of such specific mutated genes is critical for understanding the pathogenesis of cancer. More than 100 oncogenes have been identified in animal systems, but only a small subset of genes have been found consistently as mutated genes in human cancers.[7–10] Those involved in multiple tumor types include those encoding the growth factor receptors erbB and erbB2, ras, myc, Bcl-1and, Bcl-2.[9] Knowing that these genetic alterations are patient specific, personalized treatment based on tumor genotyping will probably be most effective way to combat cancers.

Personalized medicine (PM) refers to a new paradigm based on improved integration of the biological background of both the host and tumor for enhanced cancer diagnosis, prognosis and therapy.[11-13] For some, PM is guided not only by biology, but by modern functional and molecular imaging also. PM has the potential to help change the landscape of oncology. Targeted therapy has implications not only for systemic therapy but for radiotherapy and surgery as well. To make such targeted therapies a reality, better interventional radiology and endoscopic procedures and the establishment of partnerships with patient on 'the tissue quest' will enable acquisition of high-quality tumor material, truly reflecting the disease at stake.

Obviously optimal analysis of the tumor biopsies requires high through-put platforms, system biology knowledge with strong computational medicine. Targeted therapies reinforce and expand the multidisciplinary approach through the introduction of a new player; the cancer biologist. Such specialists will become mandatory members of clinical team within the next five years. Therefore PM will become integral to everyday clinical practice within the next five to ten years.

Using cancer biomarkers enables clinicians to make better treatment decisions based on the specific characteristics of individual patients and their tumors.[14] There have been significant advances in refining the prognosis of NSCLC by gene expression signatures,[15-17] most notably the 5-gene signature from Chen et al(18) and the 133-gene signature from Potti et al.[19] Gene expression-based diagnosis of lung adenocarcinomas[20] has already been incorporated in clinical settings to treat this deadly disease.

Personalized medicine has always been the ultimate goal and self-understanding of the medical community. Only through better understanding of oncogenic drivers and processes, and successful development of molecular biological techniques will we come closer to achieving this goal. A preliminary example of this approach has been provided recently by Von Hoff et al,[21] using molecular profiling of patients' tumors to identify targets and select treatments for their refractory cancers.

In this study, we determine the eleven gene signature of lung biopsy specimens for diagnostic, prognostic and therapeutic purposes. Our eleven gene panel include, five well known and studied therapeutic target genes, h-ras, k-ras, bcl-2, c-myc and her2;[22-28] a tumor growth factor, TGFα involved in most cancers including lung carcinomas;[29] a well studied tumor suppressor gene, p53 and its regulator, MDM2;[30-33] (two well studied genes involved in metastasis, mmp1and mmp14;[34,35] finally a multiple drug resistant gene, MDR1.[36,37] The rational for selecting these genes is the availability of gene targeted anticancer drugs developed in Tofigh Daru for personalized cancer therapy. The eleven-gene signature will be used to screen cancer patients before treatment. In addition to biopsy specimens which is the main focus of this study, we examined 16 surgically removed lung carcinoma tumors and 16 adjacent normal tissues previously acquired from our colleagues at Masih Daneshvari Hospital, to have relatively positive and negative controls. Our goal is to help clinicians to select a better treatment regimen, particularly when gene targeted therapy is available. The

main focus of this study is to develop a workable and quick specimen characterizing assay for small biopsy specimens

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Materials and Methods

Patient samples

Doctors performed bronchoscopy to collect fresh specimens from 51 suspected lung cancer patients at Masih Daneshvari Hospital, a Shahid Beheshti Medical University affiliate. Two specimens were obtained from each patient, one for histological examination and one for gene expression profiling. Total of 102 specimens were collected from June 2010 to January 2011. The 32frozen tumor and normal tissues were available at Tofigh Daru from previous year.The 32 tissues had been removed surgically from 16 lung cancer patients; one specimen from tumor and one fromthe adjacent normal lung tissue of the same patient. The histological analysis of all tissues was performed by pathologists at the hospital and gene expression profiling was done in the molecular research laboratory at Tofigh Daru. This study was approved by the Institutional Review Board and ethic committee of Shahid Beheshti Medical University. The clinical characteristics of these patients are described in supplementary **table S1** and **S2**.

Method of collecting specimen for molecular analysis

Fresh biopsy samples were submerged in RNAlater®, RNA Stabilizing Reagent (Qiagen, Hilden, Germany) and carried from hospital to the research facilities at Tofigh Daru. They were stored at –20°C until use.

RNA Isolation

Total RNA from each specimen, weighting 5-30 mg,was extracted and purified using the RNeasy mini kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). A polytron homogenizer was used for tissue disruption andpurified RNA concentration was measured by spectrophotometer.

Reverse Transcription

Purified RNA from each sample was used to generate complementary DNA using a Revert-Aid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol.

Table S1: The clinical and pathological characteristics of biopsy specimens.

	Cancerous	Non Cancerous	
Total number of patients	18	33	
Mean age (range)	63±10	53±21	
Sex			
Male	12	21	
Female	6	12	
Histological type			
ADC	3	Fibrosis	1
SQCC	6	Granulomatous	8
NSCC	2	Inflammation	15
SCC	4	Others	9
Others	3		

(a) ADC: Adenocarcinoma.
(b) SQCC: Squamous cell carcinoma.
(c) NSCC: Non small cell carcinoma.
(d) SCC: Small cell carcinoma

Table S2: The clinical and pathological characteristics of surgical specimens

	Cancerous specimens	Non Cancerous specimens
Total number of patients	16	16
Mean age (range)	57.53	57.53
Sex		
Male	9	9
Female	7	7
Histological type		
ADC	6	-
SCC	3	-
TC	4	-
Lymphoma	3	-

(a) ADC: Adenocarcinoma.
(b) SCC: Small cell carcinoma.
(c) TC: Typical Carcinoid.

Real-time RT-PCR

The generated cDNAs were used for qRT-PCR analysis using, ABI Step OnePlusTM (Applied Biosystems). The endogenous control gene, human β-actin had relatively constant expression level in all samples. Expression of mRNA for 12 selected genes was measured in each specimen with the use of Power SYBR Green PCR master Mix (ABI). Relative quantitation of gene expression was determined, using comparative CT method of (ΔΔCT). All samples were measured in triplicate and each PCR run was accompanied by a no-template control.

Primer design

The primers were designed using the Primer3 primer design software (Rozen & Skaletsky, 2000) and synthesized using Polygen DNA Synthesizer at Tofigh Daru. Sequence variability among individual subjects was avoided by selecting sequence regions free of known polymorphisms.

Data analysis

The threshold cycle (CT) and the PCR reaction efficiency were calculated for each sample based on its amplification curve. In all subsequent analysis, the average CT value from each sample's PCR triplicates and average efficiency calculated from all samples were used for each gene. Bio Pronet Software was used to analysis gene expression values based on the ΔΔCt (delta-delta cycle threshold) method. The housekeeping gene β-Actin expression level was used for data normalization.

Statistical analysis

T test independent was performed using SPSS19 software to evaluate the association between expression of cancer related genes and clinico-pathologic information.

Results

The gene expression analysis of 51 lung biopsy specimens was performed prior to receiving pathological evaluation. This is gene expression-based comparison of cancerous tissues versus non-cancerous tissues that suffer from other lung abnormalities. Pathological analysis showed that only18 specimens were cancerous while 33 were related to other lung diseases such as in flammations, fibrosis etc.We also examined 16 surgically removed lung cancer tissues and 16 adjacent normal tissues from the same patients to compare with biopsy gene expression results. The mean age of cancer patients was 63.9±10 and for other diseases was 53.2±21. It may also worth mentioning that 31.4% of tissues were from female patients and 66.7% belonged to males. We observed no association between diagnostic signature and patient age or sex. **Figure 1** Shows that the expressions of 3 oncogenes known to be involved in lung cancers, bcl-2, h-ras and k-ras are significantly ($p<0.05$) high in surgically removed cancerous tissues (red), andbiopsy specimens (green, purple) compare to adjacent normal tissues (blue).Two other oncogenes in this panel, Her-2 and TGF-alpha are significantly ($p<0.05$) elevated only in surgically removed tumors. C-myc on the other hand is not significantly expressed in any lung specimen. The metastatic gene, mmp1 expression is elevated in cancerous biopsies while mmp14 is highly expressed in surgically removed tumor tissues. The expression of tumor suppressor gene, P53 is significantly ($p<0.05$) high in all specimens compared to normal tissues. Differential expressions of bcl-2, h-ras k-ras as well as mmp14 arestrikingly higher in smokers than non-smokers specimens. As mentioned above, 18 collected biopsy specimens were diagnosed to be cancerous; among these 18 cancer patients, 65% were cigarette users and 35% of them were non-smokers (**Figure 2**). We

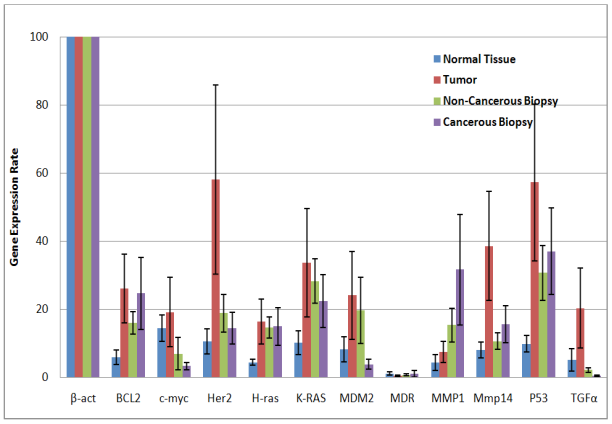

Figure 1: Eleven gene expression signatures in various human lung tissues are determined using real-time RT-PCR. The gene expression values are calculated relative to our reference gene expression, β-actin.

compared the expression of genes in biopsies collected from patients who smoke to those of non-smokers, regardless of their pathoclinical results. **Figure 3** clearly shows that all of our oncogenes except for TGF-α have significantly ($p<0.05$) elevated expression. Interestingly the result shows that, while p53 expression is elevated, the expression of its regulator gene, MDM2 is decreased. This may be a cellular defensive respond to cancer causing chemicals in cigarettes to raise the level of tumor suppressor gene p53. The expression signature clearly confirms once again that cigarette smoking plays a major role in oncogenic mutations and/or amplifications. Bcl-2 and k-ras oncogenes, known to be involved in lung cancers are overexpressed in more than 50% of lung biopsy specimens. TGF-α, a tumor growth factor gene; MDM2, p53 regulator gene; and MDR1, the drug resistant gene, had no or very low expression in all samples.

Conclusion

Lung cancer is a dynamic and diverse disease associate with numerous somatic mutations, deletions and amplifications of genes. Traditional diagnostic markers and procedures can not stratify patients with molecularly distinct diseases. Since every cancer patient has distinct molecular abnormalities, it is a critical issue to reliably identify specific therapeutic targets to address personalized cancer therapy. There are not many gene tests around to stratify cancer patients and prescribe gene-targeted drugs. In this study we have employed an 11-gene expression signature that includesfive well studies oncogenes that are believed to be excellent therapeutic targets. Compare to other gene expression profiling techniques, real-time Rt-PCR is more efficient and consistent. This technique also requires small amount of samples that makes it possible to test biopsy specimens. The 11-gene panel did not stratify our patients into distinct groups based on cancer and non-cancerous abnormalities.However the expressions of bcl-2, k-ras and h-ras are significantly ($p<0.05$) higher in both surgically removed tumor tissues and biopsy specimens compare to adjacent normal tissues. Furthermore, tissues received from cigarette user patients have shown significant elevation in the expression of these genes compare to non-smokers.The failure to stratify patients into two distinct cancerous and non-cancerous groupsbased on

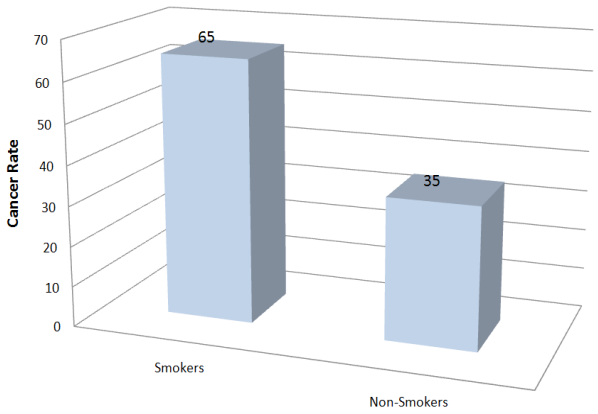

Figure 2: The data collected from cancer patients (18), shows the striking effect of smoking cigarette on cancer development.

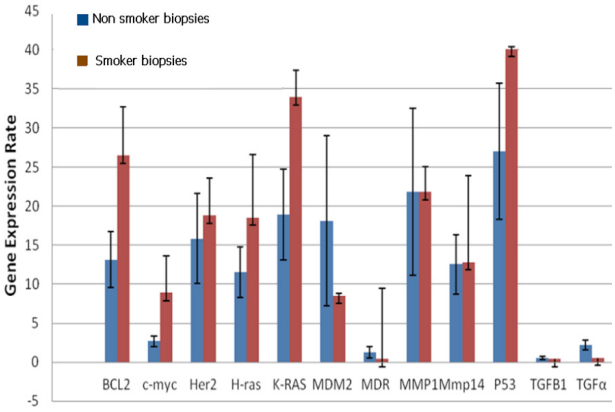

Figure 3: The oncogene expression signatures stratify patients into smokers and non-smokers group. The expressions of some oncogenes such as, bcl-2, c-myc, h-ras, k-ras and p53 as a tumor suppressor are significantly ($p<0.05$) higher in the biopsy specimens of smoker patients compare to non-smokers. The values represent the expression of each gene relative to β-actin expression.

this gene expression signature could be related to small number specimens tested. We can also postulate that other lung tissue abnormalities may elevate the expression of some oncogenes prior to cancer development. However this study verifies that smoking can definitely cause oncogenic mutation, activation and/or amplification; which is now agloballyproven fact.Overall, our data show the feasibility of a relatively simple diagnostic testfor lung biopsy specimens as small as 2-3 mm in diameters. Based on the data presented in this paper, it seems advisable to focus determining the expression profiling of other can-

cer related genes by examining large number of specimens if available.

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