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Gene Expression Profiling of Non-Small Cell Lung Cancer

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Purpose: **cDNA microarray provided a powerful alternative, with an unprecedented view scope, in monitoring gene expression levels, and led to the discovery of regulatory pathways involved in complicated biological processes. This study was performed to gain better understanding of the molecular mechanisms underlying the carcinogenesis and progression of lung cancer.**

Materials and Methods: **Using a cDNA microarray, representing 4,600 cDNA clusters, we studied the expression profiles in 10 non-small cell lung cancer (NSCLC) samples and the adjacent noncancerous lung tissues form the same patients. The alterations in the levels of gene expression were confirmed by reverse-transcription PCR in 10 randomly selected genes.**

Results: **Genes that were differently expressed in the cancerous and noncancerous tissues were identified. One hundred and nine genes (of which 68 were known) and 69 cDNAs (of which 32 were known) were up- and down-regulated in >70% of the NSCLC samples, respectively. In the cancerous tissues, the genes related to the cell cycle, metabolism, cell structure and signal transduction, were mostly up-regulated. Furthermore, we identified a few putative tumor suppressor genes that had previously been proposed by other workers.**

Conclusions: **These results provide, not only a new molecular basis for understanding the biological properties of NSCLC, but also useful resources for the future development of diagnostic markers and therapeutic targets for NSCLC.** ***(Cancer Research and Treatment 2003;35:154-160)***

Key Words: **cDNA microarray, Gene expression, Non-small cell lung cancer**

INTRODUCTION

Globally, lung cancer is the leading cause of cancer deaths, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of cancers (1). Recent molecular analyses have clarified many genetic alterations in lung carcinogenesis, but this is hardly sufficient for understanding the common pathway of the carcinogenesis and progression of lung cancer. Furthermore, lung cancer shows diverse clinical properties, such as histological type, metastatic status, invasiveness and responsiveness to chemotherapy. However, little is known about the genes associated with these characteristics.

Array technologies are accurate and comprehensive ways of simultaneously analyzing the expressions of thousands of genes, and have been applied in many research fields (2). There is now strong evidence that global gene expression profiling can reveal subtypes of cancer, on the basis of the underlying heterogeneity in the transformation mechanisms, differentiation states or cell types (3~6). The clinical use of gene expression profiles could result in more accurate and objective diagnoses of cancers, as well as better prognoses of disease or responses to treatment.

To investigate the changes in gene expressions common in NSCLC tissues, lung cancer tissues were analyzed by a cDNA microarray, representing approximately 4,600 unique genes. We identified genes that were differentially expressed between cancerous and noncancerous tissues, and a few genes suggested to be associated with tumor invasion or as being tumor suppressor genes.

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MATERIALS AND METHODS

1) Tissue samples and RNA preparation

Ten pairs of NSCLC tissues, and the corresponding adjacent noncancerous lung tissues, were obtained from patients who had undergone a lobectomy or a pneumonectomy at the Dong-A University Medical Center. The informed consent of the patients was obtained to allow the use of their operated

Table 1. Clinicopathologic characterization of 10 non-small cell lung carcinoma samples

Case No.	Age/Sex	Histology	T stage	LN* mestasis
1	65/Male	Squamous†	T1	Negative
2	49/Male	Squamous	T2	Negative
3	63/Male	Squamous	T2	Positive
4	67/Male	Squamous	T2	Negative
5	49/Male	Adenoca‡	T2	Positive
6	61/Female	Adenoca	T1	Positive
7	65/Female	Adenoca	T2	Negative
8	54/Male	Adenoca	T2	Positive
9	63/Female	Adenoca	T1	Positive
10	68/Male	Adenoca	T1	Negative

*Lymph node, † squamous cell carcinoma, ‡ adenocarcinoma

specimens and clinicopathological data for research purposes. The samples were studied anonymously. A pathologist (M.S.R) dissected the tissue samples from the surgical specimens, with special care to minimize potential contamination, and the samples immediately frozen in liquid nitrogen, and stored at -80°C until required. Total RNA was isolated from the frozen tissues using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). The remaining parts of the tumors were fixed with 10% buffered formalin, and the paraffin sections stained with hematoxylin/eosin. The histological classifications for each case were determined by a pathologist (M.S.R) based on the WHO criteria (7). The postoperative pathological staging was determined according to the guidelines of the AJCC (8). These clinical and histopathological features are summarized in Table 1.

2) Microarray analysis

10 pairs of cancerous and corresponding adjacent noncancerous lung tissues were analyzed using the *MAGIC*™ microarray (Macrogen, Inc., Korea). This array contains 4600 cDNAs, isolated from the cDNA library of human breast cancer, and is composed of 4,370 known and 230 unknown genes (EST). For synthesizing a double-stranded cDNA probe, a labeling kit, provided by Macrogen, was used. Microarray hybridization was performed according to manufacture's instructions.

3) Data analysis

Images were analyzed using the ImaGene™ analysis software (BioDiscovery, Inc., Marina del Rey, CA). Spots showing no signals, or obvious defects, were excluded from the analysis. The local background was subtracted from the remaining spots, and the ratios of the net fluorescence from the Cy5-specific channel, to those from the Cy3-specific channel, were calculated for each spot, representing the cancerous mRNA expression relative to the corresponding noncancerous lung tissue. The ratios were log-transformed and normalized, to give average log-transformed ratios equal to zero. The genes with good data present in 70% of the experiments, and with expression ratios that varied at least 2-fold between the cancerous and noncancerous tissues, were selected for the identification of the genes that were differentially expressed between the cancerous and noncancerous tissues. The differentially expressed genes, identified from cDNA microarray comparisons, were assigned to a modification of the NCBI Clusters of Orthologous Gene classification, by searching the OMIM and PubMed databases, by gene name (9). The genes were then assigned to 10 functions (cell cycle regulator, transcription, oncogene/tumor suppressor gene, metabolism, cell structure/motility, signal transduction, gene/protein expression, DNA repair, angiogenesis and immunology) and 1 miscellaneous category.

4) Semiquantitative RT-PCR

Single-stranded cDNA was synthesized, with the oligo (dT) primer in a 20-μl reaction, from 5 g of total SuperScript Preamplication for First Strand cDNA Synthesis System (Life Technologies, Inc., Rockville, MD), and diluted to 80μl. PCR was then performed with 1μl of cDNA, for 1 cycle of 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, using a gene-specific primer and Taq polymerase.

Amplification of the correct target DNA was confirmed by the mobility of gel electrophoresis and sequencing, following sub-cloning into pGEM-T easy vector (Promega, Madison, WI). β-actin was used as an internal control to confirm equal amounts between the templates.

RESULTS

1) Expression profiling revealed differentially expressed genes in NSCLC and noncancerous tissues

The fluorescence-intensity ratios were calculated, and gene-expression profiles generated, for each sample. The cancerous and noncancerous tissues were successfully distinguished (data not shown). However, this algorithm, using most of the genes on the array, did not classify the cancer tissue samples by the subgroups associated with histopathological features.

The gene-expression profiles were used to determine the differentially expressed genes between the cancerous and noncancerous tissues. When the cutoff value was set to 2.0, for the ratio between the cancerous and noncancerous tissues, 109 genes (of which 68 were known) and 69 (of which 32 were known) cDNAs were up- and down-regulated, respectively. These genes were classified in terms of 10 functions (cell cycle regulator, transcription, oncogene/tumor suppressor gene, metabolism, cell structure/motility, signal transduction, gene/protein expression, DNA repair, angiogenesis and immunology), as shown in Table 2. In the cancerous tissues, the genes related to the cell cycle, metabolism, cell structure and signal transduction, were mostly up-regulated. As expected, the cyclin-dependent kinase 6 and weel+ homolog genes, involved in cell cycle regulation, were up-regulated.

The growth of cancer is accompanied by the progressive infiltration, invasion and destruction of the surrounding tissue. Invasion and metastasis are biological hallmarks of malignant tumors, and are the major cause of cancer-related morbidity and

Table 2. Representative differential expression in non-small cell lung carcinoma, defined a 2-fold or greater change relative to expression in noncancerous tissues, for various functional classes of genes

Function	Accession No.	Gene	2 log (T/N)*	Frequency
Cell cycle regulator				
	D14678	kinesin-like2	3.908	9
	X62048	weel+ (S. pombe) homolog	3.55	8
	AF068007	microsperule protein 1	3.241	9
	X66365	cyclin-dependent kinase 6	3.096	8
	U40705	Telomeric repeat binding factor (NIMA-interacting)1	2.74	9
	M87339	replication factor C (activator 1) 4	1.645	7
	D79995	KIAA0173 gene product	-1.5275	8
	U41816	prefoldin 4	-1.918	8
	AJ245553	zinc finger protein	-2.4425	8
	AF206019	REV1 protein	-2.806	8
Transcrition				
	U09412	zinc finger protein 134 (clonen pHZ-15)	3.732	10
	U09825	zinc finger protein 173	2.9525	8
	NM_013242	similar to mouse GLt3 or D. melanogaster trancription factor IIB	2.683	8
	Y14443	zinc finger protein 200	1.505	8
	X66079	Spi-B transcription factor (Spi-1/PU.1 related)	-2.243	8
Oncogene/tumor suppressor gene	U76248	seven in absentia (Drosophila) homolog 2	3.08	9
	U82130	tumor susceptibility gene 101	2.584	9
	AJ250014	KIAA0849 protein (familial cylindromatosis (cyld) gene)	-1.58	10
	U57962	Human BRCA2 region, mRNA sequence CG018	-1.696	8
Metabolism				
	AF064254	VLCS-H1 protein	3.0675	8
	X59960	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)	2.713	9
	AK000759	hypothetical protein	2.382	10
	Z46376	hexokinase 2	2.27	9
	L06419	procollagen-lysine, 2-oxoglutarate 5-dioxygenase	2.054	9
	U68140	nuclear VCP-like protein NVLp.2	1.9975	8
	D14697	farnesyl diphosphate synthase	1.856	8
	AF039704	ceroid-lipofuscinosis, neuronal 2, late infantile (Jansky-Bielschowsky disease)	1.66	8
	D00723	glycine cleavage system protein H (aminomethyl carrier)	1.595	9
	NM_020379	1,2-alpha-mannosidase IC	-1.4825	8
	S69232	electron-transferring-flavoprotein dehydrogenase	-1.652	7
	AB018551	chromosome 16 open reading frame 7	-2.648	8
	M98045	folypolyglutamate synthase	-3.4725	8
Cell structure/ Motility	AF054910	tektin 2 (testicular)	3.415	8
	X14420	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type, autosomal dominant)	3.2175	8
	U33931	erythrocyte membrane protien band 7.2 (stomatin)	3.05	8
	Z74615	collagen, type 1, alpha 1	2.855	8
	Al031058	desmoplakin	2.813	8
	M94345	capping protien (actin filament), gelsolin-like	2.626	8
	Z15008	laminin, gamma 2	2.37	8
	Z19574	keratin 17	1.9675	8
	J000124	keratin 14 (epidemolysis bullosa simplex, Dowling-Meara, Koebner)	1.882	9
	AL110225	drebrin 1	1.877	7
	AJ250562	transmembrane 4 superfamily member 2	1.621	8
	NM_018477	uncharacterized hypothalamus protein HARP11	1.474	7

Table 2. Continued

Function	Accession No.	Gene	2 log (T/N)*	Frequency
Cell structure/ Motility	AB009799	nidogen 2	1.465	7
	NM_020125	BCM-like membrane protein precursor	-1.792	10
	AL023653	hypothetical protein	-3.055	8
	AB006780	lectin, galactoside-binding, soluble, 3 (galectin 3)	-4.078	8
	U61234	tubulin-specific chaperone c	-4.287	9
Signal transduction	AB000887	small inducible cytokine subfamily A (Cys-Cys), member 19	3.15	8
	U10099	POM (POM121 rat homolog) and ZP3 fusion protein	3.12	9
	U02680	protein tyrosine kinase 9	2.986	8
	NM_019892	phosphatidylinositol (4,5) biphosphate 5-phosphatase homolog	2.94	8
	AB014561	95 kDa retinoblastoma protein inding protein	2.91	8
	AF082657	era (E. coli G-protein homolog)-like 1	2.798	8
	M16660	heat shock 90KD protein 1, beta	2.769	10
	AL049654	protein kinase C, alpha binding protein	2.486	9
	AF070599	protein phosphatase 1, regulatory (inhibitor) subunit 11	2.327	8
	L13738	activated 021 cdc42Hs kinase	2.14	8
	M61906	phosphoinisitol-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.03	7
	AF035947	cytokine-inducible SH2-containing protein	1.657	7
	AF060798	serine/threonine kinase 16	1.567	9
	AF189009	ubiquilin 2	-1.407	7
	AL157424	synaptojanin 2	-1.5	10
	AB040890	KIAA1457 protein	-2.1925	8
	AF263541	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	-3.162	9
	M60922	flotillin 2	-3.483	8
Gene/protein expression	U40763	Clk-associating RS-cyclophilin	3.06	8
	NM_007230	mannosidase, alpha, class 1B, member 1	2.77	9
	AJ132712	nuclear RNA export factor 1	1.7475	8
	AF000982	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3	-1.525	9
	U46571	tetratricopeptide repeat domain 2	-3.58	9
	X70944	splicing factor/glutamine rich	-3.965	10
DNA repair	NM_016042	CGI-102 protein	3.763	9
	U85625	ribonuclease 6 precursor	2.966	8
	L24498	growth arrest and DNA-damage-inducible, alpha	2.9225	8
	M74905	N-methylpurine-DNA glycosylase	2.701	9
	U12134	RAD52 (S. cerevisiae) homolog	2.56	8
Angiogenesis	U03644	CBF1 interacting	3.763	9
	AF240635	protocadherin 12	2.245	9
	AF035121	kinase insert domain receptor (a type III receptor tyrosine kinase)	-1.66	9
Immunology	D12614	lyphotoxin alpha (TNF superfamily, member 1)	4.464	9
	AF053004	class 1 cytokine receptor	2.8575	8
	U05875	interferon gamma receptor 2 (interferon gamma transducer 1)	2.213	10
	L19185	peroxiredoxin 2	1.4575	8

Table 2. Continued

Function	Accession No.	Gene	2 log (T/N)*	Frequency
Immunology				
	AF240467	toll-like receptor 7	-1.506	8
	L41067	nuclear factor of activated T-cells, cytoplasmic 3	-3.1625	8
	U60800	sema domain, immunoglobulin domain (Ig)	-3.956	8

*2 log (Tumor/Noncancerous tissue)

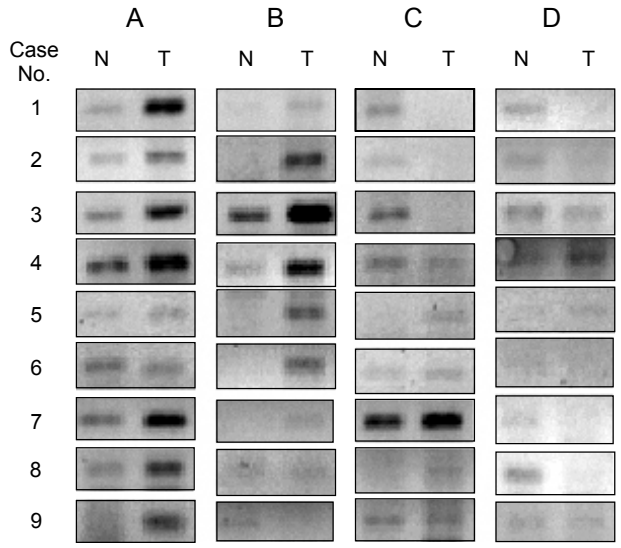

Fig. 1. The results of semiquantitative RT-PCR for the 4 tested genes in the 9 non-small cell lung cancer tissues and the noncancerous tissues were consistent with those from the microarray studies. N: normal tissue, T: tumor tissue, A: U82130 (TSG11), B: U76248 (hSIHS), C: X66079 (Spi-B), D: D79995.

mortality. A few genes, such as cyclin-dependent kinase 6, keratin 14, Retinoblastoma-binding protein, and serine/threonine kinase, were identified, and up-regulated in the cancerous tissues, which were previously reported, by Chen et al., as positive invasion-related genes (10).

Because the products of tumor suppressor genes apply brakes to cell proliferation, the loss of these genes is a key event in many human tumors. We identified a few tumor suppressor genes that had previously proposed by other workers, including BRCA-2 (11) hHDC (12), CYLD (13) and TSG101 (14). As described in table 2, these genes, with the exception of the TSG101, were down regulated in the cancerous tissues.

2) Validation of DNA microarray by semiquantitative RT-PCR

To verify the reproducibility of these gene lists, we performed semiquantitative RT-PCR using the same RNA used in the microarray analysis. Nine pairs of cancerous, and corresponding noncancerous, tissue samples were analyzed using 4 randomly selected genes. Of these genes, three putatively associated with tumorigenesis, sina, TSG101 and Spi-B, were included. Most of the lung cancer genes found from the semiquantitative RT-PCR analyses were consistent with those from the microarray studies (Fig. 1). The amplification of those specific genes failed in one sample, possibly due to degradation of the RNA.

DISCUSSION

The gene expressions of lung cancer and noncancerous tissues were analyzed to elucidate the characteristic changes associated with the carcinogenesis and progression of lung cancer. The cancerous and noncancerous tissues were distinguished by gene expression profiling. In the cancer tissues in this study, the genes related to the cell cycle, metabolism, cell structure and signal transduction, were mostly up-regulated.

Most of the genes associated with the cell cycle were up-regulated, and exemplified by the cyclin-dependent kinase 6 (CDK6) and wee1+ (S.pombe) homolog genes. CDK6 is considered as a cdc-2 related kinase, which can play roles in the regulation of the mammalian cell cycle. The cyclin D/CDK6 complex phosphorylates the retinoblastoma protein. Under-phosphorylated pRb binds to the E2F family of transcription factors. Phosphorylation of the pRb unshackles the E2F proteins, which in turn activate the transcription of several genes whose products are essential for progression through the S phase (15). Because the state of the pRb phosphorylation is crucial in determining the cell cycle progression, the up-regulation of CDK6 in cancer tissues was not unexpected. The wee1+ gene is a mitotic inhibitor that controls the G2 to M transition of the fission yeast, Schizosaccharomyces pombe, and encodes a protein kinase with both serine- and tyrosine-phosphorylating activities. The human gene, wee1Hu, is similar to the wee1+ of the yeast, and encodes a protein that is homologous to the S. pombe wee1+ and mik1+ kinases (16). Igarashi et al (16) reported that the overexpression of the wee1Hu in fission yeast generates very elongated cells, as a result of inhibition of the G2-M transition, in the cell cycle. However, in our study, the weel+ was up-regulated in the cancerous tissues, which may allow the tumor cell to survive for long periods, followed by the accumulation of additional carcinogenic effects.

The early detection of lung cancer is the key to long-term survival. However, the early diagnosis of lung cancer patients has remained an elusive goal (1). Patients with preinvasive and microinvasive cancers, which are found by cytological examination of sputum, have a high survival rate (>90%) following

surgical removal or localized therapy, but constitute less than 1% of the newly diagnosed cases (1). Therefore, the development of a new diagnostic method, specifically for the very early stage of lung cancer, is urgently required. The differential expressions of multiple genes, and their dynamic interaction between various proteins, have been well documented, and are involved in the multiple steps of invasion and metastasis. We identified a few putative genes associated with invasion, such as cyclin-dependent kinase, keratin 14, Retinoblastoma-binding protein and serine/threonine kinase, which were suggested by a study of a lung cancer cell line model (10). It has also reported that genes related to signal transduction, cell adhesion, motility and angiogenesis might play significant roles in the invasion and metastasis processes (10). In a murine model of osteosarcomas, the genes that were assigned to the motility, cytoskeleton, adherence and angiogenesis categories were considered most likely to define the differences in the metastatic behavior (17). Keratin 14, a gene associated with the cytoskeleton, represents a marker for the basal cell compartment in lung bronchi and bronchioles (18). In immunohistochemical studies, keratin 14 is usually only displayed in squamous cell carcinomas of the various lung cancer subtypes (19). The study to identify molecular profiles, and the gene cluster, specifically associated with squamous cell carcinomas and adenocarcinomas showed the under expression of the keratin 14 in the adenocarcinomas (20). However, in our study, the keratin 14 was up-regulated in both the squamous cell carcinomas and the adenocarcinomas. It has previously been reported that keratin 14 was one of the positive invasion-related genes (10), and that its expression may be a marker of tumor progression (21). Our study also suggested that keratin 14 may have a certain role associated with tumor invasion.

The loss of a tumor suppressor gene is a key event in many, possibly all, human tumors, as the physiological function of these genes are in the regulation of normal cell growth, including the cell cycle and nuclear transcription. We have identified some of the tumor suppressor genes that have previously been proposed by other research, including BRCA-2 (11), hHDC (12), CYLD (13) and TSG101 (14). As described in table 2, these genes, with the exception of the TSG101, were down regulated in the cancerous tissues. Although its favorable role in the progression of other tumors has been proposed, the information on the expression pattern of these genes in lung cancer is limited. The human homolog, TSG101, which has been mapped to chromosome 11, bands 15.1～15.2, a region proposed to contain tumor suppressor gene(s), is mutated, at high frequency, in human breast cancer (14). However, it has been suggested that the TSG101 is not mutated in lung cancer, but that aberrant splicing of the TSG101 occurs in small cell lung cancer (22). In this study of NSCLC, the TSG101 was up-regulated in the cancerous tissues, which agrees with the results from a previous study (22).

This study focused on the analysis the whole cancer tissues, rather than on only the cancer cells, in order to better describe all aspects of lung cancer, as lung cancer tissues, as with other cancers, generally contain multiple nonepithelial cell types, such as fibroblast, smooth muscle cell, endothelial cell, infiltrating lymphocyte and macrophage. Recent advances in cancer research have revealed the relevance of epithelial-stromal interactions, including extracellular matrices, matrix metalloproteinases and angiogenic factors, in cancer progression (23). Accordingly, the targets of cancer therapeutics have been recently extended from the molecules of cancer origin, to the molecules of stroma origin, such as those related to angiogenesis and matrix remodeling (24, 25). As this study was based on whole tissue samples, the list of genes up-regulated in our cancerous tissues contained, and may still contain, many genes for the stroma. With further functional studies, potentially therapeutic target molecules of the stroma might be identified. Furthermore, clinical outcomes might show, more precisely, the prognostic gene marker of lung cancer.

CONCLUSIONS

We have used a cDNA microarray to identify molecular profiles in human lung cancer. Most genes associated with the cell cycle, exemplified by the cyclin-dependent kinase 6 (CDK6) and wee1+ (S.pombe) homolog genes, and a few putative genes associated with invasion, such as cyclin-dependent kinase, keratin 14, Retinoblastoma-binding protein and serine/threonine kinase, were up-regulated in the cancerous tissues. A few tumor suppressor genes, previously proposed from other research, including BRCA-2, hHDC, CYLD and TSG101, were down regulated in the cancerous tissues. Although biologically significant, and highly reproducible, the gene expression profiles described here may only represent the basic molecular features of lung cancer. The list of genes described as expressing a high degree of differentiation are likely to provide valuable resources, not only for basic studies, such as understanding the molecular mechanism of carcinogenesis, progression and metastasis, but also for clinical applications, such as the development of novel diagnostic markers and the identification of therapeutic targets, for lung cancer.

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