· Basic Research ·

Genetic Fingerprint Concerned with Lymphatic Metastasis of Human Lung Squamous Cancer

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Abstract

Background and objective With the most recent introduction of microarray technology to biology, it becomes possible to perform comprehensive analysis of gene expression in cancer cell. In this study the laser microdissection technique and cDNA microarray analysis were combined to obtain accurate molecular profiles of lymphatic metastasis in patients with lung squamous cell carcinoma.

Methods Primary lung squamous cancer tissues and regional lymph nodes were obtained from 10 patients who underwent complete resection of lung cancer. According to the source of lung cancer cells, the samples were classified into three groups: the primary tumor with lymphatic metastasis (TxN+, *n*=5), the primary tumor without lymphatic metastasis (TxN-, *n*=5) and matched tumor cells from metastatic lymph nodes (N+, *n*=5). Total RNA was extracted from laser microdissected tumor samples. Adequate RNA starting material of mRNA from primary tumor or metastatic nodes were labeled and then hybridized into the same microarray containing 6 000 known, named human genes/ESTs. After scanning, data analysis was performed using GeneSpring™6.2. **Results** A total of 37 genes were found to be able to separate TxN+ from TxN-. TxN+ have higher levels of genes concerned with structural protein, signal transducer, chaperone and enzyme. TxN- have higher levels of genes coding for cell cycle regulator, transporter, signal transducer and apoptosis regulator. Interestingly, there were no differentially expressed genes between N+ and TxN+.

Conclusion The acquisition of the metastatic phenotype might occur early in the development of lung squamous cancer. We raise the hypothesis that the gene-expression signature described herein is valuable to elucidate the molecular mechanisms regarding lymphatic metastasis and to look for novel therapeutic targets.

Key words Lung neoplasms; Gene-expression; Lymphatic metastasis

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【摘要】背景与目的 筛选肺鳞癌患者淋巴转移差异表达基因群。方法 原发癌组织及区域淋巴结取自10例接受完全性肺癌切除手术的肺鳞癌患者。根据组织来源将标本分为三组：不伴淋巴转移的肺鳞癌组织（TxN-, *n*=5）、伴有淋巴转移的肺鳞癌组织（TxN+, *n*=5）及相应转移淋巴结中的肺鳞癌细胞（N+, *n*=5）。对各组进行激光显微切割以获得纯净癌细胞，T7RNA线性扩增获取足够量的RNA，实验通道和参照通道分别标记以后与含6 000个已知人类基因或表达序列标签的cDNA基因芯片杂交，扫描荧光信号以后进行数据分析。结果 共有37个基因可将TxN+组与TxN-组区分开，其中在TxN+组高表达的基因有8个，主要涉及蛋白合成、信号传导、伴侣蛋白和酶等。有29个基因在TxN-组高表达，这些基因主要编码细胞周期调节子、转导子、信号传导蛋白以及细胞凋亡调节蛋白。比较N+组与TxN+组却没有发现具有显著性的差异表达基因。结论 肺鳞癌的淋巴转移表型的获得可能是早期事件。这些差异基因的发现有助于阐明肺鳞癌淋巴转移的分子机制和寻找新的治疗靶点。

【关键词】肺肿瘤；淋巴转移；基因表达

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Introduction

The primary tumor may result from mutations in the growth control genes; meanwhile metastasis probably results mainly from change in gene expression pattern in the cell. Thus it is important to use new technologies for the identification and characterization of such genes in order to understand metastasis and design better strategies to combat it[1]. Biological experiments are frequently conducted in a manner that probes a single or a limited number of molecular markers that usually fail to provide satisfactory results for the studied procedure. With the most recent introduction of microarray technology to biology, it becomes distinctly possible to perform comprehensive analysis of gene expression in cancer cells, and can reveal detailed phenotypic and biological information about cancer cells[2,3]. In this study we coupled the laser microdissection (LMD) technique with cDNA microarray analysis that enable us to obtain accurate molecular profile of lymphatic metastasis in patients with lung squamous cancer without contamination of tumors by stromal cell etc.

Materials and methods

Patients and tissue sample

Primary lung squamous cancer tissues and regional lymph nodes were obtained with informed consent from 10 patients who underwent radical resection in our hospital in 2004. Clinical and pathological feature of the patients investigated are presented in Tab 1. According to the source of cancer cells, the cases were classified into three groups: the primary tumor with lymphatic metastasis (TxN+, *n*=5), the primary tumor without lymphatic metastasis (TxN-, *n*=5) and matched tumor cells from metastatic lymph nodes (N+, *n*=5). No significant differences were seen among the two groups of patients as far as age, sex, smoking history and type of tumor is concerned. None of the patients had a previous history of lung cancer or a concurrent malignancy, nor had they been exposed to any chemoradiotherapy. Tissues were snap frozen in liquid nitrogen and then stored at –80 °C. For all cases, the diagnosis was established on routine formalin-fixed paraffin-embedded material.

Laser Microdissection

Frozen section from primary and metastatic lesions were stained with haematoxylin and eosin to verify the presence of viable tumor. Serial 8-μm thick sections were prepared with cryostat (Leica CM1900, Germany), and mounted on slides for membrane-based laser microdissection (Leica, Germany). The sections were laser microdissected with an LMD system (Leica Microsystems, Wetzlar, Germany). The fragments dissected were collected in cups and 20 μL lysis solution were added (from RNAqueous-Micro Kit). An electronic image of the H&E stained section was used as a template to guide dissection of adjacent section. Fig 1 shows a typical procedure of microdissection of lung cancer cells. After cutting, 80 μl of lysis solution was added into the tube to dissolve the connective tissue in order to release the cells which is collected at the bottom of tube.

T7-based RNA amplification

Total RNA was extracted from microdissected lung squamous cancer samples using the RNAqueous-Micro Kit (Ambion, Austin, Texas), according to manufacturer instruction. Adequate RNA starting material was generated using a T7 RNA polymerase-catalyzed linear amplification method. The Amino Allyl MessageAmp™ aRNA kit (Ambion, Austin, Texas) was used in 2 successive rounds on all LMD-isolated RNA samples, according to manufacturer instruction. The Universal Human Reference total RNA (StrtageneR, Amsterdam, The Netherland) was used.

Labeling, hybridization and scanning

Ten-microgram aliquate of mRNA from primary tumor or metastatic nodes were labeled with Cy3 monofunctional dye (Amersham Biosciences UK Ltd., Bukes, United Kingdom) for the sample RNA and Cy5 monofunctional dye for the reference RNA and then hybridized onto the same microarray. The microarray used in this study were human cDNA microarrays, from the Sanger Center (Hixton, Cambridge, UK) as part of the LICR (the Ludwig Institute of Cancer Research)/CRUK Consortium. Each array contains 10 750 spots representing 9 932 sequence-validated cDNA elements for 6 000known, named human genes/ESTs. cDNA microarray chips were hybridized for 18 h in a 42 °C incubator. After hybridization, the slides were washed once with 2×standard saline citrate (SSC) for 10 min at room temperature, 0.1×SCC/0.1% sodium dedecylsulfate three times for 20 min at 55 °C, and then 0.1× SCC twice for 5 min at room temperature. Both Cy3 and Cy5 fluorescent images of hybridized microarray were captured using GSI Lumonics 4 000 scanner and ScanArray software (Perkin-Elmer Life Sciences Inc., Boston, Massachusetts)

Data Analysis

After scanning, data were recorded as paired 16-bit TIFF images. Raw images were quantified using QuantArray 3.0 software (Perkin-Elmer Life Science Inc., Boston, Massachusetts). Data analysis was performed using GeneSpring™6.2 (Silicon Genetics, Redwood City, California). For each microarray, the median intensity of all spots in each channel was normalized to the median intensity of the spots in the control channel. The spots intensities used to calculate the median value for normalization were always background subtracted. The intensity dependent LOWESS normalization was then applied followed by per-chip normalization. The normalized intensity measurements from each experiment were log transformed (base 2) and the log ratio of experimental vs. reference channel was used as the measure of gene expression. Then the normalized log ratio of the data was ready for further analysis. The significance of altered expression of each gene was evaluated by one-way ANOVA Welch t-test. We used the Bonferroni procedure

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Tab 1 The clinical and pathological feature of the studied cases

Sample No.	Source	T-stage	Smoking	Age/Sex
1#	TxN+, N+	T1	Y	72/M
2#	TxN+, N+	T3	Y	69/M
3#	TxN+, N+	T2	N	62/M
4#	TxN+, N+	T2	Y	76/F
5#	TxN+, N+	T1	Y	61/M
6#	TxN-	T2	Y	75/M
7#	TxN-	T3	Y	66/M
8#	TxN-	T2	Y	62/M
9#	TxN-	T1	N	72/M
10#	TxN-	T2	N	51/M

TxN+, primary tumor with lymphatic metastasis; TxN-, primary tumor without lymphatic metastasis; N+, tumor from metastatic lymph nodes; Y, Yes; N, No; M, Male; F, Female.

(Multiple Testing Correction) to control the false discovery rate (FDR).

An agglomerative hierarchical clustering was applied for both genes of interesting and samples from comparison of TxN+ *vs* TxN-.

Results

There were 8 genes that were significantly overexpressed in TxN+, which mainly concerned with structural protein, signal transducer, chaperone and enzyme. On the other hand, there were 29 genes that were significantly up-regulated in TxN-, which mainly code for cell cycle regulator, transporter, signal transducer and apoptosis regulator (Tab 2). A scatter plot of microarray analysis and a gene/condition tree were showed in Fig 2 and Fig 3, respectively. Interestingly, according to the current filters, there were no differentially expressed genes between N+ and TxN+.

Discussion

Microarray technologies, based on hybridization methods first employed nearly 30 years ago[4], have facilitated the analysis of expression level of thousands of genes in a single experiment. This technology is a powerful tool for analyzing gene expression profiling and has exerted profound impact on cancer studying. This tool is uniquely suited for molecular characterization: large numbers of genes can be simultaneously investigated, and the resulting gene expression patterns can be correlated with certain clinical parameters[5]. Since then, researchers have used gene expression pattern to reveal previously unknown cancer categories[6]. The clinical outcome of individuals with cancer can be predicted using the gene-expression profiles of primary tumors at diagnosis[7-11]. In addition,

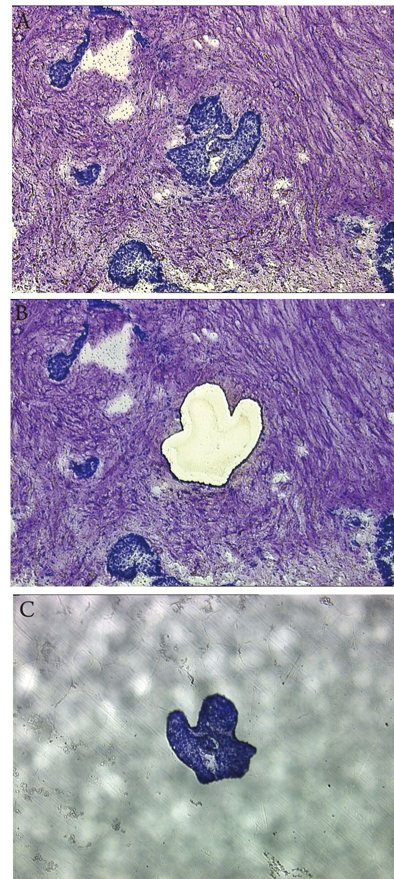

Fig 1 A typical procedure of microdissection for lung cancer cells

A: Before cutting the cancer cells; B: After cutting the cancer cells; C: the dissected cancer cells.

analyses of gene expression profiles could reveal the identify of genes involved in malignant transformation, progression, and/or metastases of tumors.

Tumor consists of mixed populations of carcinoma cells and host cells. It is imperative to study the gene-expression profile of each population consisting of tumor tissue to clearly understand the molecular changes in the procedure of metastasis. The cDNA microarray method is now widely used to analyze expression of thousands of gene simultaneously in cancer tissue and so on. But some of the gene expression differences that distinguished primary and metastatic tumor were contributed by the small amount of contaminating nonneoplastic lung tissue present in the primary tumor samples[12]. As expected, microdissection of tumor tissues can provide stroma-free tumor material in small amounts that are adequate for the molecular genetic analysis of particular mechanisms responsible for HLA class I alterations[13].

One of the challenges of LMD is to obtain sufficient amount of RNA from captured cell populations. Total RNA (10 μg) from 50 000 cells was reported to be the detection limit for direct-labeling approaches[14]. The most critical hurdle is to

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Tab 2 List of genes with altered expression between primary lung squamous cancer with lymphatic metastasis (TxN+) and without lymphatic metastasis (TxN-)

Systematic name	Symbol	Description	Classification of molecular function	Ratio
52277_A	HKR3	GLI-kruppel family member HKR3	Nucleic acid binding	0.433
41853_A	TK2	Thymidine kinase 2, mitochondrial	Enzyme	0.357
323346_C	RGS3	Regulator of G-protein signaling 3	Enzyme; Signal transducer	0.283
40266_A	FRAP1	Fk506 binding protein 12-rapamycin associated protein 1	Cell cycle regulator; enzyme; nucleic acid binding	0.472
299994_A	TNNI3	Troponin I, cardiac	Unclassified	0.401
33803_A	MLH3	Mut L homolog 3 (E.coli)	Nucleic acid binding	0.239
365687_B	IL18BP	Interleukin 18 binding protein	Unclassified	0.390
282055_A	PHKA2	Phosphorylase kinase, alpha 2 (liver)	Enzyme	2.018
28488_A	HIP2	Huntingtin-interacting protein 2	Unknown	0.371
669500_A	HIPK1	Homeodomain interacting protein kinase 1	Enzyme; nuleic acid binding	0.156
262407_A	NPC2	Niemann-Pick disease, type C2	Unclassified	0.355
38829_A	PTPRR	Protein tyrosine phosphatase, receptor type, R	Enzyme; structural protein	2.774
813179_A	IGF1	Insulin-like growth factor 1 (Somatomedin C)	Nucleic acid binding; signal transducer	2.518
267950_A			Unknown	0.462
33182_C			Unknown	0.197
41984_A	G3BP2	Ras-GTPase activating protein SH3 domain-binding protein 2	EST	3.377
1839895_A	PSMB2	Proteasome (prosome, macropain) subunit, beta type, 2	Ubiquitin	2.970
280890_A	EXT2	Exostosin 2	EST	0.173
457710_A	SV2A	Synaptic vesicle glycoprotein 2A	Transporter	0.196
297676_A	FBLN2	Fibulin 2	Structural protein	0.309
428420_B			Unknown	0.261
34313_A	OGT	O-linked N-acetylblucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase)	Enzyme	0.211
40608_A	HCRTR1	Hypocretin (orexin) receptor 1	Signal transducer; structural protein	0.262
324225_B	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	Unclassified	0.258
295568_A	HIP2	Huntingtin interacting protein 2	Enzyme; nucleic acid binding; ubiquitin	0.300
40699_A	PPP2R2B	Protein phosphatase 2, regulatory subunit B, beta	EST	0.203
343142_A	MGC40157	Hypothetical protein MGC40157	Tumor suppressor	2.725
741459_A	ATP5G3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C (subunit 9) isoform 3	Enzyme; transporter	0.215
1874367_A	CCL20	Chemokine (C-C motif) ligand 20	Immunity protein; signal transducer	0.336
470031_A	PLA2G2A	Phospholipase A2, group ILA (platetets, synovial fliud)	Enzyme; signal transducer	0.140
346956_A	APOC1	Apolipoprotein C-I	Transporter	0.210
788591_A	LTBP1	Latent transforming growth factor beta binding protein 1	Signal transducer; structural protein	0.177
51532_A	ARL6IP	ADP-ribosylation factor-like 6 interacting protein	EST	2.042
46100_B	KIAA0753	KIAA0753 gene product	Unknown	0.351
26888_A	CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	Chaperone; enzyme; structural protein	2.506
29786_A	SON	SON DNA binding protein	Apoptosis regulator; nucleic acid binding	0.278
111974_B	HMGCS1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1(soluble)		0.218

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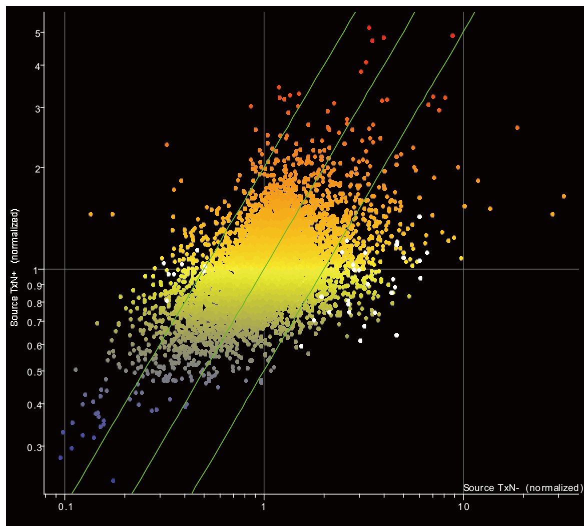

Fig 2 A scatter plot of microarray data analysis between TxN+ and TxN- in patients with primary lung squamous cancer. The differentially expressed genes remained as white, containing genes whose difference in expression between TxN+ and TxN- condition is statistically significantly. The remaining genes do not meet *one-way ANOVA* test.

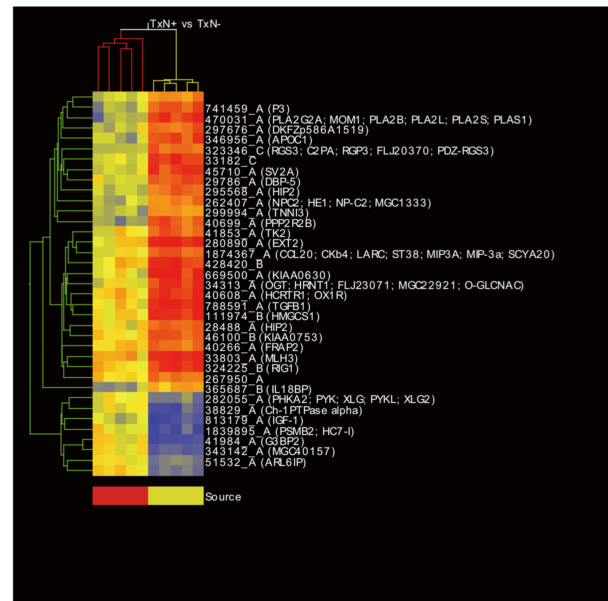

Fig 3 A gene/condition tree of samples from TxN+ or TxN- condition in patients with primary lung squamous cancer. Data are presented in a matrix format: each row represent a gene on the microarray and each column an individual mRNA sample. The results presented represent the ratio of hybridization of fluorescent cDNA probes prepared from each experimental mRNA sample to a reference mRNA sample. These ratios are a measure of relative gene expression in each experiment samples. All TxN+ samples are represented by a red branch and red block, all TxN- samples by yellow branch and block.

establish an unbiased global amplification procedure. However PCR is unsuitable to globally amplify the entire transcriptome of cells[15]. Currently, most microarray amplification methods make use of a linear-based amplification method using T7RNA polymerase, resulting in an amplified RNA (aRNA) that can be used for hybridization analysis[16]. Most microarray studies that utilize the aRNA synthesis protocol require one amplification round. Due to the small amount of cellular material in this study, we performed a second round of amplification for all the microdissected cells and used the antisense RNA for hybridization analysis successfully. It was reported, at this level of amplification, the transcriptome of a single cell can be analyzed[17].

Expression analysis with cDNA arrays showed distinct profile between TxN+ and TxN- in patients with lung squamous cancer; however there was no differentially regulated genes between N+ and TxN+. This interesting finding supports the notion that some primary lung squamous cancer are pre-configured to metastasize, and that this propensity is detectable at the time of initial diagnosis. We insisted on that acquisition of the metastatic phenotype might not necessarily occur late in the development of lung squamous cancer. Some primary lung squamous cancer may actually start out deadly to begin with. Bernards and Weinberg[18]recently posited that combination of early oncogenic alterations, not specific events that promote metastasis, determine metastatic potential. This might explain why metastasis occurs in some individuals with small, low-stage tumor or metastases could be found in the absence of clinically detectable primary tumor.

Metastasis, the major cause of cancer morbidity and mortality, is a multiple process requiring a variety of genetic alterations in the tumor cells. Comprehensive analysis of gene expression profiles between primary tumors with or without metastasis or a primary tumor and its derived tumors can identify differential genes associated with the metastatic phenotype, which help to elucidate the molecular mechanism of cancer metastasis[19]. In the study, comparison of primary lung squamous cancer with/without lymphatic metastasis revealed 37 significant alterations in the expression of metastatic-related genes, including up-regulation of IGF1, PTPRR and down-regulation of PLA2G2A, FBLN2 as well as CCL20. IGF1 may promote invasiveness in lung cancer cell through their ability to induce not only cell migration, but also by enhancing the expression and activity of matrix degrading MMP-2 and MMP-9[20]. For the patients with lung cancer, the serum IGF1 level was higher in cases with lymphatic metastasis[21]. The circulating IGF-1 levels play an important role in colon cancer development and metastasis[22]. PTPRR (protein tyro-

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sine phosphatase, receptor type, R) was strikely up-regulated in highly metastatic mouse hepatocarcinoma cell lines, suggesting it is key gene responsible for lymphatic metastasis[23]. PLA2G2A, a gene previously implicated as a modifier of the Apc (Min/+) (multiple intestinal neoplasia 1) mutant phenotype in the mouse, may suppress progression or metastasis of human gastric cancer[24]. As an important regulator of invasion and metastasis, the invasive ability of PLA2G2A-expressing AGS cells was enhanced by PLA2G2A silencing, whereas cellular migrate in non-PLA2G2A-expressing N87 cells was inhibited by enforced PLA2G2A expression, indicating that PLA2G2A is both necessary and sufficient to function as an inhibitor of gastric cancer invasion *in vitro*. The antiinvasive effect of PLA2G2A occurs, at least in part, through its ability to inhibit the S100A4 metastasis mediator gene. Consistent with its invasion inhibitor role, PLA2G2A expression was elevated in metastatic and late-stage tumors[25]. According to Wiese *et al*[26], FBLN2 was associated with stromal cells. In our study, the gene was down-regulated in TxN+ group. We speculated that this gene is related to stroma-tumor interactions in lung cancer. Expression of the chemokine receptor CCR6, associated with the local production of CCR ligand, CCL20, decressed the metastatic potential of the Lewis lung carcinoma cell line, indicating CCL20 is an important regulator of metastasis[27]. Interestingly there are no de-regulated genes in comparison of primary lung squamous cancer and its derived tumors in regional lymph nodes.

Lymph-node metastasis is a key step in tumor progression and a risk factor for recurrence after surgery. These metastasis-associated genes might serve as molecular marker for lymphatic metastasis, which may provide clues to reveal patients with increase risk of developing metastasis, and to identify novel therapeutic targets for the treatment of metastasis.

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