RESEARCH ARTICLE

Screening of Differentially Expressed Genes among Various TNM Stages of Lung Adenocarcinoma by Genomewide Gene Expression Profile Analysis

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Abstract

Background: To further investigate the molecular basis of lung cancer development, we utilize a microarray to identify differentially expressed genes associated with various TNM stages of adenocarcinoma, a subtype with increasing incidence in recent years in China. Methods: A 35K oligo gene array, covering about 25,100 genes, was used to screen differentially expressed genes among 90 tumor samples of lung adenocarcinoma in various TNM stages. To verify the gene array data, three genes (Zimp7, GINS2 and NAG-1) were confirmed by real-time RT-PCR in a different set of samples from the gene array. Results: First, we obtained 640 differentially expressed genes in lung adenocarcinomas compared to the surrounding normal lung tissues. Then, from the 640 candidates we identified 10 differentially expressed genes among different TNM stages (Stage I, II and IIIA), of which Zimp7, GINS2 and NAG-1 genes were first reported to be present at a high level in lung adenocarcinoma. The results of qRT-PCR for the three genes were consistent with those from the gene array. Conclusions: We identified 10 candidate genes associated with different TNM stages in lung adenocarcinoma in the Chinese population, which should provide new insights into the molecular basis underlying the development of lung adenocarcinoma and may offer new targets for the diagnosis, therapy and prognosis prediction.

Keywords: Lung adenocarcinoma - TNM stage - gene expression profile - differential gene expression

Asian Pac J Cancer Prev, **14 (11)**, 6281-6286

Introduction

Lung cancer is one of the most common malignancies with high incidence and mortality worldwide. The 5-year survival is 15% for Americans and 10% for Europeans, while it is 8.9% in developing countries. In China, there are about 400,000 new cases and 360,000 deaths of lung cancer per year, accounting for one third of the overall population of this cancer worldwide (Parkin et al., 2002). Although there has been exposure about some mechanisms underlying the development of lung cancer, it is far from what is needed to significantly improve upon the current diagnosis and treatment practices. Therefore, it is crucial that more lung cancer-related genes be identified to provide new markers or targets for the diagnosis, treatment and prognosis prediction of this disease.

Gene array, as a powful tool for the gene expression profile analysis of the whole genome, becomes a preferred strategy to quickly identify the differentially expressed genes in cancers. It has been widely used to identify the tumor-associated genes in various types of cancer including lung carcinomas. Most of the gene array-based expression studies of lung cancer, however, have ignored the differences of patients in clinical features, for example the Tumor-Node-Metastasis (TNM) stages of the cancer (Hofmann et al., 2006; Singhal et al., 2008). If we can identify the differentially expressed genes in lung cancers at different clinical status, it may provide new opportunities to improve the diagnosis and treatment of this cancer.

The main histological type of lung cancer is non-small cell lung carcinomas (NSCLC) which is composed of squamous cell carcinoma, adenocarcinoma and large cell lung cancer. Based on our clinical practice and published data in Chinese, lung adenocarcinoma, which is an aggressive paradigm with a high chance of relapse and early metastasis, has been on the rise as one of the most common subtypes of lung cancer in China, accounting for

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30%-40% of the overall cases.

In this study, we used a 35K Oligo gene array to identify the differentially expressed genes associated with different TNM stages in lung adenocarcinomas. Our results may provide new information to decode the molecular pathogenesis of lung cancer, and may offer new targets for the diagnosis, treatment and prognosis assessment of this cancer.

Materials and Methods

Specimens

Lung adenocarcinoma specimens were collected from total of 240 patients receiving thoracic surgery in our hospital. All the patients were primary cases without any history of other malignancies and chemo- or radiotherapies. In addition to the cancer tissues, the surrounding normal lung tissues were also collected as the control. A 1.0 cm3 volume of tissue was further cut into 0.5 cm3 blocks which were then put into 5-fold volumes of RNAlater. After freezing for 30 minutes in liquid nitrogen, the tissues were kept at -80°C for later use. Part of each cancer tissue was prepared for routine H&E staining to confirm the percentage of cancer cells. Only the samples, in which the proportion of cancer cells was more than 80%, were used to prepare RNA. The specimens were reviewed pathologically to confirm the diagnosis of lung adenocarcinoma. There were 90 samples (30 each for stage I, II and IIIA) for the gene array, and 150 samples (50 each for stage I, II and IIIA) for the further validation by qRT-PCR. The protocol for this study has been approved by the Ethics Committee of South West Hospital and it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). Informed consent has been obtained from all the study subjects.

Preparation of total RNA

The total RNA from each tissue sample was extracted with a TripureTM Isolation Reagent kit from Ambion Inc., according to the instructions. The RNA samples were purified with a Nucleospin RNA clean-up kit from MN Inc. and the manual instructions were followed. The concentration and purity of the RNA samples were assayed with a Nanodrop spectrophotometer. The integrity of the RNA was confirmed by electrophoresis of RNA in agarose gels containing formaldehyde.

Gene array hybridization

35K Oligo gene array: The Jingxin® 35K Oligo gene array (CapitalBio Inc.) included 35,000 70-mer oligonucleotide probes (human genome-wide Oligo library-- human_V4.0 from Operon Inc.), covering about 25,100 genes.

Fluorescent-labeling of RNA samples: The Jingxin® cRNA linear amplification and labeling kit (CapitalBio) was used. The RNA was first reverse transcribed to 1st-strand cDNA which was then used as a template to synthesize the 2nd-strand cDNA. The 2nd-stand cDNA was used as a template to synthesize the cRNA with T7 Enzyme Mix. Again, the cRNA was reverse transcribed to cDNA with Random Primer. The purified cDNA was used as the template to synthesize the fluorescence-labeled complimentary DNA chain with Klenow enzyme. Cy5 dCTP and Cy3 dCTP were used to label the test sample (tumor or surrounding normal tissues) and Universal Human Reference RNA, respectively. The final labeled products were purified with PCR NucleoSpin Extract II Kit (MN Inc.). A Nanodrop spectrophotometer was used to determine the concentration and labeling efficiency of the products. The fluorescent intensities of Cy3 and Cy5 should be comparable.

Hybridization: The labeled products were added to the gene arrays after being mixed with a hybridization buffer. Then the arrays were incubated in the CapitalBio® BioMixer™ II microarray hybridizing incubator overnight at 42°C. After hybridization, the gene arrays were washed and scanned with CapitalBio® LuxScan™ 10K-A scanners. The data was extracted and analyzed with LuxScan 3.0, BoaoAnalyzer6_step1.pl and BoaoAnalyzer6_step2.pl software products, and then the fluorescent intensity was normalized before analysis.

Identification of differentially expressed genes

The genes differentially expressed between tumor tissues of lung adenocarcinoma and surrounding normal lung tissues, and those between tumors at various TNM stages, were analyzed with the significance analysis of microarrays (SAM) software.

qRT-PCR

To verify the data from gene array, we chose Zimp7, GINS2 and NAG-1 candidates to be confirmed by real-time RT-PCR in a different set of tumor samples from gene array assay. There were 50 samples each for stage I, II and IIIA, respectively. The primers were designed and synthesized by TaKaRa Inc. (Table 1). GAPDH was used as the internal control (from Takara). The amplification was performed according to the instructions outlined in the SYBR® Premix Ex TaqTM Real Time RT-PCR Kit manual (TaKaRa Inc.). The 25μl volume of amplification included 1μl cDNA, 0.5μl forward and reverse primers each, 12.5μl 2×SYBR Premix, 0.5μl ROX Reference Dye I and 10μl dH_2O. Each sample repeated amplification three times. The reactions were run on Rotor gene 6300 and conditions were as follows: predenaturation at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 52.5°C for Zimp7, 59°C for GINS2 and 62°C for NAG-1 for 15 s, 72°C for 20 s. The relative expression was quantified with the double delta Ct method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Table 1. Forward and Reverse Primers of Zimp7, GINS2 and NAG-1 Genes for Real-time PCR Amplification

Primer	Sequence (5'→3')
Zimp7 Forward	CGGGTCACCATTTCCTCCAGTC
Zimp7 Reverse	GGGGCAACGCTCACACCAGATAC
GINS2 Forward	CAGACGAATGGCATGGCTTTTAC
GINS2 Reverse	GCGGGTGCTCTTAGGCTCTC
NAG-1 Forward	GCCCGCCAGCTACAATCC
NAG-1 Reverse	GGCAGGAATCGGGTGTCTCA

Table 2. 10 Differentially Expressed Genes Identified in This Study to Be Associated with Different TNM Stages of Lung Adenocarcinoma

Symbol	GB. accession	Description	Fold change	Contrast-1	Contrast-2	Contrast-3
DUSP1	NM_004417	Dual specificity protein phosphatase 1	0.41↓	1.91	-1.5	-0.3
Zimp7	NM_031449.3	human zinc finger-containing, Miz1, PIAS-like protein on chromosome 7	2.39↑	2.66	-1.3	-1.01
EST	NP_937824	34 kDa protein	2.36↑	2.16	-1.52	-0.47
EST	XM_498632	hypothetical gene supported by BC022385; BC035868; BC048326	3.67↑	2.1	-1.18	-0.2
GINS2	NM_016095.2	GINS complex subunit 2	5.44↑	-2.08	2.02	0.04
LY6K	NM_017527.3	lymphocyte antigen 6 complex, locus K	12.96↑	-0.96	1.5	-0.4
NAG-1	NM_004864.2	NSAID activated gene 1	4.81↑	-1.03	0.56	1.64
MAGE-A3	NM_005362.3	Melanoma-associated antigen 3	6.30↑	-1.02	0.87	1.97
MALAT-1	NR_002819.2	Metastasis associated lung adenocarcinoma transcript 1	4.03↑	-1.44	0.74	1.9
MMP12	NM_002426.4	Matrix metalloproteinase-12	5.42↑	-2.08	0.94	2.02

fold change, tumor tissues / normal tissues, fold change ≥2.0 or ≤0.5; ↑↓, up- or down-regulated in lung adenocarcinoma; Contrast-n, the significance of difference between stage I, II and IIIA groups of samples analyzed by the SAM software; GB, Gene Bank

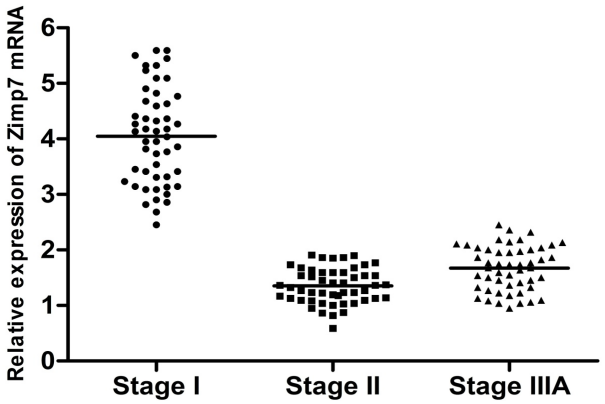

Figure 1. Determination of Zimp7 mRNA Level in 150 Patients with Lung Adenocarcinoma at Different TNM Stages by qRT-PCR. Data was calculated with the $2^{(-\Delta\Delta Ct)}$ formula and analyzed with the ANOVA's LSD test. Horizontal bars indicate median values. Compared with that in stage II and IIIA, Zimp7 was expressed at a significantly higher level in stage I lung adenocarcinoma (both of the *P* values were less than 0.01)

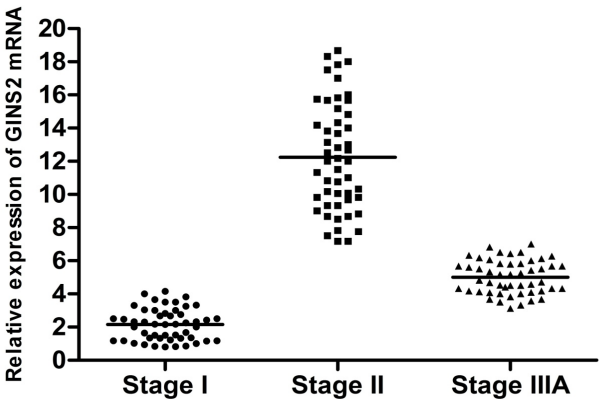

Figure 2. Determination of GINS2 mRNA Level in 150 Patients with Lung Adenocarcinoma at Different TNM Stages by qRT-PCR. Data was calculated with the $2^{(-\Delta\Delta Ct)}$ formula and analyzed with the ANOVA's LSD test. Horizontal bars indicate median values. Compared with that in stage I and IIIA, GINS2 was expressed at a significantly higher level in stage II lung adenocarcinoma (both of the *P* values were less than 0.01)

Statistical analysis

The numbers of Zimp7, GINS2 and NAG-1 transcripts between tumor samples at various TNM stages were compared using ANOVA's least significant difference test using SPSS software v. 18.0, and $p < 0.05$ was considered as significant.

Results

Screening of the differentially expressed genes

A two class unpaired method in the SAM software was adopted to screen the differentially expressed genes between 90 tumor samples of lung adenocarcinoma and their surrounding normal lung tissues. The criteria to define the difference were as follows: (1) q-value <5%; (2) the ratio of the fluorescent intensity of Cy5 to Cy3 (i.e. fold change) >2 or <0.5. Overall, 640 candidate genes were identified to be differentially expressed in tumor tissues compared to normal tissues, among which 289 genes were upregulated and 351 downregulated. Then, we used a multiclass method of the SAM software to identify the differentially expressed genes among tumor samples at different TNM stages (i.e. stage I, stage II and stage IIIA) from the 640 candidates with criteria of: (1) q-value<15%; (2) fold change >2 or <0.5. Finally, total of 10 genes were obtained (Table 2), among which 4 genes were expressed at a higher level in samples of stage I than those of stage II and IIIA (DUSP1, Zimp7, EST NP_937824 and XM_498632), and 2 genes showed a higher expression in stage II than in stage I and IIIA (GINS2 and LY6K), and there were 4 genes with a higher expression in stage II and IIIA than in stage I (NAG-1, MAGE-A3, MALAT-1and MMP12).

Confirming of the expression of certain candidate genes by real-time quantitative RT-PCR

With GAPDH as an internal control, the Ratio (T/N) of the candidate gene transcript between tumor and their matched normal tissues was calculated. If a ratio was over 2, the target gene was considered to be upregulated in the tested tumor sample, compared to its matched normal sample. The results showed that Zimp7, GINS2 and NAG-1 transcripts were upregulated in lung adenocarcinoma, and the mean Ratio(T/N) of the three genes in 150 matched tumor and normal samples were 3.86±2.09, 6.80±4.55 and 10.80±5.61, respectively, with the upregulation positive

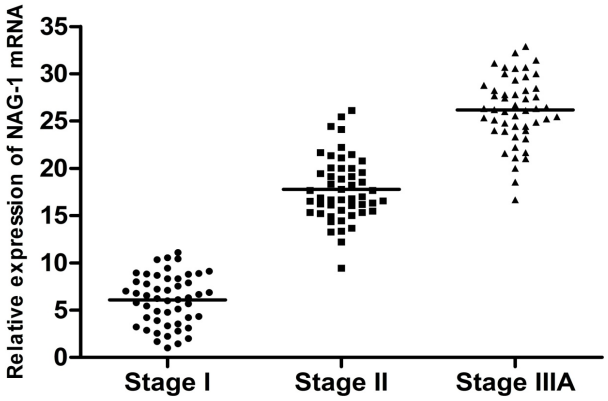

Figure 3. Determination of NAG-1 mRNA Level in 150 Patients with Lung Adenocarcinoma at Different TNM Stages by qRT-PCR. Data was calculated with the $2^{(-\Delta\Delta Ct)}$ formula and analyzed with ANOVA's LSD test. Horizontal bars indicate median values. Compared with that in stage I, NAG-1 expression was significantly increased in stage II and stage IIIA lung adenocarcinoma (both of the *P* values were less than 0.01)

percentages being 85.33% (128/150), 88.67% (133/150) and 96.67% (145/150), respectively. The results of qRT-PCR were consistent with those from the gene array.

The relative expression levels of Zimp7 mRNA in lung adenocarcinoma patients at stage I, II and IIIA were 4.04±0.86, 1.35±0.32 and 1.67±0.40, respectively, and for GINS2, the levels were 2.15±0.95, 12.23±3.27 and 5.01±1.02, respectively; for NAG-1, they were 6.08±2.67, 17.77±3.40 and 26.16±3.55, respectively (Figure 1, 2, 3). Zimp7 showed a significantly higher expression level in stage I than in stage II and IIIA, while GINS2 showed a higher level in stage II than in stage I and IIIA. NAG-1 expression was significantly increased in stage II and IIIA compared to stage I. These results were consistent with those from the gene array.

Discussion

In this study, we totally identified 10 genes differentially expressed among 90 tumor samples of lung adenocarcinoma at different TNM stages (30 cases of each stage) using a 35K Oligo gene array. Of these 10 candidates, DUSP1 was downregulated in lung adenocarcinoma and the other 9 were upregulated.

Compared with stage II and stage IIIA, stage I lung adenocarcinoma was marked by 4 differentially expressed genes, including DUSP1, Zimp7, EST NP_937824 and XM_498632.

Dual-specificity MAP kinase phosphatases 1 (DUSP1), also known as MKP1, negatively regulates the activity of extracellular regulated kinases (ERKs) and mitogen-activated protein kinases (MAPKs). It has been shown that MKP1 expression was progressively reduced with the development of epithelial carcinomas (Loda et al., 1996), and it was one of the 20 most significantly downregulated genes in colorectal cancers (Zhang et al., 1997). Recent studies suggest that the induction of MKP1 could significantly suppress the proliferative and metastatic abilities of NSCLC both in vitro and in vivo. Therefore, MKP1 could be considered a potential therapeutic target in NSCLC therapy (Tai et al., 2010). In our study, we showed that DUSP1 mRNA was significantly downregulated in lung adenocarcinoma, and the levels declined progressively with the development of this cancer, which was consistent with what reported in epithelial carcinomas. We suggested that analysis of DUSP1 expression might be useful to evaluate the disease extent and prognosis of lung adenocarcinoma.

Zimp7 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 7), also named Zmiz2, is a novel PIAS-like protein and functions as a transcriptional co-activator. Transient transfection into prostate epithelial cells showed that hZimp7 augments the transcriptional activity of the androgen receptor (AR) which was known to be important in the survival of prostate cancer cells (Huang et al., 2005). Here, we first reported the higher expression of Zimp7 mRNA in stage I lung adenocarcinomas. Whether the consequence of its high expression is also to augment the nuclear hormone receptor-regulated transcription similar to that in prostate cancer awaits further study.

Moreover, we identified 2 more genes bearing unknown functions which were expressed at higher levels in stage I lung adenocarcinomas: EST NP_937824 and XM_498632. Their potential value in the early diagnosis of lung adenocarcinoma would be further evaluated.

Compared to stage I and IIIA, stage II lung adenocarcinoma was marked by 2 differentially expressed genes: GINS2 and LY6K.

GINS complex subunit 2 (GINS2) is a member of the tetrameric complex termed GINS, composed of GINS1, GINS2, GINS3, and GINS4, which most likely serves as the replicative helicase. As it has been reported that DNA replication–associated proteins have diverse functions in different cells, GINS components, especially GINS2, has been recently suggested to have a function in cell division, more precisely in choromosome segregation in cancer cells (Hanissian et al., 2004). A high level of GINS2 expression was found among metastasizing breast tumors. Bioinformatic analysis of published gene expression and DNA copy number studies of clinical breast tumors suggested GINS2 to be associated with the aggressive characteristics of a subgroup of breast cancers in vivo (Thomassen et al., 2009). In our study, GINS2 mRNA was observed to be significantly highly expressed in stage II lung adenocarcinoma for the first time. Together with its expression in breast cancer, GINS2 is speculated to be a potential metastasis-promoting gene, and its overexpression may also participate in lung adenocarcinoma metastasis.

Lymphocyte antigen 6 complex locus K (LY6K) is a cancer testis antigen located on chromosome 8q24.3. Gene expression profile analyses of NSCLC revealed that LY6K was specifically expressed in testis and transactivated in a majority of NSCLCs. Immunohistochemical staining confirmed that LY6K overexpression was associated with poor prognosis for NSCLC (Nobuhisa et al., 2007). Cell viability assays demonstrated that significant inhibitions of cell growth, migration, and invasion occurred in LY6K knockdown bladder cancer cell lines (Matsuda et al., 2011). Our results showed that LY6K

transcript was significantly overexpressed in stage II lung adenocarcinomas, suggesting that upregulation of the LY6K gene may contribute the development of this cancer, and thus LY6K could be used as a potential prognosis predictor for lung adenocarcinoma, as previously reported in NSCLC.

Compared with stage I, there are a total of four genes, inlcuding NAG-1, MAGE-A3, MALAT-1 and MMP12, to be significantly upregulated in stage II and IIIA.

The NSAID activated gene 1 (NAG-1) was identified as a divergent member of the TGF-β superfamily. Reports of NAG-1 expression in tumors have been inconsistent. Kim (2002) has shown by immunohistochemistry that expression of NAG-1 is downregulated in colon tumors. No expression of NAG-1 was found in squamous metaplastic tracheal epithelium, whereas positive expression was observed in the normal tracheobronchial epithelia (Newman et al., 2003). In contrast, high expression of NAG-1 is also frequently observed in tumors. NAG-1 was highly expressed in melanoma, and the metastatic melanoma biopsies had strong expression of MIC-1 compared to the primary melanoma biopsies (Boyle et al., 2006). David (2003) reported that NAG-1 was markedly up-regulated in colorectal cancers, and serum NAG-1 levels were higher in patients with higher TNM stage. The apparent dichotomy in NAG-1 expression in tumors raises the possibility that NAG-1 plays distinctly different roles at different stages of tumor progression: suppression of tumorigenesis at the early stages of cancer development, and promotion of tumor invasiveness and survival at more advanced stages of disease. This is not unexpected since NAG-1 is a member of the TGF-β superfamily, but an explanation for this change in biological activity is not clearly understood at the present time. Our data first revealed that NAG-1 transcript was upregulated in lung adenocarcinomas in contrast to no expression of it reported previously in squamous metaplastic tracheal epithelia, and its expression level was markedly increased with the progression of the disease similar to that in colorectal cancer reported by David et al. (2003). Thus it may be used as a potential marker to asses the prognosis for lung adenocarcinoma.

Melanoma associated antigen-A3 (MAGE-A3) is a cancer testis antigen expressed in cancer cells but not in normal tissues. It is a promising target for anticancer immunotherapy because it is exclusively presented on the cell surface of cancer cells and might be associated with an aggressive cancer phenotype. Sienel (2004) compared the expression of MAGE-A3 in Stage I and Stage II NSCLCs, and found that in comparison to Stage I, the rate of MAGE-A3 positive tumors was significantly increased in Stage II, in agreement with its possible role in the tumor metastasis. Consistent with what reported, we found that the expression of MAGE-A3 mRNA was significantly increased in lung adenocarcinomas at stage II and IIIA compared with stage I. MAGE-A3 may also be a candidate target for the immunotherapy of lung adenocarcinoma.

Metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) is a novel non-coding RNA of more than 8000 nt expressed from chromosome 11q13. Short-interfering RNA-mediated MALAT-1 silencing impaired in vitro cell motility of lung adenocarcinoma cells (Tano et al., 2010). MALAT-1 was demonstrated to be significantly associated with metastasis in NSCLC patients, and can be used as prognosis-predicting parameters in early-stage NSCLC (Ji et al., 2003). We showed that the expression of MALAT-1 was significantly higher in stage II and IIIA than in stage I lung adenocarcinoma, which was consistent with the published data. Our results demonstrated again the important value of MALAT-1 in the prediction of metastasis and prognosis of lung adenocarcinoma.

MMP-12, a member of the matrix metalloproteinases (MMPs) family, is also termed as macrophagic elastase, whose substrates include elastin, type IV collagen and fiber junction protein. Much has been reported regarding the roles of MMPs in the invasion and metastasis of different cancers (Nabeshima et al., 2002). We had ever compared the MMP-12 mRNA levels between cancer tissues and matched surrounding normal tissues, between TNM stage I and stage II/III, as well as between tumors with lymph node metastasis and without, in cases of NSCLC. The data showed that MMP-12 was present in all the tested cancer tissues but not in normal tissues. There was a significantly higher expression level in stage II/III than in stage I, and a higher level in cancers with lymph node metastasis than those without. Consistent with what reported, this gene array based study of TNM stages in lung adenocarcinoma further confirmed the role of MMP-12 in the metastasis of NSCLC and the value as a potential marker for prognosis prediction.

In summary, we established an association of the genome-wide gene expression profiles with lung adenocarcinoma at different TNM stages in the Chinese population, giving a preliminary study of the TNM stage-related genes in lung adenocarcinomas. A majority part of the 10 genes obtained in this study have been reported in lung cancer, and our results further demonstrate their essential roles in the development of lung adenocarcinoma. The other three genes that have never been reported previously in lung cancer however, have been suggested to be involved in cancers at different levels. Further revelation regarding the roles of these genes in lung cancer would be of great value.

Acknowledgements

This work was supported by two grants from the Chongqing Local Natural Sciences Foundation of China (2007AC5067 and 2010BB5192). The author(s) declare that they have no competing interests.

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