Genome-wide cDNA microarray screening of genes related to survival in patients after curative resection of non-small cell lung cancer

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Abstract. We conducted a study to determine whether the expression levels of genes in tumors were correlated with the survival of patients after complete resection of non-small cell lung cancer (NSCLC). The expression levels of 1176 genes in resected tumor specimens from 28 patients were analyzed using the Atlas™ Human Cancer 1.2 Array. The pathological stages of the resected tumors were I, II and III in 14, 5 and 9 patients, respectively. We compared the differences of gene expression between patients who survived (n=12) and those who died (n=16). The expression levels of cyclin-dependent kinase 8, phosphoinositide-3-kinase, interferon regulatory factor 3 and tubulin were significantly higher in the tumors of surviving patients with stage I lung cancer ($p<0.01$). The expression levels of 12 genes, including the interferon-stimulated genes, were significantly higher in surviving patients with stage II or III lung cancer ($p<0.01$). Stepwise multivariate regression analysis revealed that 4 and 12 genes in stage I and stage II/III cancers, respectively, were independent prognostic factors ($p<0.01$). In conclusion, these survival-related genes are considered to be possible targets of adjuvant therapy after surgical resection of NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in Japan. To improve the prognosis of patients with NSCLC, attempts have been made to develop tests that will facilitate early diagnosis and treatment, and thereby decrease patient mortality. Although many chest roentgenogram-negative lung cancers can be detected by chest computerized tomography, a significant number of patients with early-stage disease show aggressive tumors. Although locoregional control of NSCLC can be achieved by surgery, more than 70% of relapses in patients with stage I disease occur at distant sites (1). Thus, most patients with NSCLC have systemic disease, even at the earliest stage. Recent efforts at improving the management and outcome of patients with this disease have been directed at induction and adjuvant chemotherapy to reduce the high systemic relapse rate.

Cancer cells with genetic alterations at the early stage are considered to subsequently acquire other gene alterations, resulting in the progression to locally advanced or metastatic tumors. Many genetic alterations related to cell proliferation, apoptosis, vascularization and tumor invasion have been reported to be prognostic factors in resected NSCLC. A study comparing the survival of 72 patients with small adenocarcinomas of the lung according to the expression of individual genes found that the overall survival of patients showing positive expression of survivin, cyclin D1 and integrin ß1 was significantly worse than that of patients whose tumors did not express these genes (2). It was concluded that multiple, but not single, oncogene expression in tumor cells is an indicator of poor prognosis in patients with this type of cancer. However, no previous study has investigated the gene alterations that mostly influence tumor progression and metastasis in early-stage NSCLC. Clarification of the gene alterations that influence tumor progression from the early to the advanced stage in NSCLC is required when considering new therapeutic strategies for patients with resectable tumors. Some treatments directed against molecular targets in NSCLC are currently undergoing clinical trials. Gefitinib is a molecular target drug that suppresses tyrosine kinase of the EGFR receptor, and is active in patients with EGFR mutation in lung cancer cells (3,4). Also, a randomized study showed that bevacizumab, an antibody against VEGF, prolongs overall survival significantly in patients with non-squamous cell carcinoma treated with paclitaxel plus carboplatin (5). Unfortunately, however, these molecular targeting drugs have been shown to be ineffective in patients after resection of NSCLC. Moreover, the key molecular targets in patients after resection of NSCLC have not been clarified. Using the cDNA microarray technique on samples of advanced or metastatic NSCLC, we previously identified three independent genes, each of which is correlated with tumor chemoresistance and patient survival (6,7). Therefore, in the present study, we investigated which cancer-related genes influence the prognosis of patients after

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curative resection of NSCLC using the cDNA microarray method. We compared the differences in gene expression between patients who survived and those who died due to tumor relapse and progression.

Patients and methods

This study was approved by the Institutional Review Board of Kanagawa Cancer Center.

Patients. Patients with histologically proven lung cancer who underwent curative surgical resection of their lung tumors were entered into the present study. None of the patients had received prior chemotherapy for the primary lesion. Written informed consent for genetic analysis of their tumor tissue was obtained from all the patients.

Tumor samples. Specimens of tumors were obtained just after surgical resection and frozen immediately for storage at -80˚C until genetic analysis. The other resected tumors were fixed in formalin for pathological diagnosis.

Extraction and purification of RNA and preparation of probes. The total RNA of each sample was isolated and treated with DNase I to avoid contamination with genomic DNA by silica membrane affinity chromatography using a total RNA isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). One hundred nanograms of total RNA from each sample was reverse-transcribed into cDNA and amplified by polymerase chain reaction (PCR) using a Super SMART™ PCR cDNA synthesis kit (BD Biosciences Clontech, CA, USA) according to the manufacturer's instructions. Each cDNA sample was subjected to microarray expression profiling using the BD Atlas™ Human Cancer 1.2 Array (Clontech) based on the manufacturer's protocol described previously (6,7).

cDNA microarray. Each labeled probe was then hybridized into a separate Atlas Array. The signal intensity for each spot, corresponding to each gene examined, was determined using a STORM image analyzer (Amersham Bioscience, Picataway, NJ, USA). The hybridization pattern and signal intensity were analyzed to determine the changes in gene expression levels using AtlasImage™ 2.01 software (Clontech, Laboratory, Inc., Japan).

Statistical methods. Differences in the mean levels of gene expression between patients who survived and those who died were examined by the t-test. The influence of the expression of each gene on patient survival after resection was examined by multivariate analysis. Differences at $p<0.01$ were considered significant.

Results

Twenty-eight patients among those who underwent a lobectomy with mediastinal lymph node resection for NSCLC between February 2000 and October 2001 gave approval for their resected tumor tissue to be used for this study. Nineteen of the patients were male and nine were female, with a median age of 70 years (range 35-85 years). Seventeen patients had adenocarcinoma, and the others squamous cell carcinoma. The pathological stages of the resected tumors were I, II and III in 14, 5 and 9 patients, respectively.

Table I. Patient characteristics.

	No. of patients
Total	28
Age (years)	
Mean 70	
Range 35-85	
Gender	
Male	19
Female	9
Pathology	
Adenocarcinoma	17
Squamous cell carcinoma	11
Stage (pathological)	
IA	9
IB	5
IIB	5
IIIA	6
IIIB	3

The expression levels of 1176 genes in the resected tumor specimens were analyzed using cDNA microarray screening. Six housekeeping genes that were expressed in all 28 tumor samples were used as controls for gene expression: Ubiquitin C, liver glyceraldehyde 3-phosphate dehydrogenase, major histocompatibility complex class I C, cytoplasmic beta-actin, 60S ribosomal protein L13A, and 40S ribosomal protein S9.

Among the patients, 6, 4 and 6 patients with stage I, II and III disease died, respectively. We then compared the differences in gene expression between the patients who died and those who survived. The gene expression levels of cyclin-dependent kinase 8, phosphoinositide-3-kinase, interferon regulatory factor 3 and tubulin were significantly higher in the tumors of patients who survived after resection of stage I lung cancer (t-test, $p<0.01$; Table II). Only the expression of the thymine-DNA glycosylase gene was significantly higher in the tumors of patients with stage I lung cancer who subsequently died ($p<0.01$). The expression levels of 12 genes, including the interferon-stimulated genes, were significantly higher in patients with stage II or III lung cancer who survived after tumor resection (t-test; $p<0.01$; Table III). None of the genes showed a higher expression in the tumors from patients who subsequently died. Stepwise multivariate regression analysis revealed that 4 and 12 genes in stage I and stage II/III tumors, respectively, were independent prognostic factors.

Table II. Genes closely associated with survival of patients in stage I.

Description	Alive (n=8)	Dead (n=6) (mean±SE)	P-value
Cyclin-dependent kinase 8	6.5±1.7	0.3±0.3	0.0093
Phosphoinositide-3-kinase, class 3	18.5±4.0	0.8±0.7	0.0026
Thymine-DNA glycosylase	0.4±0.3	2.0±0.4	0.0061
Interferon regulatory factor 3	14.9±2.7	3.2±1.0	0.0037
Tubulin, alpha, ubiquitous	36.0±3	20.0±2.4	0.002

Table III. Genes closely associated with survival of patients in stage II and III.

Description	Alive (n=5)	Dead (n=9) (mean±SE)	P-value
Cyclin-dependent kinase (CDC2-like) 10	16.0±4.8	3.9±1.4	0.0094
Zinc finger protein 173	12.0±3.3	3.1±1.1	0.008
Topoisomerase (DNA) III alpha	15.8±4.4	1.7±1.3	0.0023
Proliferating cell nuclear antigen	15.0±4.9	2.6±1.0	0.0065
Activin A receptor type II-like 1	16.2±4.7	3.1±1.2	0.0047
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	16.8±5.3	2.7±1.1	0.0049
Interferon gamma antagonist (IFN-gamma antagonist)	8.8±2.9	0.8±0.8	0.0049
Insulin-like growth factor I (IGF1); somatomedin C	20.2±8.3	0.7±0.7	0.0072
Interferon-stimulated gene (20 kD)	36.6±8.6	3.0±1.9	0.0003
Ste20-related serine/threonine kinase	11.8±3.6	1.4±1.0	0.004
Dihydroorotate dehydrogenase	14.8±5.3	2.1±1.1	0.0093
Eukaryotic translation initiation factor 2 alpha subunit (EIF2-alpha)	13.2±4.4	1.6±1.0	0.0054

Discussion

We analyzed the differences in the expression of cancer-related genes in the resected specimens of NSCLC between patients who survived and those who died due to cancer relapse and progression. We previously reported some genes whose expression levels in the tumor specimens obtained by transbronchial biopsy were correlated with the survival of patients who received chemotherapy (7). In both studies, such genes included cyclin-related genes and protein kinase. In the present study, for every gene that showed a significant difference in expression, except for thymine-DNA glycosylase, the expression was higher in tumor tissue from patients who survived than in patients who died. Among these survival-related genes, cyclin-dependent kinase and interferon-related genes were commonly expressed in early (stage I) and advanced (stage II and III) cancers. Most of these survival-related genes showed a higher expression in patients who survived, suggesting that these genes may suppress tumor progression. The cell cycle regulator cyclin-dependent kinase and intracellular signaling factor phosphoinositide-3-kinase play an important role in tumor progression. Tubulin is a target of some anticancer drugs, and its expression is reported to be a prognostic factor in patients receiving vinorelbine therapy (8). Interferon regulatory factor 3 has been shown to regulate the host immune system and apoptosis via inhibition of NF-kappaB activity (9). The present study suggests that this factor has an inhibitory effect against lung cancer progression. Thymine-DNA glycosylase interacts with the mismatch repair protein and is implicated in the suppression of neoplasia (10). The expression of this gene was significantly higher in patients who died, but relatively low in comparison with other survival-related genes, so the difference might only be coincidental.

Eukaryotic translation initiation factor 2α is a heat-shock protein whose phosphorylation is necessary to inhibit protein synthesis initiation in arsenite-treated cells and is essential for stress granule formation (11). This anti-stress effect may suppress tumor progression or metastasis. Azadihydroorotate-ethylester and azadihydroorotate-hydrazide, formed by the dehydrogenation of dihydroorotate analogues by dihydroorotate dehydrogenase, inhibit the growth of Lewis lung carcinoma (12). A potent inhibitor of dihydroorotate dehydrogenase exerts tumoricidal effects by inhibiting a step in *de novo* pyrimidine biosynthesis (13). Akt, a serine/ threonine protein kinase, mediates growth factor-associated cell survival. An examination of surgically resected NSCLC demonstrated that phosphorylated Akt is a significant independent factor associated with favorable prognosis (14). It has also been demonstrated that interferon-

transfected tumor cells stimulate a high level of nitric oxide production, which is correlated with vigorous non-specific antitumor activity (15). Another study has shown that intravenous injection of lipoplexes containing plasmids, regardless of interferon gene insertion, results in a significant reduction of lung metastatic nodules, probably due to the action of proinflammatory cytokines such as TNFα and interferon-γ (16). Insulin-like growth factor-I modulates cell growth and survival, and is thought to be important for tumor development. An animal study demonstrated that tumor incidence and growth rate were markedly reduced in mice inoculated with H-59 murine lung carcinoma cells expressing the receptor for type 1 insulin-like growth factor (17). However, insulin-like growth factor-1 is known to promote NSCLC survival, and its cooperation with amphiregulin inhibits bax- and bad-mediated apoptosis via the protein kinase C-dependent pathway in NSCLC cells (18). Thus, in the present study, it is unclear why this gene showed a high expression in tumors from patients who survived. Activin A, a homodimeric protein and a member of the TGF-ß superfamily, is involved in the inflammatory repair process. It promotes the proliferation of human airway smooth muscle cells (19), and also contributes to human lung fibroblast activity (20). However, its effect on tumor progression or suppression has not been clarified. The proliferating cell nuclear antigen has been examined as a prognostic factor in various tumors, but its role in NSCLC remains controversial. DNA topoisomerases play important roles in DNA metabolism through their ability to catalyze the inter-conversion of topological isomers of DNA. While the functions of topoisomerase I and II are quite well established, and are now targets of anticancer drugs, the function of topoisomerase III is not fully understood, although recently a role in the maintenance of nuclear structure has been suggested (21). The data from the present study are consistent with the notion that topoisomerase III has a key function in homologous recombinational repair during the S phase that is essential for ensuring the subsequent fidelity of chromosome segregation.

The functions of some prognosis-related genes can be understood in terms of the suppression of tumor progression through immune- or apoptosis-mediated actions. However, cyclin- or repair-related genes appear to have antagonistic functions. Genes such as these, exerting opposite effects, will require further study to clarify whether or not they fulfill a normal function. The tumor cells of patients showing an unfavorable course may have abnormal functions and show a decreased gene expression. In fact, homozygous deletion of cyclin-dependent kinase has been demonstrated in tumor cell lines from patients with stage III or IV NSCLC showing an unfavorable outcome, whereas this feature is absent in tumor cell lines from patients with stage I or II NSCLC showing a favorable outcome (22). Thus, it appears that these survival-related genes show a relatively higher expression in tumors from patients with a favorable outcome. In conclusion, it has been shown that the expression levels of survival-related genes are increased in NSCLCs from patients with a favorable outcome. These survival-related genes may be promising targets for molecular therapy aimed at increasing their expression in cancers. Also, as high expression of some immune-related genes was observed in patients with a favorable outcome, adjuvant immunotherapy may be useful in some patients after tumor resection.

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