Antigens Recognized by IgG Derived from Tumor-infiltrating B Lymphocytes in Human Lung Cancer

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Abstract. *Background: Lung cancer tissues are often infiltrated by B lymphocytes, but it is not clear whether these infiltrations represent tumor-specific immune response or a nonspecific reaction. Materials and Methods: The serological analysis of recombinant cDNA expression libraries (SEREX) were previously modified using a severe combined immunodeficient (SCID) mice model engrafted with fresh human lung cancer. Here, a panel of antigens recognized by tumor-infiltrating B lymphocytes (TIB) in human lung cancer were characterized. Results: The modified SEREX analysis identified 22 distinct antigens in a large cell carcinoma of the lung. Sequence analysis and real time-PCR analysis showed that 55% of isolated antigens were overexpressed in tumor tissues and 9% had mutation. Conclusion: The results of this study indicate that the humoral immune response of TIB in lung cancer patients can be detected in the xenotransplanted SCID mouse model and our modification shows high sensitivity and specificity for identification of tumor antigens.*

The serological analysis of recombinant cDNA expression libraries of human tumors with autologous serum, so-called SEREX, was developed to standard techniques for identification of tumor antigens (1). Several SEREX studies of lung cancer have been reported, both on non-small (2-4) and small cell lung cancer (5). Brass *et al.* (2, 3) detected 35 positive clones from the screening of cDNA library from a squamous cell lung carcinoma. Six clones coded for eIF-4 gamma, a eukaryotic translation initiation factor, are amplified in 30% of squamous cell lung cancer (6). In another study, Güre *et al.* (4) analyzed an adenocarcinoma of the lung with autologous serum and isolated 20 positive clones. One of those was aldolase A, expressed at high levels in most lung cancers (7). However, 50% of the antigens identified by SEREX were recognized in an apparently non-cancer-related manner and can be classified as naturally occurring auto-antigens expressed in normal cells and tissues (8), since many gene products were recognized by the immune system of cancer patients including auto-antigens expressed in normal cells and tissues. Over 1,000 antigens have been identified to date in various malignancies by using SEREX, but to select these antigens by tumor specificity, many steps are necessary.

In the microenvironment of many solid tumors, lymphocytes are the major component of mononuclear cell infiltrations. The immune system is known to act against tumors and the presence of tumor-infiltrating lymphocytes is considered to reflect the host tumor-related immune response. Lung tumors are often infiltrated by B lymphocytes, however, it is not clear whether these tumor-infiltrating B cells (TIB) represent a tumor-specific immune response or nonspecific lymphocyte recruitment (9). To investigate the potential role of TIB in lung cancer, we and other investigators previously examined antibody production by TIB in fresh human lung cancer tissue engrafted in SCID mice (10-13). To rule out the possible reaction of antigens with non-specific antibodies, the original SEREX method was modified. The first modification was the establishment of an autologous lung cancer cell line, A904L, as the pure source of cDNA for SEREX analysis. Another modification was the utilization of IgG produced by TIB as a probe instead of the patient's serum.

The present study is an extension of our previous studies (10-12) and was designed to determine the profile of antigens recognized by TIB in a patient with large cell carcinoma of the lung.

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Key Words: Lung cancer, tumor microenvironment, tumor-infiltrating B lymphocytes, SEREX.

Materials and Methods

The study protocol was approved by both the Human and Animal Ethics Review Committees of the University of Occupational and Environmental Health, Japan, and a signed consent form was obtained from the patient prior to tissue sampling in the present study.

Engraftment of human lung tumor tissue into SCID mice. Female SCID mice (C.B.-17 scid/scid, 6-week old) were obtained from Charles River Inc. (Tokyo, Japan) and were maintained in specific pathogen-free environment throughout the study. Part of the surgically resected specimen from the patient (A904) with lung cancer was prepared for implantation as described previously (10-12). The tumor-engrafted SCID mice were bled by retro-orbital venipuncture every 2 weeks and the serum was collected for analysis of human Ig. Human Ig titers were measured by the latex agglutination method (10). Serum samples were collected and stored at –20˚C until used.

Lung cancer cells and tissues. The A904L (lung cancer cell line) was established from the tumor as described previously (14). Panels of control cDNAs of normal tissues, commercially available (Clontech Laboratories, Palo Alto, CA, USA), were used for the analysis of mRNA expressions of the target genes in normal tissues.

Immunoscreening and DNA sequencing. mRNA was extracted from the A904L cells. The cDNA libraries of these cells were constructed in a lambda ZAP Express vector using a cDNA library kit (Stratagene, La Jolla, CA, USA), and immunoscreening of the library was performed as described previously (11). The sequencing reactions were performed using ABI PRISMTM 310 (PE Biosystems, Tokyo, Japan) automated sequencers (11).

Real-time quantitative RT-PCR. To examine the mRNA levels of each cDNA coding antigen in normal and lung cancer tissues, tissues were obtained from 5 patients with primary lung cancer, who underwent surgery at our department. None of the patients had received any anticancer treatment prior to surgery. The histopathological types included three adenocarcinomas and two squamous cell carcinoma. Complementary DNA was synthesized as described earlier (11). Quantitative RT-PCR was carried out in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). The relative amount of mRNA was measured as detection of intercalated SYBR green. PCR was done with the 25 ml SYBR GREEN PCR Master Mix (Applied Biosystems, Foster, CA, USA), either 1 ml of cDNA or 1 ml of water and each primer set described below in a total volume of 50 ml. The PCR cycles were 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 67˚C for 1 min. The PCR primer sequences of β-actin were the same as those of β-actin screening. The concentration of each primer set was 200 nM and 100 nM for the identified genes and β-actin. These samples were obtained the threshold cycle number (C_T) indicating that the amount of amplified target product reached a fixed threshold. DC_T was obtained by comparing the C_T of identified antigens with the C_T of β-actin in same amount of template. Relative quantification was achieved by comparison with the DC_T of A904L cells. The relative expression was calculated by the following formula:

$$\text{Relative expression} = 2^{-(\text{DCT sample} - \text{DCT A904L cells})}$$

Results

Sequence analysis of SEREX-defined cDNA clones. A cDNA expression library of 2.3×10^6 primary clones was prepared from A904L and phage plaques were immunoscreened with human IgG derived from TIB at 1:200 dilution as described previously (11). A total of 119 positive cDNA clones were obtained and all clones were sequenced. These 119 positive clones were represented by 22 different antigen genes. A homology search through the BLAST database revealed that the 22 antigen genes corresponded to previously identified genes, including 9 genes of unknown function (Table I). Sequence analysis of each gene identified mutations in 2 out of the 13 known genes (Table I).

mRNA expression levels of SEREX-defined cDNA clones. To examine whether isolated genes were overexpressed in the A904L cells, their mRNA expression profiles were analyzed by real time RT-PCR. The mRNA levels of 7 out of 13 known genes and 5 out of 9 unknown genes were overexpressed compared to mRNA levels of autologous normal lung tissues (Table I). As shown in Table II, five genes also showed higher mRNA levels in other lung cancer tissues compared to matched normal lung tissues. These results indicated that overexpressed antigens could be well recognized by TIB, and some of the antigens had tumor-specific overexpression.

JNK 2 (C-Jun N terminal kinase 2). In 119 positive cDNA clones, JNK 2 (*C-Jun N terminal kinase 2*), was represented repeatedly in 74 clones. Sequence analysis of these cDNA clones indicated the wild-type JNK 2. In the next step, the mRNA levels of JNK 2 were examined in the autologous cell line (A904L cells) and other lung cancer specimens by real time PCR. The mRNA overexpression of JNK 2 was detected only in the A904L cells (Tables I and II). These results suggest that overexpressed JNK 2 might play important role in the growth of A904L cells.

p53. p53 was also repeatedly represented in 13 clones. These clones had a single base substitution (CGC to CTC), which alters codon 158 from Arg to Leu as described previously (11). Among the isolated antigens in this study, the IARC (International Agency for Research on Cancer) TP53 Mutation Database indicated that 38 cases with the same mutation among 1,893 lung tumors (including large cell carcinoma) had the same mutation (15). These results indicate that our modification of the SEREX method showed high sensitivity for identification of tumor antigens.

GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classic glycolytic protein. The presence of high levels of

Table I. *Characteristics of genes isolated from large cell carcinoma of the lung (A904).*

Gene	Identified antigens	Function	Mutation	Relative expression of mRNA (folds)*
1	c-Jun N-terminal kinase 2 (JNK2)	signal		3.8
2	p53	signal/ transcription	+	
3	weekly similar to nucleolar protein NOP4	ribosome biogenesis		2.5
4	human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	glycolysis		8.5
5	3' similar to phosphatidylinositol 5-phosphate 4-kinase gamma	signal	+	N.D.**
6	kinectin	signal		5.8
7	ubiquitin specific protease 16 (USP 16)	mitosis		4.0
8	PHD finger protein 3 (PHF 3)	transcription		1.4
9	growth arrest-specific 11 (GAS 11)	mitosis/ cytoskeleton		0.9
10	huntington interacting protein 1(HIP 1)	cytoskeleton/ apoptosis		0.7
11	a disintegrin metalloproteinase with thrombospondin repeats (ADAMTS9) precursor	remodeling		2.4
12	Rho protein-binding protein kinase (ROK)	signal		1.0
13	acetyl-coenzyme-A acetyltransferase 2	signal		2.2
14	KIAA1574 protein	unknown		5.4
15	HSPC028 protein	unknown		3.0
16	KIAA0373 gene product	unknown		3.2
17	KIAA1387 protein	unknown		0.4
18	clone MGC: 1954 mRNA	unknown		2.0
19	protein X	unknown		4.5
20	clone DKFZp686J12242	unknown		N.D.
21	clone RP-112c15	unknown		N.D.
22	mitochondrial DNA	unknown		1.1

*overexpression of A904L cells compared with autologous normal lung tissue as estimated by real time quantitative RT-PCR.
**N.D.; not done.

GAPDH mRNA in lung, cervical, breast and pancreas cancers was reported (16) and GAPDH expression was suggested to be associated with cancer cell proliferation and with tumor aggressiveness. Although the five isolated GAPDH-encoding cDNA clones were of the wild-type, GAPDH was overexpressed in A904L cells and in 2 out of 5 other lung cancer tissues (Tables I and II).

Table II. *Comparison of the mRNA expressions of isolated antigens in lung cancer and normal lung tissues.*

Identified antigen	Overexpression in lung cancer tissues (n=5)*
c-Jun N-terminal kinase 2 (JNK2)	0/5
weakly similar to nucleolar protein NOP4	2/5
human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	2/5
kinectin	3/5
a disintegrin metalloproteinase with thrombospondin repeats (ADAMTS9) precursor	1/5
acetyl-coenzyme-A acetyltransferase 2	0/5
KIAA1574 protein	0/5
HSPC028 protein	0/5
KIAA1387 gene product	1/5
clone MGC: 1954 mRNA	1/5

*squamous cell carcinoma of the lung=2, adenocarcinoma of the lung=3.

Kinectin. This protein was reported recently as the most frequently identified antigen in human hepatocellular carcinoma by SEREX (17, 18). In our study, sequence analysis revealed that cDNA clones encoding kinectin derived from tumor tissue were identical with autologus cells and other lung cancer cells. However, real-time PCR analysis showed that overexpression was seen in A904L cells and 3 out of 5 other lung cancer tissues (Tables I and II). These results also suggest that the overexpression in the tumor might elicit humoral immunity and that our modification of maintained the sensitivity and specificity for the identification of tumor antigens.

ADAM TS-9 (a disintegrin metalloproteinase with thrombospondin repeats) precursor. ADAM-TS/metallospondin genes encode a new family of proteins with structural homology to the ADAM metalloprotease-disintegrin family. Members of the ADAM-TS family have been implicated in the cleavage of proteoglycans, the control of organ constitution during embryonal development and the inhibition of angiogenesis. ADAMTS-9 has a metalloprotease domain, a disintegrin-like domain, one internal TSP1 motif and three carboxy-terminal TSP1-like submotifs. In contrast to other ADAM-TS family members, ADAMTS-9 is expressed in all

fetal tissues examined, as well as some adult tissues (19). In the present study, the isolated cDNA clone encoding ADAMTS-9 precursor had no mutation, however it was overexpressed in A904L cells and in 1 out of 5 other lung cancer tissues (Tables I and II).

GAS 11 (growth arrest-specific 11). The growth arrest-specific 11 (GAS11) gene, a potential tumor suppressor gene (20), consists of 11 exons spanning approximately 25 kb. Mutations of this gene were reported in breast cancer (20). However, in the present study, the isolated cDNA clone encoding GAS 11 had no mutation and showed no overexpression in cancer cells. These results suggested that this cDNA clone was a naturally occurring auto-antigen.

Other genes. Rho protein-binding protein kinase (ROK) and PHD finger protein 3 (PHF 3) showed no mutation and no overexpression. These clones were also categorized as auto-antigens. Acetyl-coenzyme-A acetyltransferase 2 and weakly similar to nucleolar protein NOP4, which are associated with malignancy (21, 22), showed high mRNA levels in A904L cells and in 2 out of 5 of the other lung cancer tissues (Tables I and II). Although 3 genes, similar to phosphatidylinositol 5-phosphate 4-kinase gamma, had a single base substitution (C to T), the mutation did not alter codon 44 (silent mutation). This single base substitution was found in 7 out of 10 cases of lung cancer (data not shown).

Discussion

In the present study, 119 positive clones were isolated from the cDNA library and these antigens were recognized by TIB in human lung cancer. Sequencing and real time PCR analysis for isolated genes revealed that 14 out of 22 genes (63.6%) showed abnormal expression (mutation or overexpression). Previously, the specificity of the SEREX method described that 50% of isolated antigens were non-cancer-related antigens based on the results of seroreactivity (8). Our results indicate that TIB may elicit a humoral response to these abnormal expression of genes, such as mutation and overexpression in the tumor microenvironment.

Although numerous antigens were reported by the SEREX method, it is not clear why such genes elicit a humoral immune response. It would be important to distinguish tumor antigens from self-antigens that have no direct relevance to cancer. For instance, mutational events in the tumor may elicit antibodies that cross-react with the corresponding non-mutated counterparts in normal cells. We previously isolated p53 with a single base substitution (CGC to CTC), which alters codon 158 from Arg to Leu (11) and this mutation resulted in overexpression of the p53 protein. The overexpressed p53 protein was recognized by TIB and may play an important role in tumorigenesis. Overexpression of the tumor-related protein appears to be one of the reasons for the immunogenicity of antigens isolated by SEREX.

The gene encoding kinectin, isolated by SEREX in the present study, was overexpressed in autologous cancer cells and in 3 out of 5 other lung cancers. Kinectin is a conserved integral membrane protein anchored in the endoplasmic reticulum *via* a transmembrane domain (23). Authors of recent studies reported that kinectin was isolated by SEREX in hepatocellular carcinoma (17, 18). Furthermore, Wang *et al.* (18) found that kinectin has variants with tumor-associated splicing. Considered together, these results suggest that overexpressed kinectin may play a role in the carcinogenesis or the cancer phenotype of hepatocellular carcinoma, indicating a possible linkage to carcinoma of the lung.

In our screening, JNK 2 was the most frequently detected cDNA clone. In the present study, JNK 2 had no mutation but was overexpressed in autologous lung cancer cell lines. JNK (*C-Jun NH2-terminal kinase*) was implicated in numerous cell functions including stress responses, apoptosis, transformation and cell proliferation (24, 25). Two genes, JNK 1 and JNK 2, are commonly expressed in mammalian cells whereas a third gene, JNK3, is principally expressed in the brain, testis, and heart tissues. Recently, Jnk2-knockout mice were generated (26) and Yang *et al.* (27) suggested that JNK2 plays a critical role in the tumor promotion process.

In conclusion, our results indicate that humoral immune response of TIB in lung cancer patients is poised to respond to mutations and amplifications of cancer-related genes. Our SCID mice model proved a useful tool for identifing tumor antigens, because of the diverse cancer-related immune responses. Using the same model, we are currently investigating the roles of antibodies against the identified antigens in the suppression and/or progression of tumor growth. We produced polyclonal antibodies for protein X, which has an extracellular domain and treatment of A904 tumors xenografted into SCID mice with these antibodies is now under study.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

We thank Dr. A. Nagashima (Department of Chest Surgery, Kitakyushu Municipal Medical Center, Japan, Kitakyushu, Japan) for helpful discussion and Ms K. Noda for her excellent technical assistance.

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Received March 30, 2006
Accepted June 2, 2006