Antitumor Effect of Antibody against a SEREX-Defined Antigen (UOEH-LC-1) on Lung Cancer Xenotransplanted into Severe Combined Immunodeficiency Mice

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Abstract

We previously reported the humoral immune response of tumor-infiltrating B lymphocytes in a lung cancer patient and 22 genes coding tumor-associated antigens identified using the serological identification of antigens by recombinant expression cloning method. In this study, we investigated one of these genes, designated University of Occupational and Environmental Health—Lung cancer antigen-1 (UOEH-LC-1), which has an extracellular domain. Quantitative reverse transcription-PCR revealed that UOEH-LC-1 was expressed ubiquitously in the normal tissues tested. However, it was overexpressed in 5 of 11 (45.5%) lung cancer cell lines and also in 9 of 15 (60%) lung cancer tissues compared with the paired normal lung tissues. A sequence analysis revealed that UOEH-LC-1 has a transmembrane domain. Flow cytometry analysis using a polyclonal antibody against UOEH-LC-1 revealed positive staining on lung cancer cell lines that were positive for expression of mRNA of UOEH-LC-1. Phage plaque assay showed the specific reactivity of anti–UOEH-LC-1 antibody against UOEH-LC-1 protein derived from the antigen encoding phage. By immunohistochemical staining with the anti–UOEH-LC-1 antibody, 7 of 28 (25.0%) lung cancer specimens showed positive staining on the cell surface. The administration of anti–UOEH-LC-1 antibody inhibited the growth of the UOEH-LC-1−positive tumors that were xenotransplanted into severe combined immunodeficiency mice. Complement-dependent cytotoxicity was one of the mechanisms to suppress the tumor growth. These results suggest that the antibody against UOEH-LC-1 therefore seems to have a promising therapeutic potential as a treatment for lung cancer. [Cancer Res 2007;67(17):8351–7]

Introduction

Various kinds of tumor-associated antigens in human cancer have been identified over the past 15 years. These discoveries have made it possible to apply various kinds of immunotherapy for the treatment of cancer, such as peptide vaccination (1, 2), peptide pulsed dendritic cell vaccination (3, 4), adoptive immunotherapy using tumor-infiltrating lymphocytes (5, 6) and antibody therapy (7, 8).

Recently, a number of monoclonal antibodies have been approved for cancer patients. Trastuzumab is a monoclonal antibody against the extracellular domain of the human epidermal growth factor receptor (HER2) protein and has been reported to improve the prognosis of patients with metastatic breast cancer (9, 10). The addition of bevacizumab, a monoclonal antibody against vascular endothelial growth factor, to fluorouracil-based combination chemotherapy has been reported to induce a significant improvement in the survival among patients with metastatic colorectal cancer (11). Cetuximab is a monoclonal antibody directed against the ligand binding site of the epidermal growth factor receptor (EGFR). A European randomized phase II trial investigated the efficacy of cisplatin and vinorelbine with versus without cetuximab in patients with advanced non–small cell lung cancer as a first-line therapy, and the cetuximab arm showed a slight degree of superiority (12). Lung cancer is known to be one of the intractable cancers. Much effort has been paid to overcome the poor prognosis of lung cancer. In addition to standard therapies such as surgery, chemotherapy, and radiotherapy, antibody therapy is expected to become another treatment modality to improve the prognosis of lung cancer patients.

We previously reported that tumor-infiltrating B lymphocytes in lung cancer patient (A904) produced immunoglobulin G (IgG) antibodies and identified 22 tumor-associated antigens by the IgG antibodies (13, 14). We focused on one of these antigens as a target for antibody therapy, which is highly expressed in lung cancer tissues and has extracellular domain. The antigen reveals high homology to the function-unknown gene registered in GenBank (accession no. AK 024297), which is located in chromosome 14q32 and encode 821 amino acids. We designated it as University of Occupational and Environmental Health—l lung cancer antigen-1 (UOEH-LC-1). In this study, we investigated the mRNA expression of the antigen in lung cancer cell lines and tissue specimens of lung cancer and examined antigen specificity of the polyclonal antibody against UOEH-LC-1. Finally, a suppressive effect of the antibody on A904L tumor growth was examined in a xenotransplanted model of severe combined immunodeficiency (SCID) mice.

Materials and Methods

The study protocol was approved by the Human and Animal Ethics Review Committee of the University of Occupational and Environmental Health, Japan, and written informed consent was obtained from all patients regarding the usage of surgical specimens.

Cell lines and tissues of lung cancer. Surgically resected tumor and paired normal lung tissue specimens were frozen and kept at −80°C until analysis. The histologic types of lung cancer cell lines used are adenocarcinoma (A110L, A129L, B90I, C422L, D611L, and H1224L),...
squamous cell carcinoma (PC10 and QG56), large cell carcinoma (A904L and C831L), and adenosquamous carcinoma (A529L). A904L is a lung cancer cell line that originated from a lung cancer tissue of patient A904, in which case UOEH-LC-1 has been identified by the serological identification of antigens by recombinant expression cloning (SEREX) method. The cell lines were cultured in a complete culture medium consisting of RPMI 1640 (Life Technologies-BRL) supplemented with 10% heat-inactivated FCS (Equitech-Bio), 10 mmol/L HEPE, 100 units/mL penicillin G, and 100 mg/mL streptomycin sulfate.

**Reverse transcription-PCR.** Total RNAs from the frozen tissue specimens and cells lines were obtained using the RNeasy kit (Qiagen). A panel of total RNA from 20 normal tissue specimens comprising the adrenal gland, bone marrow, brain, fetal brain, fetal liver, heart, kidney, liver, lung, placenta, prostate, salivary gland, skeletal muscle, testis, thymus, thyroid gland, trachea, uterus, colon, and spinal cord were purchased (Clontech Laboratories). RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). These cDNAs were used as templates for PCR amplification. Gene-specific oligonucleotide primers were designed to each DNA segment at the estimated proper melting temperature. The gene-specific primers for UOEH-LC-1 were commercially synthesized by Hokkaido System Science. The sequence of the UOEH-LC-1 primers were 5'-ACGGTCTCTACTTATTAG-3' (forward) and 5'-TTTTCACAAAGTTACGTAGCC-3' (reverse). The expected size of the PCR products for UOEH-LC-1 was 2,359 bp. The integrity and quantity of the cDNAs were determined by the amplification of β-actin. The PCR products were analyzed by 1.2% gel electrophoresis and ethidium bromide visualization.

**Real-time PCR.** Quantitative reverse transcription-PCR (RT-PCR) was carried out in ABI PRISM 7000 (Applied Bioscience). The relative amount of UOEH-LC-1 mRNA was measured by means of a detection system using intercalated SYBR green. PCR was done with 10 µl of SYBR green PCR Master Mix (Applied Bioscience), either 5 µl of cDNA or 1 µl of water and each primer set described below in a total volume 20 µl. The PCR cycles were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 67°C for 1 min. The primer sequence for UOEH-LC-1 were as follows: sense, 5'-TTCAAGGTCITCTTCTCGC-3' and antisense, 5'-GAATCTCACCAGC-CAGGAG-3'. The threshold cycle number (Ct) was defined as a fractional cycle number at which the amount of amplified target product reaches a fixed threshold. ΔCt was obtained by comparing Ct of UOEH-LC-1 with Ct of β-actin in the same amount of templates. The relative quantification was achieved by comparison with ΔCt of normal lung. The relative expression was calculated by the following formula: relative expression = 2-ΔΔCt, where ΔΔCt was calculated by the following formula: ΔΔCt = ΔCt tumor cells – ΔCt normal lung.

**Antibody against UOEH-LC-1.** A rabbit polyclonal antibody against UOEH-LC-1 was commercially produced by repeated injections with a 15-amino-acid hydrophilic peptide corresponding to the unique region in the carboxyl terminus (CETDLPSSKAKFDS) of UOEH-LC-1, coupled to keyhole limpet hemocyanin, and the sera were subsequently purified by means of a synthesized peptide-affinity column by the Asahi Techno Glass Corporation.

**Fluorescence-activated cell sorting analysis.** Staining and flow-cytometric analysis of cancer cells were done using fluorescence-activated cell sorting (FACS) scan (Becton Dickinson). Cancer cells (5 × 10⁶) were incubated with the anti-UOEH-LC-1 antibody (1:200) or pooled normal rabbit serum, Cedarlane Laboratories Ltd.) for 4 h at 37°C. The supernatant (100 µl) was collected and counted on a gamma counter. The percent specific lysis was calculated using the following formula:

$$\text{% specific lysis} = \left( \frac{\text{observed cpm of test sample} - \text{spontaneous cpm of target cells with medium}}{\text{maximum cpm of target cells with 0.5% Triton X-100} - \text{spontaneous cpm of target cells with medium}} \right) \times 100$$

**Statistical analysis.** The Wilcoxon signed-rank test was done using the StatView software program (SAS Institute). The findings were considered to be significant if the P value was <0.05.

**Results**

**mRNA expression of UOEH-LC-1 in lung cancer cell lines, lung cancer tissues, and normal tissues.** To verify an over-expression of UOEH-LC-1, we carried out the quantitative RT-PCR in 11 lung cancer cell lines and 3 normal lung tissues apart from the primary lesion derived from three patients with lung cancer. In 5 of 11 cell lines, A904L, A110L, A129L, C422L, and C831L, the expression levels of UOEH-LC-1 mRNA were approximately 2-fold higher than those of normal lung tissues (Fig. 1A). The mRNA expression level of lung cancer tissue specimens were compared with their paired normal lung tissues in 15 patients (Fig. 1B). UOEH-LC-1 was overexpressed among the 9 of 15 cancer tissues (60%). mRNA of UOEH-LC-1 was expressed in all of 20 normal human tissues tested. It was expressed highest in the testis and more than twice as high in the heart, kidney, liver, prostate, and thyroid gland as that of normal lung (Fig. 1C).
Figure 1. The expression of UOEH-LC-1 was analyzed by quantitative RT-PCR. The expression of \( \beta \)-actin was used as an internal control. A, the mRNA levels were measured in 3 normal lung tissue specimens derived from each individual and 11 human lung cancer cell lines by quantitative real-time RT-PCR. The relative UOEH-LC-1 mRNA levels were evaluated as the fold increase in comparison with normal lung tissues. Columns, mean of triplicates. B, the intensities of the UOEH-LC-1 expression were compared in the 15 pairs of normal lung and lung cancer tissue of each patient. The expressions of \( \beta \)-actin were standardized in each sample. mRNA expression of UOEH-LC-1 level of lung cancer tissue is higher than the paired normal lung. C, the UOEH-LC-1 expressions in the panel of normal tissues were evaluated. Lane 1, lung; lane 2, adrenal gland; lane 3, bone marrow; lane 4, brain; lane 5, fetal brain; lane 6, fetal liver; lane 7, heart; lane 8, kidney; lane 9, liver; lane 10, placenta; lane 11, prostate; lane 12, salivary gland; lane 13, skeletal muscle; lane 14, testis; lane 15, thymus; lane 16, thyroid gland; lane 17, trachea; lane 18, uterus; lane 19, colon; lane 20, spinal cord; lane 21, A904L (control).
FACS analysis and phage plaque assay. To assess the specific binding of the rabbit polyclonal anti–UOEH-LC-1 antibody using UOEH-LC-1–positive cell lines, flow cytometry was done. Two UOEH-LC-1–positive cell lines (A110L and A904L) and one negative cell line (H1224L) were assessed for antibody binding. Strong anti–UOEH-LC-1 antibody binding was revealed with the two lung cancer cell lines (A110L and A904L; Fig. 2A). However, UOEH-LC-1–negative cell line (H1224L) did not bind to the antibody at all. The result of FACS analysis indicated that UOEH-LC-1 protein localized on their cell surface. To investigate the specific reactivity of the anti–UOEH-LC-1 against the protein of UOEH-LC-1, phage plaque assay was done. Anti–UOEH-LC-1 antibody showed specific reactivity with the phage encoding UOEH-LC-1 but did not with phages encoding irrelevant genes at all (Fig. 2B).

Immunohistochemical analysis of UOEH-LC-1 in lung cancer. Immunohistochemical evaluation of UOEH-LC-1 was done in the 28 lung cancer tissue specimens (Table 1). The immunoreactivity of UOEH-LC-1 was weakly positive in five cases and strongly positive in two cases. Representative immunohistochemical staining is shown in Fig. 3A and B. In Fig. 3C, cancer tissue and noncancerous tissue are shown in the same field. In the region of noncancerous tissue, the majority of normal epithelial cells and lymphocytes were negative for UOEH-LC-1, whereas UOEH-LC-1 was positively stained in cancer cells.

Growth-suppressive effect of anti–UOEH-LC-1 antibody in the transplantation model with A904L cells. The growth-suppressive effect of anti–UOEH-LC-1 antibody was examined in the SCID mice model (Fig. 4). A904L cells were inoculated s.c. into the lateral flanks of the SCID mice, and the mice were treated with anti–UOEH-LC-1 antibody i.p. or at the peritumoral area on days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27. I.p. injection of anti–UOEH-LC-1 antibody results in marked growth inhibition of A904L xenograft in comparison to the control group (P = 0.02). A peritumoral injection also significantly inhibited the growth of tumor xenografts in comparison to the control group (P = 0.03).

Cytotoxic effect of polyclonal antibody of UOEH-LC-1 against lung cancer cells in vitro. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are known to be major mechanisms of antibody-dependent cytotoxicity. Therefore, we examined the anti–UOEH-LC-1 antibody-dependent CDC activity against UOEH-LC-1–expressing cell lines (A904L, A110L) and UOEH-LC-1–negative cell lines (H1224L)

Table 1. UOEH-LC-1 expression in lung cancer tissue specimens

<table>
<thead>
<tr>
<th>UOEH-LC-1</th>
<th>Histology</th>
<th>n</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(n = 17)</td>
<td>(n = 11)</td>
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<tr>
<td>Strongly positive (+++)</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
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<tr>
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<td>2</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Negative</td>
<td>21</td>
<td>15</td>
<td>6</td>
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in the presence of the rabbit complement. As shown in Fig. 5, the anti–UOEH-LC-1 antibody showed a significant cytotoxicity against UOEH-LC-1–positive A904L (A) and A110L (B) but did not against UOEH-LC-1–negative H1224L (C). ADCC activity of the antibody was not detected against these lung cancer cell lines (data not shown).

Discussion

The clinical application of humanized or murine/human chimeric monoclonal antibodies has been well established for the treatment of various human cancers. However, there were a limited number of reports that showed the effectiveness of antibody therapy against lung cancer (15, 16). The identification of cancer-related molecules on the cell surface is important to develop antibody therapy against cancer. The ideal targets should preferentially be highly expressed on the surface membrane of tumor cells, genetically stable, not be shed or secreted, and play a critical role in tumor development and/or proliferation (17). In our previous study, we found 22 antigen-coding genes from a lung cancer cell line (A904L), which were recognized by the antibody derived from tumor-infiltrating B cells (13, 14). In this study, we characterized one of these antigens designated UOEH-LC-1, highly homologous to the function-unknown gene registered in GenBank (accession no. AK 024297), which encodes 821 amino acids. From a bioinformatic analysis, UOEH-LC-1 has a transmembrane domain, 7 N-glycosylation sites, 10 protein kinase C phosphorylation sites, and 2 tyrosine sulfate sites. N-glycosylation is important for membrane protein for protein stability, activity, and specificity of interaction. In addition, it is also related to the adherence of cell-cell and cell-matrix (18–20).

The mRNA expression of UOEH-LC-1 in normal human tissues and lung cancer cell lines was analyzed by quantitative RT-PCR. mRNA of UOEH-LC-1 was expressed ubiquitously among 20 normal human tissues. Expression levels were relatively low in 14 of the 20 normal tissues; however, it was expressed more than twice as high in the heart, kidney, liver, prostate, testis, and thyroid gland as normal lung. The quantitative RT-PCR revealed that more than a 2-fold up-regulation of UOEH-LC-1 to be observed in 5 of 11 (45.5%) lung cancer cell lines in comparison to normal lung tissue. We also showed that the mRNA of UOEH-LC-1 was overexpressed in 9 of 15 (60%) lung cancer tissue specimens in comparison to paired normal lung tissues. In an immunohistochemical analysis, 25.0% of lung cancer tissue specimens showed a positive expression of UOEH-LC-1. These results suggest that UOEH-LC-1 might thus be a candidate molecular target for immunotherapy. FACSanalysis and phage plaque assay showed specificity of this antibody against UOEH-LC-1. The localization on the cell surface and the overexpression in lung cancer tissue imply that UOEH-LC-1 is a promising target for antibody therapy. Furthermore, in our experimental model using SCID mice, tumor growth was significantly suppressed by the antibody treatment.

Because the mRNA of UOEH-LC-1 was ubiquitously expressed in normal tissues and especially expressed in the heart, kidney, liver, prostate, testis, and thyroid gland at a higher level, we should be careful about adverse responses when we use the antibody in the

![Figure 3](image_url)

**Figure 3.** Representative immunohistochemical staining of lung cancer tissues. A, a high expression of UOEH-LC-1 with deep brown staining is shown in the case of squamous cell carcinoma (strongly positive; ++); B, a low expression of UOEH-LC-1 is shown in the case of squamous cell carcinoma (weakly positive; +). C, the specimen shows margin between cancer tissue and noncancerous tissue. In the region of noncancerous tissue, most of the normal epithelial cells and lymphocytes were negative for UOEH-LC-1, whereas UOEH-LC-1 was positively stained in cancer cells.

![Figure 4](image_url)

**Figure 4.** The antitumor activity of anti–UOEH-LC-1 antibody in A904L cell–inoculated SCID mice. A904L cells were inoculated s.c. into SCID mice on day 0. PBS (○; n = 4), or anti–UOEH-LC-1 antibody (●; n = 3) was given i.p. PBS (○; n = 4) or anti–UOEH-LC-1 antibody (●; n = 3) was given at the peritumoral site (at a distance of 5 mm from the tumor). A904L is a UOEH-LC-1–positive cell line. The results are expressed as the mean tumor volume ± SD.
were used at 1:5, 1:10, and 1:100 dilution with PBS. A904L and A110L are UOEH-LC-1 expressing cell lines. H1224L is a UOEH-LC-1 negative cell line. The antibody for UOEH-LC-1 (n) mean values of triplicate cultures are shown. Bars, SD.

**Figure 5.** The effect of anti–UOEH-LC-1 antibody on CDC against A904L (A), A110L (B), and H1224L (C). 3HClabeled cells were incubated with 100 μL of antibody for UOEH-LC-1 (●) or normal rabbit sera (○) with 50 μL of complement for 4 h as described in Materials and Methods. The antibody and normal rabbit sera were used at 1:5, 1:10, and 1:100 dilution with PBS. A904L and A110L are UOEH-LC-1 expressing cell lines. H1224L is a UOEH-LC-1 negative cell line. The mean values of triplicate cultures are shown. Bars, SD.

clinical setting. However, trastuzumab, a monoclonal antibody against HER-2/neu, is commercially used against HER-2/neu positive breast cancer without severe side effects, regardless of the modest expression of HER-2/neu in normal breast epithelial cells, thyroid, endometrium, kidney, and colon mucosa (21). Further study will be needed to clarify UOEH-LC-1 expression in the normal tissues and the potential limitation of the therapy targeting the molecule of UOEH-LC-1.

One possible mechanism of an antitumor effect of the anti–UOEH-LC-1 antibody was mediated by the CDC activity. The CDC of the anti–UOEH-LC-1 antibody was observed against UOEH-LC-1 expressing A904L and A110L cells, but not against a UOEH-LC-1 negative lung cancer cell line (H1224L). We speculated that a higher cytotoxicity against A110L than A904L corresponded to a higher expression of UOEH-LC-1 on A110L based on the results of a quantitative RT-PCR. The CDC activity by the antibody against UOEH-LC-1 suggests that the antibody could promote the assembly of the entire complement cascade. Although the contributions of the complement in the antitumor monoclonal antibody therapy are still not well understood, Imai et al. (22) showed that CDC played an important role in the antitumor effect of antiganglioside GD2 monoclonal antibody therapy of murine metastatic cancer. Macor et al. examined two chimeric monoclonal antibodies, cMOV18 and cMOV19, to recognize the distinct epitopes of the receptor highly expressed on epithelial ovarian cancer cells and, thus, showed their ability to activate the complement cascade (23). ADCC or a direct effect of the antibody against tumor growth was also reported as one of the possible mechanisms to exert the antitumor effect.

In conclusion, UOEH-LC-1 was overexpressed on the cell surface of lung cancer cells in a significant proportion of lung cancer cell lines and lung cancer tissue specimens. The inhibitory effects of the antibody against UOEH-LC-1 on xenotransplanted lung cancer growth suggest the usefulness of UOEH-LC-1 as an effective target for lung cancer treatment.

**Acknowledgments**


**Grant support:** Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and High-Altitude Research Grant from the University of Occupational and Environmental Health, Japan.

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We thank Misako Fukumoto, Yuki Goto, Kihoru Noda, Yukari Oshibuchi, and Ayako Yamasaki for their technical help.

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