# CABYR Is a Novel Cancer-Testis Antigen in Lung Cancer

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**Abstract**

**Purpose:** Cancer-testis (CT) antigens are often expressed in a proportion of tumors of various types. Their restricted normal tissue expression and immunogenicity make them potential targets for immunotherapy. CABYR is a calcium-binding tyrosine phosphorylation–regulated fibrous sheath protein initially reported to be testis specific and subsequently shown to be present in brain tumors. This study was to determine whether CABYR is a novel CT antigen in lung cancer.

**Experimental Design:** mRNA expression of CABYR-a/b (combination of CABYR-a and CABYR-b) and CABYR-c was examined in 36 lung cancer specimens, 14 cancer cell lines, and 1 normal cell line by conventional and real-time reverse transcription-PCR. Protein expression of CABYR was analyzed in 50 lung cancer tissues by immunohistochemistry. Antibodies specific to CABYR were analyzed in sera from 174 lung cancer patients and 60 healthy donors by ELISA and Western blot.

**Results:** mRNA expression of CABYR-a/b and CABYR-c was observed, respectively, in 13 and 15 of 36 lung cancer tissues as well as in 3 and 5 of 14 cancer cell lines, whereas neither of them was observed in adjacent noncancerous tissues or the normal cell line. Protein expression of CABYR-a/b and CABYR-c was observed, respectively, in 20 and 19 of 50 lung cancer tissues. IgG antibodies specific to CABYR-a/b and CABYR-c were detected, respectively, in 11% and 9% of sera from lung cancer patients but not from the 60 healthy donors.

**Conclusion:** CABYR is a novel CT antigen in lung cancer and may be a promising target for immunotherapy for lung cancer patients.

Lung cancer is among the leading causes of malignancy-related deaths worldwide. Although three therapeutic modalities (surgical resection, chemotherapy, and radiotherapy) have been established, long-term survival for lung cancer patients is still generally poor. Therefore, development of new therapeutic methods is very important to improve the cure rate. Recent advances in tumor treatment suggested that immunotherapy targeting tumor antigens, such as cancer-testis (CT) antigens, could be a promising strategy (1).

CT antigens are a class of tumor antigens with normal expression restricted to male germ cells in the testis and various types of cancer but generally not in adult somatic tissues (2, 3). Since the first CT antigen MAGE-1 (subsequently renamed MAGE-A1) was reported by Boon et al. in the early 1990s (4, 5), more than 47 CT genes or gene families have been identified, and their expression has been studied in numerous cancer types to date (3, 6–9). CT antigens become ideal targets for cancer vaccine, considering that a number of them can elicit spontaneous cellular and/or humoral immune responses in some cancer patients (3), and that the testis is an immune privileged site, as well as considering the lack of HLA class I expression on the surface of germ cells (10). However, the expression frequency of a certain CT antigen varies with different tumor types (3), and many CT antigens present heterogeneous expression in the same tumor tissue (11–13). Polyvalent cancer vaccines containing epitopes of several CT antigens may provide a way to increase the number of eligible patients and overcome the heterogeneity. Thus, it is of great value to identify and characterize new CT antigens, particularly those that are immunogenic in human (1, 14).

CABYR is a calcium-binding tyrosine phosphorylation–regulated protein isolated from human spermatozoa. It is encoded by the gene *CABYR* on chromosome 18 at 18q11.2, and the protein localizes to the principal piece of the sperm flagellum in association with the fibrous sheath and exhibits calcium binding when phosphorylated during capacitation (15). According to National Center for Biotechnology Information Reference Sequence[5], there are six transcript variants of CABYR encoding five protein isoforms: CABYR-a, CABYR-b,

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[5] http://www.ncbi.nlm.nih.gov/RefSeq/

CABYR-c, CABYR-d, and CABYR-e (two of the transcript variants encode the same isoform, CABYR-c). Although CABYR was initially reported to be testis specific (15), recently, expression of CABYR-c and CABYR-d has been observed in normal brain and especially in brain tumors using reverse transcription-PCR (RT-PCR; ref. 16), suggesting that CABYR may be expressed in some other tissues or tumors, and, furthermore, it may be a new CT antigen. For these reasons, additional studies are warranted to examine CABYR expression in other types of tumors and its capability to elicit immune responses in cancer-bearing patients.

In the present study, mRNA and protein expression of CABYR-a/b and CABYR-c was found in a number of lung cancer tissues. Moreover, specific antibodies to CABYR were also detected in some of the sera from lung cancer patients. Therefore, our data support CABYR as a novel CT antigen in lung cancer.

## Materials and Methods

***Cell lines, tumor tissues, and sera.*** The lung squamous cell carcinoma cell line NCI-H226; the lung adenocarcinoma cell lines A549, SPC-A-1, and LTEP-a-2; the melanoma cell line A375; the breast cancer cell line MCF7; the hepatocellular carcinoma cell line BEL-7402; the gastric cancer cell line BGC823; the biliary tract carcinoma cell line QBC-939; the human ovarian cancer cell line SKOV3; the cervical cancer cell line HeLa; the metastatic prostate cancer cell line PC3M; the human osteosarcoma cell line OS732; the chronic myeloid leukemia cell line K562; and the normal liver cell line L02 were maintained in our laboratory. Thirty-six lung cancer tissue specimens, 31 paired adjacent noncancerous tissue specimens, and paraffin-embedded sections of normal testis-epididymis, normal brain, 14 lung cancer specimens, and 3 brain cancer specimens were obtained from General Hospital of Beijing Unit, PLA and Peking Union Medical College and Chinese Academy of Medical Sciences. One hundred seventy-four serum samples of the lung cancer patients at the initial diagnosis of disease were collected from General Hospital of Beijing Unit, PLA and the Second Hospital of Xi'an Jiaotong University. Sixty normal control serum samples were obtained from a general health examination in staffs of General Administration of Sport. All samples under the study were collected between December 2002 and July 2004, and informed consent was signed by each donor.

***RNA isolation and reverse transcription.*** Total RNA was isolated from cell lines and tissues using Trizol reagent (Invitrogen, Carlsbad, CA), and 2 μg were reverse transcribed into single-strand cDNA in 20 μL of reaction buffer using Molony murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo (dT)15 (Promega) as a primer. Normal tissue cDNA panels (multiple tissue cDNA panels I and II) composed of brain, colon, heart, kidney, leukocytes, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, thymus, and testis were purchased from BD Biosciences Clontech (Palo Alto, CA).

***Conventional PCR.*** Specific trans-intronic primers used for conventional PCR (Table 1) were designed according to National Center for Biotechnology Information Reference Sequence. PCR was run on the ABI GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplification program for CABYR transcript variants was 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C for 35 cycles after 5 min at 94°C followed by a 10-min elongation step at 72°C. RNA integrity in each sample was checked by amplification of *β-actin* gene segment. PCR products were visualized on an ethidium bromide agarose gel (1%) using ChemiImager 4400 (Alpha Innotech Corp., San Leandro, CA).

***Real-time PCR.*** Real-time PCR reactions were done on the ABI 7300 Real-time PCR System (Applied Biosystems). Expression of CABYR-a/b was analyzed using Taqman Gene Expression Assay (Assay ID: Hs00201830_m1, TaqMan minor groove binder probes, FAM dye-labeled, Applied Biosystems). Briefly, 20 μL reaction contained 10 μL Taqman Universal PCR Master Mix (Applied Biosystems), 1 μL Taqman Gene Expression Assay Primer/Probe Premix, 2μL of cDNA or $H_2O$ as negative control, and 7 μL of $H_2O$. Thermal cycle conditions for CABYR-a/b were as follows: 2 min at 50°C for, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of CABYR-c was analyzed using 10 μL SRBY Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μL with 2 μL of cDNA (generated as described above) or $H_2O$ as negative control, 0.8 μL of forward and reverse primers (Table 1; 10 mmol/L each), and 6.4 μL of distilled $H_2O$. Thermal cycle conditions for CABYR-c were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. A uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products. β-Actin was used as an endogenous control for each sample. Standard curves with 10-fold dilutions of testis cDNA were constructed to calculate the amplification efficiencies ($E$) of the target and the endogenous control. All reactions were carried out in triplicate. The relative expression of the CABYR mRNA was calculated with the following formula (17):

$$\text{Ratio} = \frac{(E_{\text{CABYR}})^{\Delta cp_{\text{CABYR}}(testis-sample)}}{(E_{\beta-actin})^{\Delta cp_{\beta-\text{actin}}(testis-sample)}} \times 100$$

***Generation of CABYR recombinant proteins and anti-CABYR antisera.*** The nucleotide sequences coding a shared segment of CABYR-a (amino acids 181-493) and CABYR-b (amino acids 163-475) and full-length CABYR-c were amplified from human testicular cDNA (BD Biosciences Clontech) and cloned into pET30a(+) vector (Novagen, Madison, WI) to produce recombinant fusion proteins of CABYR that contained 6-histidine peptide at the $NH_2$ terminals. The N-His–tagged CABYR proteins were expressed in *Escherichia coli* BL21 derivative strain Rosetta (Novagen), purified by Ni-NTA affinity column under denaturing conditions according to manufacturer's protocol (Qiagen GmbH, Hilden, Germany), and identified by mass spectrometry. Respective five BALB/c mice were immunized with 150 μg purified CABYR-a/b and CABYR-c recombinant proteins in complete Freund's adjuvant followed by three booster injections in incomplete Freund's adjuvant at 2-week intervals. Bleedouts were done 1 week after the final booster injection followed by Western blot analysis.

***Immunohistochemistry.*** Frozen, 4-μm tissue sections were fixed with 4% paraformaldehyde for 15 min followed by the inactivation of endogenous peroxidase with 3% $H_2O_2$ for 10 min. After washing in PBS, sections were blocked in 10% goat serum for 20 min and incubated with anti-CABYR antisera at a 1:100 dilution at 37°C for 1.5 h. After washing in PBS, horseradish peroxidase–conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) was applied and incubated at room temperature for 30 min. After washing in PBS, sections were incubated with 3,3′-diaminobenzidine substrate until suitable staining developed and counterstained with hematoxylin solution. Then, sections were dehydrated and permanently mounted in nonaqueous mounting media. For paraffin-embedded tissues, sections were heated at 60°C overnight, dewaxed, and rehydrated with xylene and grades of alcohol. Antigen retrieval was carried out in an antigen retrieval buffer [10 mmol/L citrate buffer (pH 6)] at above 95°C in a microwave cooker for 10 min. Subsequent steps were carried out as above. Preimmune sera were used as negative control.

***ELISA.*** Detection and titration of antibodies specific to CABYR-a/b and CABYR-c was done by indirect ELISA. Briefly, recombinant CABYR protein solution (100 μL per well) at a concentration of 1 μg/mL in coating buffer [15 mmol/L $Na_2CO_3$, 35 mmol/L $NaHCO_3$ in distilled water (pH 9.6)] was added to 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated overnight at 4°C. Plates were washed thrice with 0.05% Tween 20/PBS and blocked with 100 μL per well of 5% bovine serum albumin/PBS for 1 h at room temperature. After washing, sera samples (100 μL per well) diluted at 1:100 with 3% bovine serum albumin/PBS were added to the plate and incubated for

**Table 1.** Information of primers

| Target | Accession no. | Primers | Product length (bp) |
|---|---|---|---|
| CABYR-a/b* | NM_012189 | Forward, 5′-GCAGTCACCACGAGTTAGTCC-3′ | 510 |
| | NM_153768 | Reverse, 5′-CCTCGTTCACTTGTTGCCAT-3′ | |
| CABYR-c | NM_138644 | Forward, 5′-CTTACTATGTATAGAGGGAATACTAC-3′ | 430 |
| | NM_153769 | Reverse, 5′-GTTCACTTGTTGCCATTGCTAA-3′ | |
| β-Actin | NM_001101 | Forward, 5′-GAGCTACGAGCTGCCTGACG-3′ | 416 |
| | | Reverse, 5′-CCTAGAAGCATTTGCGGTGG-3′ | |

*We used a pair of primers to amplify a shared segment of CABYR-a and CABYR-b sequences because of the similarity of nucleotide sequences of them, which makes it difficult to distinguish them from each other and from the other three isoforms.

1 h at room temperature. After washing, diluted goat anti-human IgG labeled with peroxidase (Sigma, St. Louis, MO) was added (100 μL per well) and incubated for 1 h at room temperature. After washing, 100 μL of substrate solution [50 mmol/L citric acid, 100 mmol/L $Na_2HPO_4$, 0.03% *ortho*-phenylenediamine, 0.1% $H_2O_2$ in distilled water (pH 5)] was added in each well and incubated for 15 min at room temperature. After adding 3 mol/L $H_2SO_4$ (50 μL per well), plates were read at 490 nm by the Model 550 microplate reader (Bio-Rad, Hercules, CA). A positive reaction was defined as an absorbance value that exceeds the mean absorbance value of sera from healthy donors ($n$ = 60) by 3 SD. In each experiment, a negative control was set up by adding 3% bovine serum albumin/PBS instead of diluted sera, and a nonspecific control was set up by adding the 6-histidine fusion protein (8 kDa) expressed by *E. coli* that was transformed with original pET-30a (+) vector (without any insert) instead of recombinant CABYR fusion protein. All results were confirmed in two independent experiments.

***Western blot.*** Purified recombinant CABYR proteins were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, United Kingdom). After blocked with 5% skim milk/Tween 20/TBS for 2 h at room temperature, the membranes were incubated with the primary antibody (patients' sera at a 1:100 dilution) overnight at 4°C and then with alkaline phosphatase–conjugated goat anti-human IgG (Sigma) as the secondary antibody for 1 h at room temperature. Color substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega) was added and incubated for 5 min for color development. A negative control was set up by adding PBS instead of primary antibody, and a nonspecific control was set up by using the 6-histidine fusion protein mentioned in the ELISA method. For assessing specificity of mice antisera, total proteins extracted from the CABYR mRNA-positive cell line A549 were used as the antigen; mice antisera at a 1:800 dilution were used as the primary antibody; and preimmune sera were used as control.

## Results

***CABYR mRNA is restrictedly expressed in testis among normal tissues but frequently expressed in lung cancer tissues and several cancer cell lines.*** We first investigated the expression profile of CABYR by conventional PCR. mRNA expression of CABYR-a/b and CABYR-c was observed only in testis among 16 normal tissues and in 13 of 36 (36%) and 15 of 36 (42%) lung cancer tissues, respectively, but was not observed in paired adjacent noncancerous tissues. In addition, mRNA expressions of CABYR-a/b and CABYR-c were also detected in 3 and 5 of 14 of various types of cancer cell lines but not in the normal liver cell line L02. Representatives of such analysis are shown in Fig. 1.

For more precise confirmation, the expression profile of CABYR in all of the tested samples was further analyzed by real-time quantitative PCR (Fig. 2). The expression level of CABYR was evaluated by calculating relative ratio to that in the human testis. Although CABYR-a/b mRNA could be detected in all 16 normal tissues, the relative ratio in testis (ratio = 100) was at least 833-fold higher than that (ratio < 0.12) in the other normal tissues except brain (ratio = 0.81). In addition to normal testis (ratio = 100), CABYR-c mRNA was detected in normal brain (ratio = 0.41), lung (ratio = 0.16), pancreas (ratio = 0.07), but not in the other 12 normal tissues. Ratios of CABYR-a/b mRNA ranged from 0.56 to 19.21 in 13 specimens that were shown to be positive by conventional PCR and ratios of CABYR-c mRNA ranged from 0.17 to 3 in 15 specimens that were shown to be positive by conventional PCR. However, ratios of CABYR-a/b mRNA were <0.17 in adjacent noncancerous tissues, and CABYR-c mRNA was detected only in four paired adjacent noncancerous tissues, of which ratios

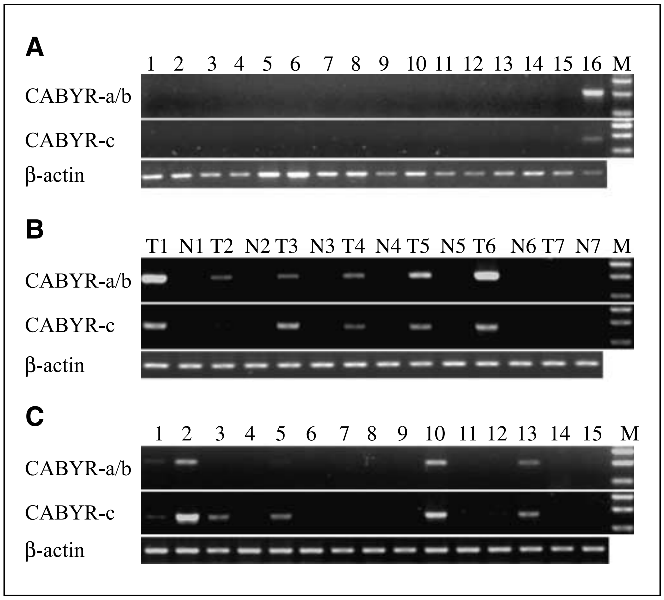


**Fig. 1.** Conventional RT-PCR analysis for expression of CABYR transcript variants in (*A*) 16 normal tissues (*lane 1,* brain; *lane 2,* colon; *lane 3,* heart; *lane 4,* kidney; *lane 5,* leukocytes; *lane 6,* liver; *lane 7,* lung; *lane 8,* ovary; *lane 9,* pancreas; *lane 10,* placenta; *lane 11,* prostate; *lane 12,* skeletal muscle; *lane 13,* small intestine; *lane 14,* spleen; *lane 15,* thymus; *lane 16,* testis; *lane M,* molecular maker), (*B*) lung tumor tissues (*T*) and the paired adjacent noncancerous tissues (*N*), and (*C*) 14 tumor cell lines and 1 normal liver cell line L02 (*lane 1,* NCI-H226; *lane 2,* A549; *lane 3,* SPC-A-1; *lane 4,* LTEP-a-2; *lane 5,* A375; *lane 6,* MCF7; *lane 7,* BEL-7402; *lane 8,* BGC823; *lane 9,* QBC-939; *lane 10,* SKOV3; *lane 11,* HeLa; *lane 12,* PC3M; *lane 13,* OS732; *lane 14,* K562; *lane 15,* L02; *lane M,* molecular maker).

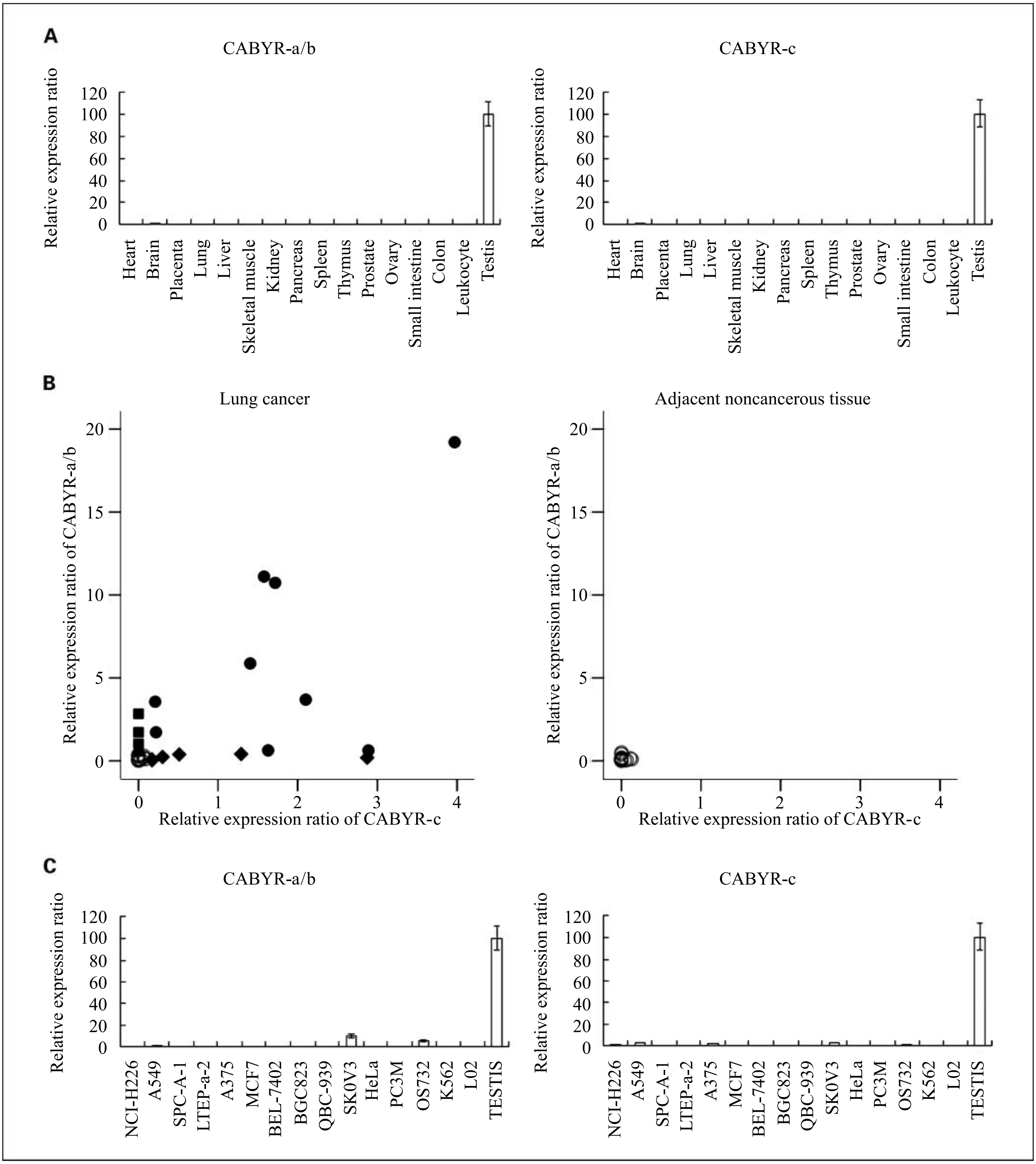


**Fig. 2.** Real-time PCR analysis of CABYR transcript variants in (*A*) 16 normal tissues, (*B*) 36 lung cancer tissues and 31 paired adjacent noncancerous tissues (●, CABYR-a/b and CABYR-c double positive; ■, CABYR-a/b single positive; ♦, CABYR-c single positive; ○, double-negative specimens by conventional RT-PCR), and (*C*) 14 tumor cell lines and 1 normal liver cell line L02.

were <0.13. In addition, mRNA expression of CABYR-a/b and CABYR-c could also be detected in several cancer cell lines.

The mRNA expression data was analyzed statistically using the software SPSS 13.0. A significant positive correlation between CABYR-a/b and CABYR-c mRNA expression was found in lung cancer tissues (Pearson's correlation test, $P = 0.011$), whereas mRNA expression of CABYR-c, but not CABYR-a/b, was significantly more frequent in squamous cell carcinoma than

**Table 2.** Summary of CABYR mRNA expression in lung cancer

| Type | Age mean | Gender (male/female) | Clinical stage | | mRNA expression (no. positive/no. tested) | |
|---|---|---|---|---|---|---|
| | | | I and II | III and IV | CABYR-a/b | CABYR-c |
| Adenocarcinoma | 57.3 | 8/5 | 7 | 6 | 4/13 | 2/13* |
| Squamous cell carcinoma | 58.0 | 7/10 | 9 | 8 | 6/17 | 11/17* |
| Large cell lung carcinoma | 64.0 | 1/0 | 0 | 1 | 1/1 | 1/1 |
| Small cell lung carcinoma | 74.0 | 1/0 | 0 | 1 | 1/1 | 1/1 |
| Adenosquamous carcinoma | 66.3 | 2/1 | 0 | 3 | 1/3 | 03 |
| Other types of lung cancer | 50.0 | 1/0 | 1 | 0 | 0/1 | 0/1 |
| Total | 58.8 | 20/16 | 17 | 19 | 13/36† | 15/36† |

*$\chi^2$ test, $P = 0.007$.
†Pearson's correlation test, $P = 0.011$.

in adenocarcinoma ($\chi^2$ test, $P = 0.007$). No correlation was observed between CABYR mRNA expression and age, gender, or clinical stage of patients (Table 2).

***Generation of CABYR recombinant proteins and anti-CABYR antisera.*** The CABYR recombinant proteins were produced by the method above, fractionated by SDS-PAGE, and stained by Coomassie blue (Fig. 3). The recombinant CABYR-c displayed an expected molecular weight of about 49 kDa, whereas the recombinant segment of CABYR-a/b showed higher apparent molecular weight (about 80 kDa) than predicted (41 kDa). The slower migration of the latter is likely due to its acidic nature (16). The purity of the isolated recombinant protein was confirmed by obtaining a single band, which was identified by mass spectrometry (data not shown). Mouse anti-CABYR antisera were prepared as described above. Considering the homology between CABYR isoforms (Fig. 4), which may cause cross-reaction between an antiserum to CABYR-a/b and to CABYR-c, Western blot was done to assess specificity of the antisera. It was shown that antisera from three of the five CABYR-a/b–immunized mice and antiserum from two of the five CABYR-c–immunized mice detected only an about 86-kDa band of CABYR-a/b and only an about 41-kDa band of CABYR-c, respectively. In contrast, the preimmune sera did not detect any band (Fig. 3). These antisera were considered to be specific to CABYR-a/b or CABYR-c and were used in the subsequent experiments.

***Immunohistochemistry showed frequent expression of CABYR proteins in lung cancer tissues.*** Immunohistochemistry was carried out to analyze frozen sections of 36 lung cancer tissues and 31 paired adjacent noncancerous specimens, which were analyzed in RT-PCR, as well as paraffin-embedded sections of another 14 lung cancer specimens, 3 brain cancer specimens, normal brain, and normal testis-epididymis as a positive control (Fig. 5). Positive staining (>10% of cancer cells were stained) was observed in 11 and 2 of 13 and 23 CABYR-a/b mRNA-positive and mRNA-negative specimens, respectively, and in 12 and 1 of 15 and 21 CABYR-c mRNA-positive and mRNA-negative specimens, respectively. In the paraffin-embedded sections, positive staining of CABYR-a/b and CABYR-c was observed in 7 and 6 of 14 lung cancer specimens and in all of the three brain cancer tissues. In total, CABYR-a/b–positive specimens included 9 adenocarcinomas, 10 squamous cell carcinomas, and 1 adenosquamous carcinoma; CABYR-c–positive specimens included 5 adenocarcinomas, 13 squamous cell carcinomas, and 1 adenosquamous carcinoma. No positive staining was observed in the adjacent noncancerous tissues (Fig. 5C and F). In the cancer tissues, staining was predominantly in the cytoplasm of cancer cells (Fig. 5A, B, D, E, G, and J). In the normal brain, staining of CABYR-c was in the cytoplasm of cells (Fig. 5K), whereas staining of CABYR-a/b was not found (Fig. 5H). In the normal testis-epididymis, CABYR-a/b and

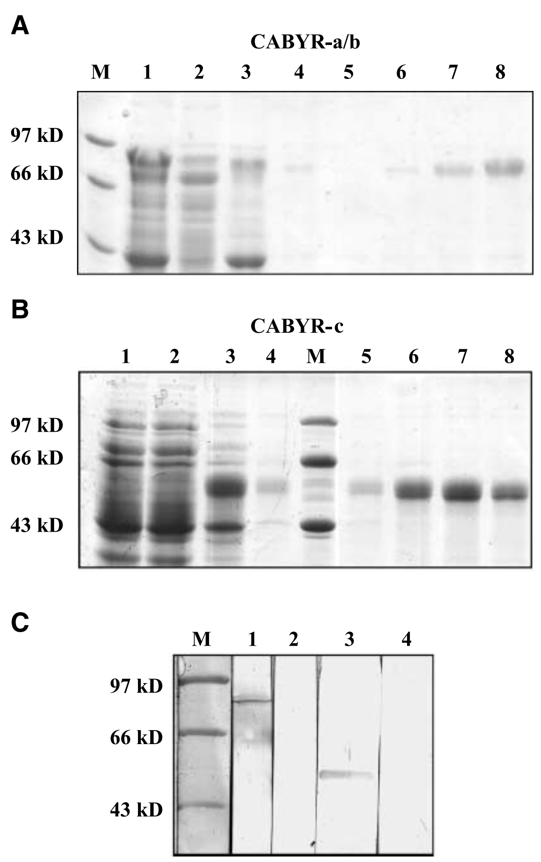


**Fig. 3.** Generation of CABYR recombinant proteins and anti-CABYR antisera. *A,* CABYR-a/b. *B,* CABYR-c. *Lane M,* protein marker; *lane 1,* whole lysate from *E. coli* transformed with recombinant pET-30a(+) vector; *lane 2,* flow through fraction; *lanes 3* and *4,* wash through fractions; *lanes 5* to *8,* different eluted fractions. *C,* Western blot analysis of the A549 cell lysate reacted with immune (*lanes 1* and *3*) and preimmune (*lanes 2* and *4*) sera to CABYR (*lane M,* protein marker; *lanes 1* and *2,* CABYR-a/b; *lanes 3* and *4,* CABYR-c).

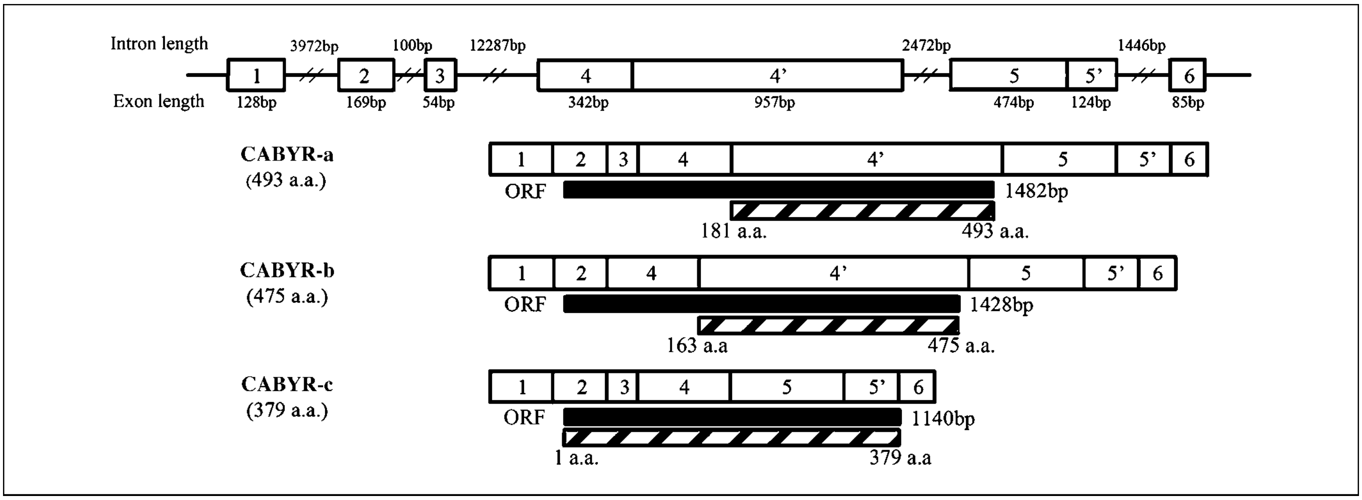


**Fig. 4.** Schematic representation of genomic structure of *CABYR* gene and transcript variants in this study. Exons are coded with numbered boxes. Introns are shown by double diagonal lines. Open reading frames are shown by black bars. Proteins involved in immunization are shown by hatched bars. Exons 4, 4′, 5, and 5′ are derived from alternative splicing.

CABYR-c showed staining of spermatids in the seminiferous epithelium, spermatozoa within the lumen of the seminiferous tubules (Fig. 5I, L, and O), and sperm flagellum within the lumen of epididymis (Fig. 5M and N). The postmeiotic expression pattern was consistent with the previous study (15). In contrast, tissue sections using the preimmune sera as negative control showed no positive staining.

***Antibody response to CABYR isoforms.*** Having shown the expression of CABYR in lung cancer, we then did ELISA to detect IgG antibodies specific to CABYR in sera from 174 patients with various types of lung cancer and 60 healthy donors. Using the mean absorbance value of healthy donors (0.25 and 0.24 for CABYR-a/b and CABYR-c, respectively) + 3 SD (0.08, 0.09) as a cutoff value (0.49, 0.51), positive immunoreactions to CABYR-a/b and CABYR-c were detected in 11% (19 of 174) and 9.2% (16 of 174) lung cancer patients, respectively, but not in the healthy donors. The absorbance value of negative control in each experiment was below 0.01, and the absorbance value of nonspecific control for each positive serum was below the cutoff value (see Fig. 6A and B and Table 3).

To further confirm the presence of CABYR antibodies in the aforementioned sera, Western blot was done using recombinant CABYR protein or a control recombinant 6-histidine fusion protein as antigen and the ELISA-positive or ELISA-negative sera as primary antibody. Only 5 of 19 CABYR-a/b antibody-positive sera and 6 of 16 CABYR-c antibody-positive sera were further confirmed to be positive by Western blot. The results may be due to lower sensitivity of Western blot than that of ELISA assay. In contrast, 20 healthy donors, 20 CABYR antibody negative sera, the negative control, and the nonspecific control still remained negative, which were consistent with ELISA assay (Fig. 6C and D).

***Association between CABYR protein expression in lung cancer tissues and antibody responses in patients' sera.*** Both tissue specimens and serum samples were available from 22 lung cancer patients (10 squamous cell carcinoma, 8 adenocarcinoma, 1 small cell lung cancer, and 3 other types of lung cancer) for immunohistochemistry and ELISA. CABYR-a/b antibodies were detected in two of the six CABYR-a/b–positive patients' sera, and CABYR-c antibodies were detected in three of the nine CABYR-c–positive patients' sera but none of the 16 CABYR-a/b–negative or 13 CABYR-c–negative patients' sera. The correlation between protein expression and presence of antibodies was statistically significant for both CABYR-a/b and CABYR-c (Pearson's correlation test, $P = 0.014$ and $P = 0.025$). However, further studies in a larger scale are needed to confirm the results.

## Discussion

Identification and characterization of new CT antigens is of great value in cancer immunotherapeutic studies. Although CABYR was originally isolated from human spermatozoa and was shown to be testis specific in normal tissues using Northern blot (15), the expression profile of CABYR in the present study suggests that it is likely to be a new CT antigen. A combination of conventional RT-PCR and real-time PCR were used to determine the expression levels of CABYR-a, CABYR-b, and CABYR-c in 16 normal tissues, 15 cell lines, and 36 lung cancer tissues. Because of the similarity of nucleotide sequences of CABYR-a and CABYR-b, which makes it difficult to distinguish them from each other and from the other three isoforms, specific primers were designed to amplify a shared segment of the two sequences. Using 35-circle RT-PCR, it was found that mRNA expression of CABYR-a/b and CABYR-c was present in several cancer cell lines, lung tumor tissues, and normal testis but not in the other 15 normal tissues. However, mRNA of CABYR-a/b and CABYR-c was also detected, respectively, in all of and 4 of the other 15 normal tissues with very low levels by real-time PCR. This may be due to the higher sensitivity of real-time PCR compared with conventional PCR. In addition, minor groove binder–labeled Taqman probe, which is more sensitive than the usual tetramethylrhodamine-labeled Taqman probe (18), was used to detect CABYR-a/b in the present study. Therefore, very low levels of mRNA expression of CABYR-a/b detected in normal tissues were reasonable. As opposed to the conventional view that CT genes are expressed only in gametogenic tissues, a recent study showed that some CT antigens, such as NY-ESO-1, CT15/Fertilin β, CT-16, CT-37, and XAGE-1, were also present in some other normal tissues (3). In most cases, this does not impair their immunotherapeutic potential because their expression levels are extremely low in non-gametogenic tissues and unlikely to result in the presence of immunologically relevant levels of MHC/CT peptide complexes (3). Notably, although the expression levels of CABYR-a/b and CABYR-c in normal brain (ratio = 0.81 and 0.41, respectively) were very low compared with testis (ratio = 100), they were obviously higher than in the other non-testicular tissues (ratio <0.12 and <0.17, respectively). This

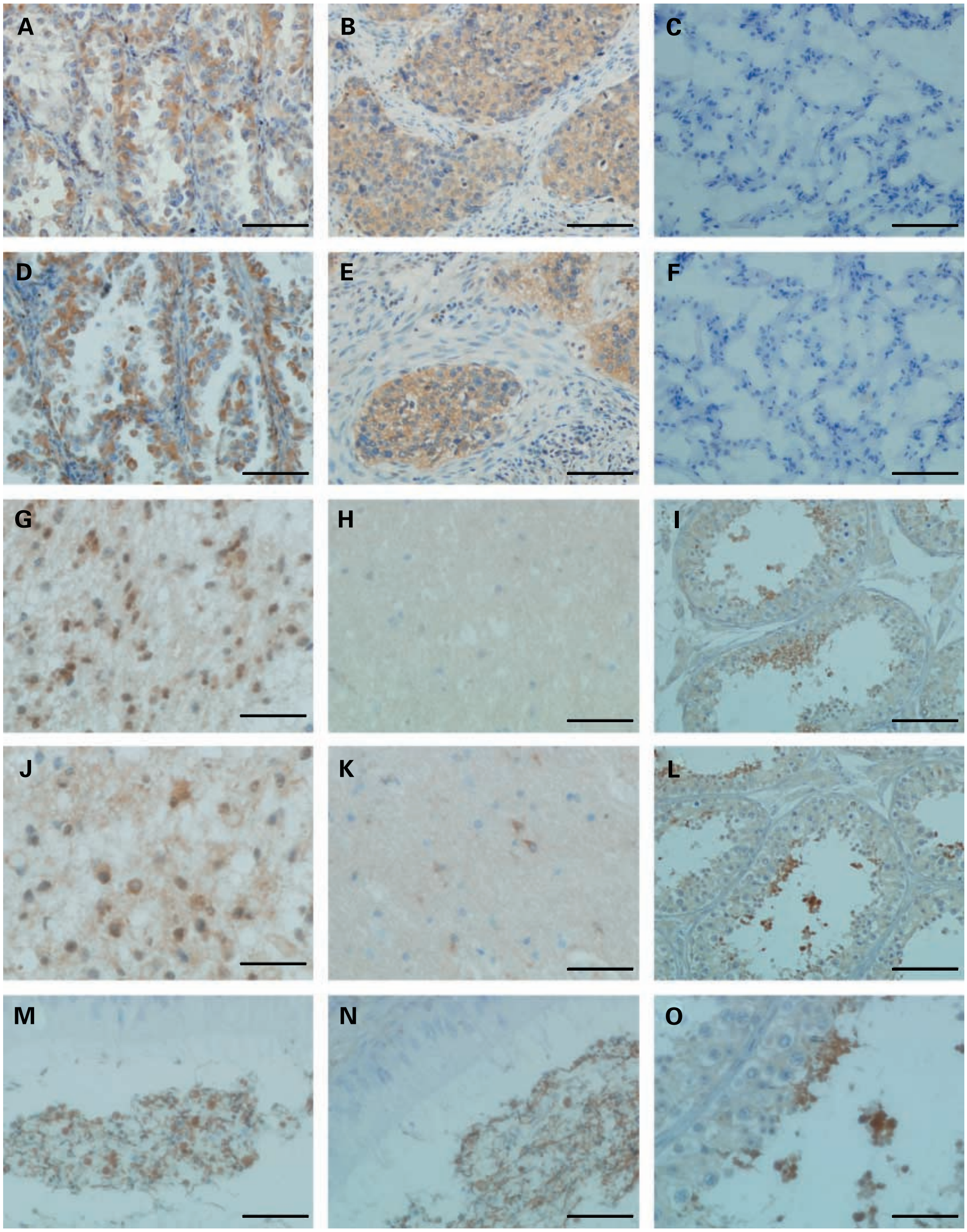


**Fig. 5.** Immunohistochemical analysis of CABYR protein expression in lung cancer (*A* and *D,* adenocarcinoma; *B* and *E,* squamous cell carcinoma), noncancerous lung tissues (*C* and *F*), brain cancer (*G* and *J*), normal brain (*H* and *K*), and testis-epididymis (*I*, *L*, and *O,* seminiferous epithelium; *M* and *N,* lumen of epididymis). CABYR-a/b (*A-C, G-I*, and *M*). CABYR-c (*D-F, J, K, L, N*, and *O*). Scale bar, 100 μm (*A-I, K*, and *L*) and 50 μm (*G, J*, and *M-O*).

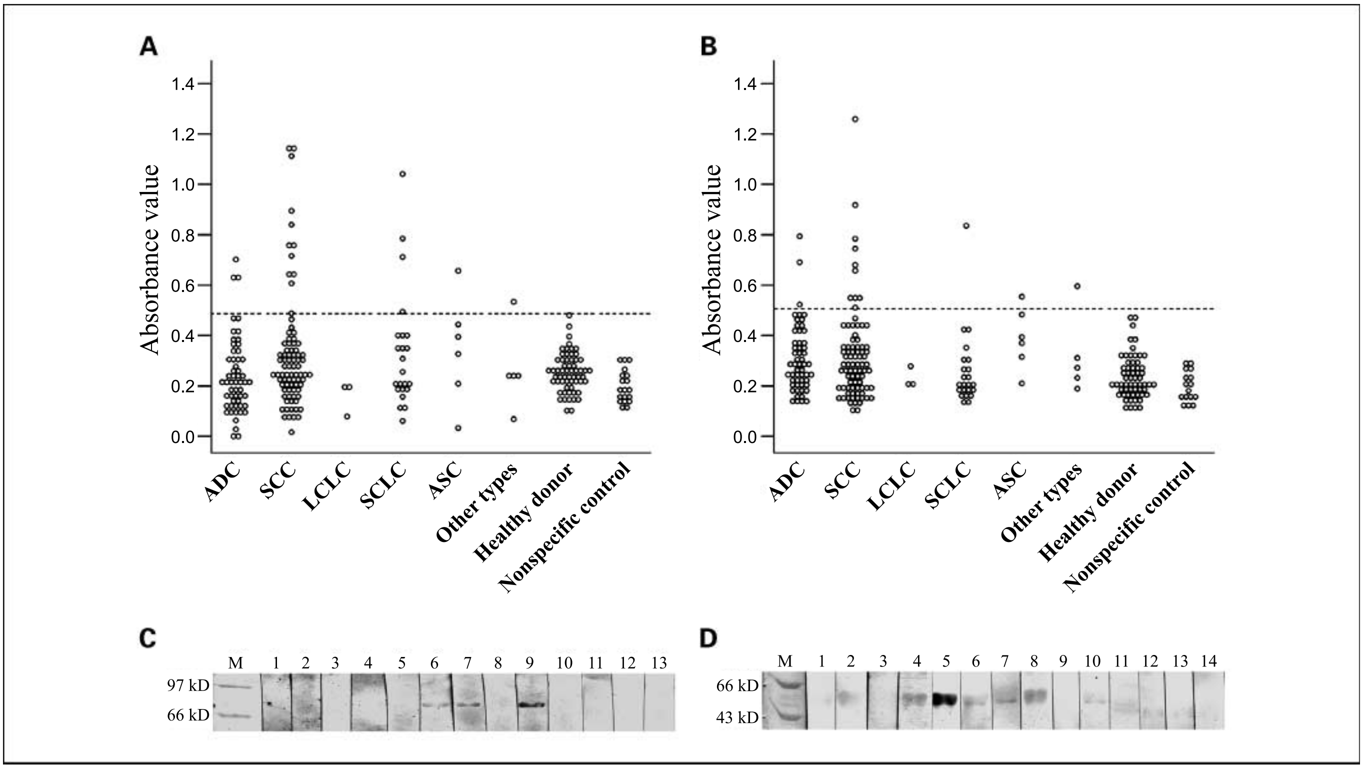


**Fig. 6.** Analysis of CABYR-a/b – specific (*A* and *C*) and CABYR-c – specific (*B* and *D*) IgG antibodies in sera from lung cancer patients and healthy donors by ELISA (*A* and *B*) and Western blot (*C* and *D*). ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCLC, large cell lung carcinoma; SCLC, small cell lung carcinoma; ASC, adenosquamous carcinoma; dashed line, cutoff value. *Lane M,* protein marker; *lanes 6, 7*, and *9,* positive reaction with sera from patients; *lanes 1* to *5* and *8,* negative reaction with sera from patients; *lanes 10* to *13,* negative reaction with sera from healthy donors (*C*). *Lane M,* protein marker; *lane 2* and *lanes 4* to *8,* positive reaction with sera from patients; *lanes 1, 3, 9*, and *10,* negative reaction with sera from patients; *lanes 11* to *14,* negative reaction with sera from healthy donors (*D*).

does not detract their potential to be targets for immunotherapy either because the central nervous system has been considered to be an immunologically privileged site where the highly specialized endothelial blood-brain barrier allows no entry of circulating lymphocytes unless inflammation occurs in the central nervous system (19). Therefore, the mRNA expression profile suggests that CABYR is likely to be a CT antigen in lung cancer and, furthermore, in other types of cancer because of its expression in several cancer cell lines such as A375, OS732, and SKOV3.

In addition to the analysis of mRNA expression, immunohistochemistry was carried out to analyze CABYR protein expression in lung cancer. The frequent expression of CABYR protein in lung cancer tissues and absence of CABYR protein in noncancerous lung tissues confirmed the result from analyzing CABYR at the mRNA level. However, it is noteworthy that some mRNA-positive specimens showed no positive staining, whereas some mRNA-negative specimens showed positive staining in immunohistochemical analysis. This discrepancy may derive from the different areas of the same tumor specimens for RT-PCR and immunohistochemistry. In addition, the protein level sometimes does not necessarily reflect the mRNA level due to mRNA degradation, different translational regulation, and protein degradation, etc. Thus, the immunohistochemical analysis of CABYR provided more evidence supporting CABYR as a CT antigen in lung cancer. Moreover, we notice that expression of CABYR in brain cancer cells was more frequent than that in normal brain cells, indicating that CABYR may be expressed in other types of cancer. Further studies are necessary to confirm this speculation.

Although the evaluation of expression levels is very important in identifying CT antigens, the *in vivo* immunogenicity of them should also be considered. Despite CT antigens, such as MAGE (20) and SSX (21), were expressed frequently in lung cancer, humoral immune responses were rarely observed in the patients (22). Recently, high frequency of antibody responses to XAGE-1b, another CT antigen, has been observed in adenocarcinoma but not in other histologic types of lung cancer (23). In attempt to analyze the *in vivo* immunogenicity of CABYR, ELISA and Western blot analysis to sera from lung cancer patients and healthy donors were further done. IgG antibodies specific to CABYR-a/b and CABYR-c were detected, respectively, in 19 of and 16 of 174 patients' sera. Besides adenocarcinoma, humoral immune responses were also found in squamous cell carcinoma and other histologic types of lung cancer (Table 2). Statistically significant correlation between CABYR expression and immune response suggested that the presence of IgG antibodies were most likely caused by the high levels of CABYR expression in the tumor cells rather than a result of immune dysregulation and spontaneous autoimmunity (24). The presence of IgG antibodies to CABYR in multiple patients implied that B cells have been activated with T-cell cognitive help in these patients, suggesting that CABYR may also be immunogenic to T cells (10, 24). However, it needs to be determined whether cellular immune response to CABYR can be elicited in sero-positive patients in further

**Table 3.** Summary for immunogenicity of CABYR-a/b and CABYR-c in lung cancer

| Type* | Antibody of CABYR-a/b | | | | Antibody of CABYR-c | | | |
|---|---|---|---|---|---|---|---|---|
| | Absorbance, mean (SD) | No. positive/ no. tested | *P* | | Absorbance, mean (SD) | No. positive/ no. tested | *P* | |
| | | | $\chi^2$ test† | *t* test‡ | | | $\chi^2$ test† | *t* test‡ |
| Healthy donor | 0.25 (0.08) | 0/60 | — | — | 0.24 (0.09) | 0/60 | — | — |
| ADC | 0.24 (0.15) | 3/55 | >0.05 | >0.05 | 0.31 (0.14) | 3/55 | >0.05 | <0.01 |
| SCC | 0.33 (0.24) | 11/82 | <0.01 | <0.05 | 0.32 (0.19) | 10/82 | <0.01 | <0.01 |
| LCLC§ | 0.16 (0.07) | 0/3 | — | — | 0.23 (0.08) | 0/3 | — | — |
| SCLC | 0.33 (0.24) | 3/23 | <0.01 | >0.05 | 0.26 (0.15) | 1/23 | >0.05 | >0.05 |
| ASC | 0.34 (0.21) | 1/6 | <0.01 | >0.05 | 0.39 (0.12) | 1/6 | <0.01 | <0.01 |
| Other | 0.26 (0.17) | 1/5 | <0.01 | >0.05 | 0.32 (0.16) | 1/5 | <0.01 | >0.05 |
| Total | 0.30 (0.21) | 19/174 | <0.01 | <0.05 | 0.31 (0.17) | 16/174 | <0.01 | <0.01 |

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCLC, large cell lung carcinoma; SCLC, small cell lung carcinoma; ASC, adenosquamous carcinoma.
*Other, other types of lung cancer; Total, total number of patients.
†$\chi^2$ test was used to assess the statistical significance of difference in ratios of sera-positive donors between patients and healthy donors.
‡Two-tailed Student's *t* test was used to assess the statistical significance of difference in absorbance values between patients and healthy donors.
§Sample size was too small for statistical analysis.

studies. Although for the CABYR-a/b and CABYR-c antibody double-positive sera, it is possible that antibody responses to CABYR-a/b and CABYR-c were overlapping in reactivity because of potential homology (Fig. 4), the CABYR-a/b and CABYR-c single-positive sera suggested that both of them could induce humoral immune response. Taken together, our results mentioned above support that CABYR is a new CT antigen in lung cancer.

Up to now, many of the hitherto known genes encoding CT antigens have been mapped to the X chromosome, whereas some of them have been mapped to autosomes (2, 3). CABYR belongs to the latter group, being mapped to 18q11.2. The biological functions of only a few CT antigens are known, such as SCP-1, which is involved in meiotic chromosome synapsis (25); OY-TES-1, which is involved in the packaging of acrosin in the sperm head (26); CT15/Fertilin β, which is involved in egg/sperm membrane interactions (27, 28); and SSX, which is involved in the transcriptional control (29). The previous studies (15, 30) suggest that CABYR-a/b, which has calcium-binding capacity, may play a role in calcium signaling pathway that regulates sperm motility during sperm capacitation, whereas the calcium unrelated function of CABYR-c is unclear.

A postmeiotic expression pattern of CABYR was observed in the previous and present study, which increases the possibility for CABYR to be an immunotherapeutic target. It is well known that testis is considered an immune privileged site due to the presence of the so-called blood-testis barrier. However, many of the known CT antigens are first expressed in spermatogonia or young spermatocytes located in the basal compartment of the seminiferous epithelium, the nonprotected side of the blood-testis barrier. Thus, immunotherapy targeting these CT antigens may result in reduction or elimination of spermatogenesis and permanent injury to the testes of the patients. In contrast, vaccines targeting CT antigens, which lie on the protected side of the blood-testis barrier (the adluminal compartment of the seminiferous epithelium), may reduce the risk (31, 32). Therefore, CT antigens expressed postmeiotically are more appropriate for immunotherapy. Immunofluorescent localization of CABYR in the human testis has shown a postmeiotic expression pattern (15), supporting that it may be a suitable CT antigen for an immunotherapeutic target.

In summary, we have identified a novel CT antigen CABYR, which was frequently expressed in lung cancer and was immunogenic in some of the cancer-bearing patients. Our results support CABYR as a candidate target for lung cancer immunotherapy.

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