

Characterization of preexisting humoral immunity specific for two cancer-testis antigens overexpressed at the mRNA level in non-small cell lung cancer

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In order to establish a rationale for immunotherapy for lung cancer, we have investigated immunological characteristics of tumor-associated antigens (TAAs) discovered through molecular approaches. Preexisting Abs specific to these predicted TAAs were examined using specimens of lung pleural effusions (LPEs) and sera in non-small cell lung cancer (NSCLC) patients. The novel cancer-testis (CT) antigens L514S and L552S were highly expressed in approximately half of the NSCLC tissues and established cell lines examined. When lung cancer patients in the USA and Japan were screened, 13%, 17%, and 5% were found to have Abs specific to recombinant L514S, L552S, and NY-ESO-1 proteins, respectively, whereas 48 normal donors had no Abs to these three CT antigens. The Ab titers specific to recombinant L552S and L514S proteins were similar to, and slightly lower than, Abs specific to NY-ESO-1 in stage IV NSCLC patients. To further characterize the preexisting specific Abs, the epitopes were analyzed using 20-aa length peptides entirely covering both antigens. An epitope common to the patients' L514S-specific Abs was identified as aa 85-100 and multiple epitopes, including a major epitope (aa 141-160), were identified for L552S-specific Abs. The Ab epitopes thus identified are not found in human, animal, or bacterial proteins, other than L514S, L552S, or XAGE-1. These data clearly demonstrate that both molecularly defined CT antigens L514S and L552S are immunogenic, at least in terms of humoral responses, suggesting that both CT antigens are promising candidates for immunotherapy.

Keywords: human, non-small cell lung cancer, tumor antigens, L514S, L552S, humoral immunity, epitope mapping

Introduction

Immunotherapy using cytokines and identified TAAs can achieve certain benefits for melanoma patients, as suggested by our accumulated immunological knowledge of TAAs (1). However, it is not yet clear that immunotherapy can be applied to patients with other forms of cancer, nor is it known which TAAs are best suited to cancers such as lung cancer. Preexisting immunity in patients is proof of the immunogenicity of TAAs and can be dramatically enhanced by vaccination (2, 3). Studies have been undertaken in order to discover novel immunogenic

TAAs and to ascertain how widely these TAAs are recognized by cancer patients. These TAAs have been categorized as follows: (i) CT antigens (4, 5, 6); (ii) oncofetal antigens (7, 8); (iii) overexpressed differentiation antigens (9, 10); and (iv) mutated antigens (11, 12). Recently, much attention has been devoted to CT antigens as they are highly expressed in a wide variety of tumors and include potent immunogenic TAAs such as MAGE, GAGE, NY-ESO-1/CT6.1, and others (4, 5, 6, 13, 14).

Our study attempts to use molecular methods to identify novel tumor-specific antigens in NSCLC patients. A couple of these TAAs had been previously classified as CT antigens by molecular characterization (15). Following that, we studied the preexisting immunity and characteristics of these CT antigens by assessing how widely and strongly they are recognized in lung cancer patients. L514S is a novel antigen expressed in cancer and in normal testis tissues at the mRNA level. L552S is a CT antigen located on the X chromosome that is an alternatively spliced form of XAGE-1/CT12.1 (16). It has already been determined that the mRNA expression of these antigens is high in NSCLC patients. These CT antigens were examined in conjunction with NY-ESO-1, the well-known CT antigen frequently associated with both humoral and cellular immune responses (5, 17, 18, 19). L514S and L552S were recognized by multiple specimens in a fashion similar to that of NY-ESO-1. Epitope analysis of the patients' specific Abs also identified a couple of major epitopes in L514S and L552S. The homology search that followed confirmed that the amino acid sequences of major epitopic peptides are fully specific to L514S, L552S, or XAGE-1.

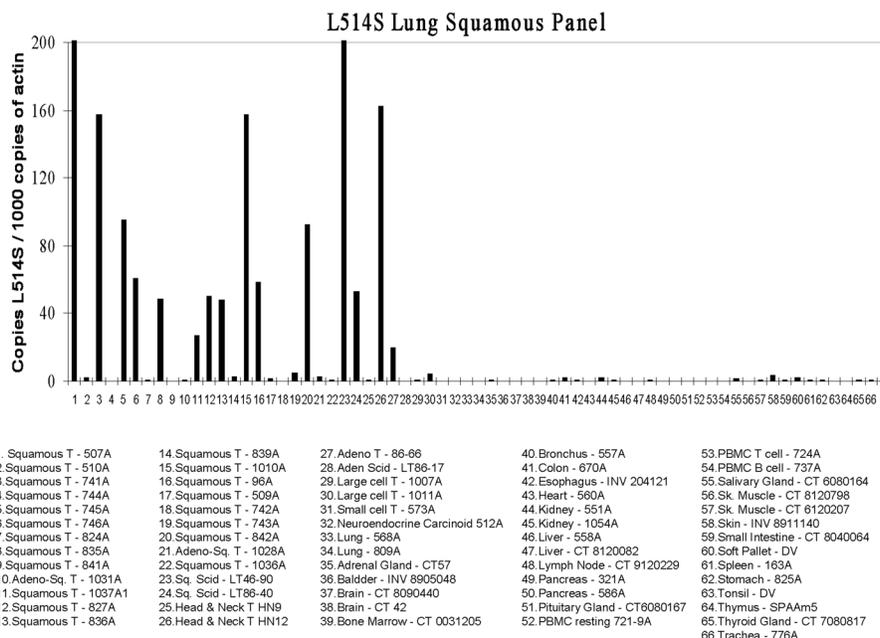
These data show that L514S and L552S induced specific Abs, as can be seen from the titer of the antibodies detected in NSCLC patients. A comparable analysis using the known CT antigen NY-ESO-1 showed that L514S and L552S are both immunogenic and are promising candidates for immunotherapeutic, as well as diagnostic, use.

Results

Molecular characterization of lung cancer antigens

L514S is an NSCLC-specific antigen that was discovered by the cDNA-subtraction method (15), although a detailed analysis of

Figure 1



Real-time PCR analysis of L514S expression in lung squamous tumors. A panel of 66 cDNA samples composed of 32 tumor specimens and 34 normal tissues was tested. L514S mRNA expression was analyzed by real-time PCR. Overexpression was defined as >10-fold expression as compared to the highest expression in normal tissue except for testis. L514S was overexpressed in 11 of 20 lung squamous tumors.

Table 1
L514S, L552S and NY-ESO-1 expression in non-small cell lung tumors

NSCLC Tumor Specimens	CT Antigen Expression		
	L514S	L552S	NY-ESO-1
All	19/40 (48%)	16/40 (40%)	3/25 (12%)
Adenocarcinoma	8/20 (40%)	12/20 (60%)	3/25 (12%)
Squamous cell carcinoma	11/20 (55%)	4/20 (20%)	-

how frequently lung cancer specimens express this antigen has not been conducted. Real-time PCR analyses were set up, and Figure 1 shows representative data for L514S in the lung squamous cell carcinoma panel. Eleven of twenty squamous cell carcinomas highly expressed L514S mRNA, and 40% of adenocarcinomas were also positive (data not shown), while normal tissues and cells other than the testis were negative for its expression. In Table 1, we summarize the mRNA expression of the three different antigens - L514S, L552S, and NY-ESO-1 - in NSCLC. About 40% of NSCLCs and more than half of the lung adenocarcinomas specimens examined highly express L552S (5, 16). NY-ESO-1 mRNA expression was observed in 12% of our adenocarcinoma panel; this frequency is similar to those reported earlier for NSCLC (17, 20).

Preexistence of Abs specific to NSCLC antigens in patients

First, the antibody responses to L514S and L552S in lung cancer patients' sera and in pleural effusions of NSCLC were examined. A total of 40 pleural effusions collected from advanced stage NSCLC patients (stage IV) were set up to analyze Abs specific to both recombinant CT antigens. Recombinant NY-ESO-1 protein was set up in parallel, as a control antigen. Pleural effusions that had Abs specific to L514S and L552S by ELISA were confirmed by Western blot analysis. Interestingly, several NSCLC patients whose pleural effusions had Abs

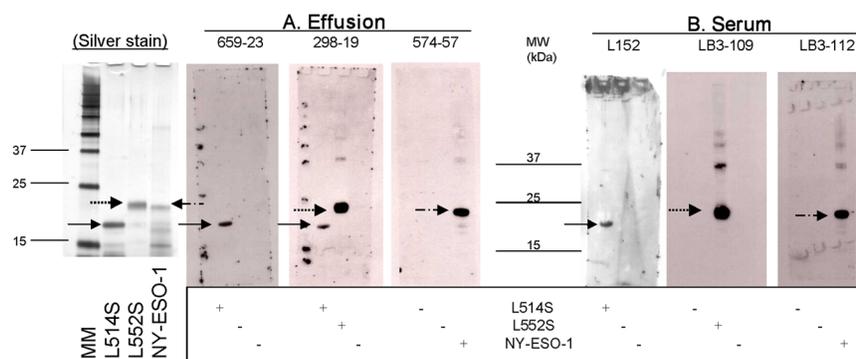
specific to L514S, L552S, and/or NY-ESO-1 were identified. Representative data are shown in Figure 2A. Pleural effusion 659-23 had Abs specific to recombinant L514S, which had the expected molecular weight of 16 kDa. The Western blot band corresponding to recombinant L514S did not disappear when *E. coli* lysate was added (data not shown). This appears to confirm that detectable Ab responses were not specific to contaminated *E. coli* proteins, since recombinant proteins were derived from *E. coli* and patients frequently had strong Abs specific to *E. coli* proteins. Pleural effusion 298-19 had Abs specific to L514S with low intensity, and to L552S with high intensity. Pleural effusion 574-57 had Abs specific to NY-ESO-1. In contrast, 6 pleural effusions of subjects without cancer had no Abs specific to these CT antigens, including NY-ESO-1 (data not shown).

Antibodies to these CT antigens were also found in the sera of multiple NSCLC patients. In Figure 2B, the results of Western blot analysis are shown for each CT antigen. The bands in the Western blot did not disappear even when *E. coli* lysate was added. Moreover, the same patient's pleural effusion and serum had Abs specific to the same cancer antigens. For example, L552S-specific Abs were detected in both the pleural effusion and the serum of one patient (data not shown). These results indicate that these three antigens are immunogenic in terms of humoral responses detected by Western blot analyses, and that the Abs detected were specific to individual CT antigens.

Comparison of patient Ab titers for the three CT antigens

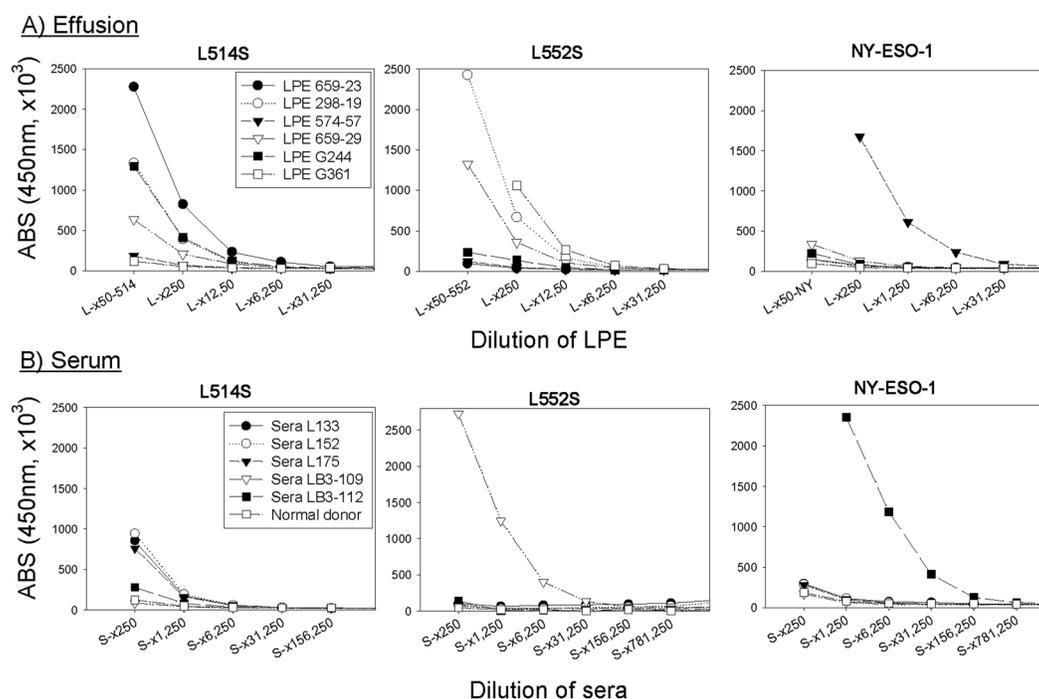
From the 130 USA patients' specimens and the 48 normal donors' sera tested, 6 patients' pleural effusions, 5 patients' sera, and 1 normal donor's serum were selected as representative samples and analysed further to determine the Ab titers towards L514S, L552S, and NY-ESO-1. As shown in Figure 3 (A and B, middle panels), Abs specific to L552S could be detected in pleural effusions (LPE 298-19 and G361) diluted 10⁴-fold and in

Figure 2



Western blot analysis of NSCLC patients' pleural effusions and sera for Abs against L514S, L552S, and NY-ESO-1. Three pleural effusions (A) and three sera (B) were selected by ELISA using His-tag recombinant proteins L514S (solid arrow), L552S (dotted arrow), and NY-ESO-1 (partially dotted arrow). These were added to a nitrocellulose membrane on which the three proteins were blotted. After washing with 0.05% Tween/PBS, the membranes were developed by HRP-conjugated anti-human IgG and chemiluminescence substrate. The molecular weights of the L514S, L552S, and NY-ESO-1 His-tag proteins were determined to be 16 kDa, 18 kDa, and 18 kDa, respectively, by silver staining (left).

Figure 3



Titration analysis of L514S-, L552S-, and NY-ESO-1-specific Abs in pleural effusions and sera of NSCLC patients. Patients' pleural effusions (A) were diluted 50- to 31,250-fold, and sera (B) were diluted 250- to 781,250-fold with PBS containing 5% goat serum and 5% non-fat dry milk (NFDM). The samples were incubated in 96-well plates that had been protein-coated and blocked with 10% NFDM/PBS. After washing with 0.05% Tween/PBS, the plates were developed by HRP-conjugated antihuman IgG and substrate.

sera (serum LB3-109) diluted 10^5 -fold. The titers were similar to those of the NY-ESO-1-specific Abs in specimen LPE 574-57, even though some pleural effusions, such as LPE 659-29, have a lower titer of L552S-specific Abs (Figure 3A, middle panel). These data clearly show that L552S-specific Ab responses were induced in NSCLC patients at a level similar to that seen against NY-ESO-1.

In the case of L514S (Figure 3, left panels), specific Ab responses were detected in pleural effusions (LPE 659-23, LPE 298-19, and LPE G244) diluted approximately 10^3 - to 10^4 -fold, similar to or slightly lower than the titer of NY-ESO-1-specific Abs of LPE 574-57. However, the titers of L514S-specific Abs in sera (L133, L152, and L175) were significantly lower than those of L552S- and NY-ESO-1-specific Abs in sera. It has been suggested that only advanced stage cancer patients have higher

Table 2
Frequency of Abs against L514S, L552S and NY-ESO-1 in the sera of stage I-IV NSCLC patients from the US and Japan

Study Group	Country	Specimens	Frequency of Abs against CT Antigen		
			L514S	L552S	NY-ESO-1
NSCLC patients	US	sera	13/90 (14%)	11/90 (12%)	3/90 (3.3%)
	Japan	sera (low titer, $> \times 10^2$)	7/60	10/60	1/60
		sera (high titer, $> \times 10^4$)	0/60	4/60	4/60
		total	7/60 (12%)	14/60 (23%)	5/60 (8.3%)
US + Japan	total	20/150 (13%)	25/150 (17%)	8/150 (5.3%)	
Normal volunteers	US	sera	0/48 (0%)	0/48 (0%)	0/48 (0%)

titers of L514S-specific Abs because all of these effusion samples were from stage IV patients. Moreover, it was observed that the specific Abs detected to the three CT antigens were of the IgG1 and IgG3 class, suggesting that these humoral responses have been class-switched with T cell help.

Incidence of Abs specific to L514S, L552S, or NY-ESO-1 in NSCLC patients in the USA and Japan

Next, we screened the sera of 90 and 60 NSCLC patients from the USA and Japan, respectively, in order to determine if these CT antigens were recognized by Abs from patients in the two countries in a similar manner. The sera of USA patients were analyzed by ELISA and confirmed by Western blot analysis (300-fold dilution); sera of Japanese patients were analyzed by ELISA and classified into two groups, low titer (>100 -fold dilution) and high titer ($>10^4$ -fold dilution). As shown in Table 2, Abs specific to NY-ESO-1 were detected in 3.3% and 8.3% of the sera of NSCLC patients in the USA and Japan, respectively. The frequency of Abs specific to the other two CT antigens assayed was high, as much as 12-14% for L514S and 12-23% for L552S in USA and Japanese patients. In contrast, the 48 normal donors assayed did not have any Abs specific to the three CT antigens tested. The absence of NY-ESO-1-specific Abs in normal donors was consistent with an earlier report (20). Moreover, high titers of L552S-specific Abs were also observed in Japanese patients, as high ($>10^4$ -fold dilution) as those seen for NY-ESO-1-specific Abs; however, only low titers of L514S-specific Abs were observed in this study. Thus far, we have not found any significant ethnic differences in the rates of Abs specific for these three CT antigens, even though L552S- and NY-ESO-1-specific Abs were relatively more frequent in Japanese patients than in patients from the USA. More importantly, it was found that 30% or more of the NSCLC patients studied had generated Ab responses specific to one of the three CT antigens evaluated.

Epitope analysis of L514S- and L552S-specific Abs

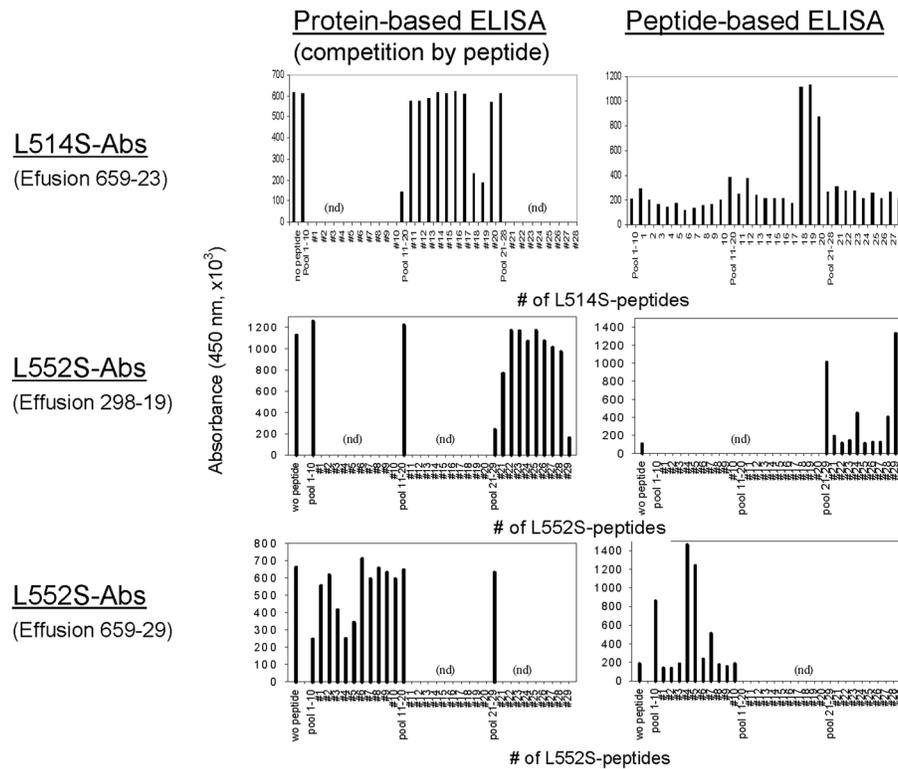
In order to characterize the Abs specific to these CT antigens, we set up experiments using peptides to identify the epitopes recognized by the L514S- and L552S-specific Abs in the patients' specimens. The ELISA plate was coated with L552S or L514S protein, and patient Abs and peptides 20-aa in length derived from these CT antigens were added simultaneously to see how they competed for Ab recognition (protein-based ELISA). In parallel, 20-aa peptides covering the entire L514S and L552S molecules and overlapping by 15 aa were also utilized for the peptide-based ELISA to see whether patient Abs bound to the individual peptides. As shown in Figure 4 (protein-based ELISA, left panels), peptide pool #11-20 of L514S blocked the

binding of Abs in pleural effusion 659-23 to L514S-protein, but peptide pools #1-10 and #21-28 did not block Ab binding to L514S-protein. This competition by a peptide pool was also observed with pleural effusion G244 (data not shown), in which the same peptide pool (#11-20) blocked the binding of Abs to L514S protein. Further breakdown into individual peptides showed that L514S peptides #18 and #19 included the major L514S-specific Ab epitope in pleural effusions 659-23 and G244. This was further demonstrated by the pooled or individual peptide-based ELISA. Namely, peptides #18 and #19, coated on ELISA plates were recognized by L514S-specific Abs in pleural effusion 659-23 (Figure 4, upper right panel). L514S-specific Abs in pleural effusion G244 also mainly recognized peptides #18 and #19 coated on ELISA plates (data not shown). Both the protein-based and the peptide-based ELISAs clearly showed that L514S peptides #18 and #19 include the common epitope recognized by multiple patients' L514S-specific Abs. Thus far, all the patients' Abs specific to L514S protein tested recognized peptides #18/#19 as the major epitope. Interestingly, rabbit Abs generated by immunization with the L514S protein also recognized peptides #18/#19 (data not shown).

In the case of L552S (Figure 4, middle and lower left panels), peptide pool #21-29 of L552S blocked Ab binding to L552S protein in pleural effusion 298-19, but peptide pools #1-10 and #11-20 did not block Ab binding to L552S protein. In contrast, peptide pool #1-10 blocked pleural effusion 659-29, but other peptide pools did not. Further breakdown into individual peptides showed that the C-terminal peptide #29 is the major epitope of L552-specific Abs in pleural effusion 298-19, and peptides #4 and #5 include the major epitope of L552S-specific Abs in pleural effusion 659-29. The peptide-based ELISA of L552S further confirmed these findings. As shown in Figure 4 (middle and lower right panels), peptide #29 included the epitope of L552S-specific Abs in pleural effusion 298-19, and peptides #4 and #5 included the epitope of the patients' L552S-specific Abs. Both the competitive analysis and the peptide-based ELISA clearly showed that peptides #4/#5 and peptide #29 included the epitope of L552S-specific Abs in the patients' pleural effusions. Interestingly, the major epitopes of rabbit Abs induced by immunization with the L552S protein were present in the C-terminal end #29 peptide of L552S. The minor epitopes localized to the N-terminal of L552S (data not shown).

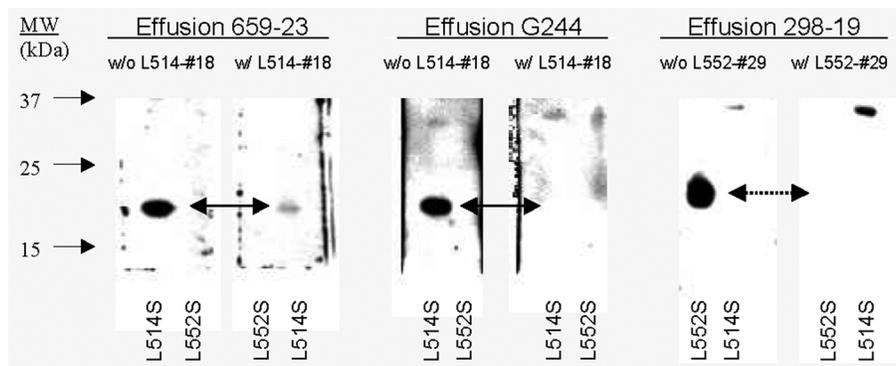
These data were confirmed by Western blot analysis. As shown in Figure 5, the recognition of L514S protein by pleural effusion 659-23 was largely, but not completely, blocked by the addition of peptide #18 (10 μ g/ml). The recognition of L514S protein by Abs in pleural effusion G244 was completely blocked by the addition of peptide #18 at a concentration of 10 μ g/ml. For L552S, the specific recognition of pleural effusion 298-19 was

Figure 4



Epitope analysis of L514S- and L552S-specific Abs in NSCLC patients. For the competitive ELISA (left panels), plates were coated with L514S (upper left panel) or L552S (middle and lower left panels) protein and blocked with 10% NFDMPBS. Diluted (300x) pleural effusion, with or without pooled or individual peptides of L514S or L552S, was then added to the plates. For the peptide-based ELISA (right panels), pooled and individual peptides of L514S (upper right panel) or L552S (middle and lower right panels) were coated on 96-well plates. After the plates were blocked with 10% NFDMPBS, diluted (300x) patients' LPEs were added. After washing, both plates were developed with HRP-conjugated antihuman IgG and substrate.

Figure 5



Competitive Western blot analysis with epitopic peptides of L514S and L552S. Patient pleural effusions (300-fold dilution; 659-23, G244, and 298-19) were incubated 30 min in the absence (left) or presence (right) of L514S peptide #18 or L552S peptide #29 at a concentration of 10 μ g/ml. They were then assessed by Western blot analysis.

completely blocked by the addition of peptide #29 (10 μ g/ml). These data were identical in both types of ELISA. These data also suggest that the Abs specific to L514S and L552S are already skewed to recognize one major epitope of L514S and L552S.

Sequence homology to the epitopes defined

We searched for amino acid sequences homologous to that of the epitopes identified in order to explain the Ab specificity to L514S and L552S. A search of the Entrez protein database showed that the amino acid sequence overlapping peptides #18

shown that almost all patients positive for NY-ESO-1-specific Abs also had Th and/or CTL responses specific to NY-ESO-1, but Ab-negative patients did not have any of these cellular responses at all, even if their tumors expressed NY-ESO-1 mRNA (17, 18). Moreover, MAGE, TRP, and gp100 originally identified by patients' TILs/CTLs have been identified serologically as well (3, 4). It is likely that these cognate T and B cell responses specific to certain TAAs occur frequently in cancer patients because intracellular TAAs overexpressed in tumors may be cross-primed through necrosis/apoptosis of tumor cells with professional APCs, or through the nonspecific inflammation that surrounds tumors, resulting in the induction of broad immunity, including Ab and CTL responses (22). In the advanced stages of lung cancer, LPEs frequently include T cells and B cells that are potentially specific to TAAs. The data reported here also show that Ab responses specific to TAAs existed in pleural effusions with infiltrating lymphocytes, as described recently (23). Interestingly, patient LPE 298-19 was found to have Abs specific to L552S and L514S, at different titers. Other patients had similar titers of both L552S- and NY-ESO-1-specific Abs (data not shown). These humoral immune responses to multiple TAAs seem to occur frequently in cancer patients.

This report explores the B cell/Ab epitopes predicted for TAAs L514S, L552S, and XAGE in several patients. L552S and XAGE-1 are alternatively spliced forms of each other. In Figure 3 we showed that one patient's Abs recognized peptide #4/#5 of L552S. Even though we have not confirmed L552S expression in this particular patient, this demonstrates that the tumor expressed the protein, including at least peptide #4/#5 of L552S, because this epitope does not have any homology to other human protein sequences. Molecular analyses and amino acid alignment of L552S also showed that only L552S, and not XAGE-1 or XAGE-1b, was the predicted protein containing peptide #4/#5 of L552S, as shown in Figure 6. This process, combined with both the epitope analysis and the homology search, is very useful for detecting and confirming humoral responses specific to TAAs in cancer patients and can also be used to assess cellular responses. The sequential analyses conducted in this report clearly demonstrate that L514S and L552S, which are overexpressed at the mRNA level in NSCLC, were recognized by specific Abs from lung cancer patients. By analogy we show that L514S and L552S are two of the most immunogenic TAAs studied in lung cancer, since NY-ESO-1 is one of most immunogenic CT antigens studied to date.

The peptide-based ELISA was able to identify minor epitopes recognized by L552S-specific Abs. This is interesting, as it may relate to epitope spreading (24, 25), and it is also quite interesting in terms of finding minor epitopes of TAAs in cancer patients. Pleural effusion 298-19 had Abs specific to L552S peptides #29 and #24. Another patient's serum specimen (J9) also had Abs specific to peptide #24, as well as Abs specific to peptide #29. Furthermore, these peptide #24-specific Ab responses were not observed in the sera of normal volunteers, nor in that of patients for which no L552S-specific Abs were detectable (data not shown). However, the Western band recognized by the L552S-specific Abs of these patients' specimens could not be cleared by peptide #24 without #29 peptide, suggesting that peptide #24 is one of the minor epitopes. It is also likely that peptides #17 and #21, which are weakly recognized by patient Abs, are also minor epitopes (Table 3). These data show that Ab responses specific to L552S have spread from one major immunogenic epitope to multiple minor epitopes. Kinetic analyses of these major and minor

epitopes in cancer patients could reveal how epitope spreading and/or skewing takes place during disease progression and regression.

In Figure 6, the sequence alignment of all three alternatively spliced forms, L552S, XAGE-1, and XAGE-1b, is shown. The shortest, XAGE-1b, is an 81-aa protein which extends from peptide #17 to the C-terminal peptide #29 of L552S (21). The epitope analysis, including the presence of minor Ab epitopes, suggests that the protein expressed is mainly the 3rd isoform XAGE-1b, and not L552S or XAGE-1a. This preferential recognition by patient Abs of the C-terminal half and not the N-terminal half was also observed among Japanese patients whose Abs were specific to L552S (Table 3). Moreover, previous data from our own and other groups showed that mRNA expression of both L552S and XAGE-1 using specific primers is much lower than that of the common portion shared between L552S, XAGE-1a and the 3rd short isoform XAGE-1b (16, 21). A recent report also observed that mRNA expression of either XAGE-1 or L552S (XAGE-1c) isoform is rare, but that XAGE-1b (26) is abundantly expressed. On the other hand, it has also been observed that the major epitope of polyclonal L552S Abs generated in L552S-immunized rabbit is skewed to the C-terminal end of the protein, even though minor epitopes exist in the N-terminal half of L552S. These data suggest that the preference of L552S-specific Abs in NSCLC patients for certain epitopes is mainly caused by the expression of the shortest isoform XAGE-1b of the three, and by the existence of an immunogenic amino acid sequence in L552S.

The proof and characterization of the preexisting, high-titer Ab responses specific to the novel CT antigens L514S and L552S have been compared to that of the known CT antigen NY-ESO-1. We deduced that both predicted CT antigens are expressed in the tumor site at the protein level, as the epitopes identified were recognized by specific Abs and have no sequence homology to other known molecules in the Entrez protein database. As these specific IgG responses are likely to be mediated by cognate T cell responses (24, 27), studies are in progress to determine whether specific T cell responses, including CTL responses, are present in Ab-positive and -negative patients.

Abbreviations

CT, cancer-testis; LPE, lung pleural effusion; NFD, non-fat dry milk; NSCLC, non-small cell lung cancer; TAA, tumor-associated antigen

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Materials and methods

Pleural effusions and sera of lung cancer patients

All pleural effusions were collected from advanced stage lung cancer patients (stage IV) according to Internal Review Board

guidelines. Fluid was centrifuged for 15 min at 300 x g to remove mononuclear cells. Supernatant was decanted into a sterile tube, passed through a 5 µm filter, and stored frozen at -20°C. Sera were obtained from NSCLC patients (stages I-IV) (Samplex Inc., Westlake Village, CA, USA and Lifeblood Biological Services, Memphis, TN, USA) and from normal donors with informed consent. Sera and pleural effusions were used at dilutions of 1:50 to 1:7 x 10⁶.

Quantitative real-time RT-PCR analysis

To compare the relative level of gene expression in multiple tissue samples, a panel of 66 cDNA samples was constructed using total RNA extracted from tissues and/or cell lines. Real-time PCR was performed using L514S-specific primers to quantify the copy number in each cDNA sample. Each cDNA sample was tested twice, and each reaction was reported as an average of the copy number of the gene of interest normalized against the average of the actin copy number in each cDNA sample. All RT-PCR reactions were performed on an ABI PRISM 7700 Detector (PE Biosystems, Foster City, CA, USA). The details as to how the RT-PCR was performed have been published previously (15, 16).

Recombinant TAA proteins and peptides

L514S was PCR-amplified with the following phosphorylated primer pair: 5'-CACACTAGTGTCCGCGTGGCGGCCTAC-3' and 5'-CATGAGAATTCATCACATGCCCTGAAGGCTCCC-3', as described in an earlier report (15). The phosphorylated PCR product was digested with *EcoRI* and ligated into a modified pET28 vector (Novagen, Madison, WI, USA) containing a His tag in-frame at the N-terminus. The ligated DNA was transformed into Top10 cells (Invitrogen, Carlsbad, CA, USA) and minipreps screened through DNA sequence analysis. The correct clone was then transformed into BL21 (DE3) CodonPlus RIL cells (Stratagene, La Jolla, CA, USA). An overnight culture was started from an isolated colony grown in LB plus kanamycin (30 µg/ml) and chloramphenicol (34 µg/ml). One-liter volume day cultures were grown in 2-L baffled flasks using 2xYT media with the same antibiotics. The cultures were grown to an OD₆₀₀ of 0.4-0.6 and induced with 1 ml of 1 M IPTG. The cultures were grown another 3 h and then harvested. The pellets were collected and washed with 1xPBS and then resuspended in lysis buffer (20 mM Tris, pH 8.0, 500 mM NaCl), and frozen at -20°C overnight. The frozen pellets were then thawed and lysed through a French press and centrifuged at high speed. The inclusion body pellet was then washed with 0.5% CHAPS, 20 mM Tris (pH 8.0), 500 mM NaCl, followed by resuspension in 8 M urea, 20 mM Tris (pH 8.0), 500 mM NaCl. The denatured pellet was then passed over a nickel column and eluted with increasing concentrations of imidazole in 20 mM Tris (8.0), 8 M urea, 100 mM NaCl buffer. The elution fractions that contained protein were dialyzed into 10 mM Tris (pH 8.0).

The L552S coding region was also PCR-amplified with the following primers, 5'-CGGTGCCACGCCCATGGACCTTC-3' and 5'-CTGAGAATTCATTAACCTGTGGTTGCTCTCACC-3' as described in a previous report (16). The PCR product was digested with *EcoRI* and cloned into a modified pET28 vector that had been cut with *Eco72I* and *EcoRI*. Once the sequence was confirmed, the construct was transformed into BL21 pLys S and BL21 CodonPlus cells. Protein purification was performed as described above.

The NY-ESO-1 coding region was PCR-amplified, and the recombinant protein purified as described in an earlier report (20).

L514S and L552S peptides, 20 aa in length and which overlap by 15 aa, were synthesized by solid phase Fmoc synthesis. Their purity was determined by HPLC. Individual peptides were dissolved in DMSO at a concentration of 10 mg/ml and stored at -20° before use.

Protein- and peptide-based ELISAs

For the protein-based ELISA, 50 µl of each protein solution (10 pmole/ml) was added to each well of a 96-well plate (Nunc, Denmark) with bicarbonate coating buffer (pH 9.5) and incubated overnight at 4°C. For the peptide-based ELISA, 50 µl of pooled or individual peptide solution (20 µg/ml) was coated in the same manner. The protein or peptide solution was flicked out, and the plates were blocked with 10% NFDM-containing PBS for at least 3 h at room temperature. The plates were then washed with 0.05% Tween-containing PBS and the samples (50 µl/well), that is the patients' pleural effusions and sera serially diluted with 5% goat sera and 5% NFDM-containing PBS, were added to the plates which were then incubated for 3 h at room temperature or overnight at 4°C. Following washing with PBS-Tween, diluted goat antihuman IgG conjugated to HRP (Jackson Laboratories, Bar Harbor, ME, USA) was added to the plates which were then incubated 30 min at room temperature. After washing, substrate solution (TMS EIA substrate, Bio-Rad Laboratories, Hercules, CA, USA) was added and the plates incubated 15 min at room temperature. After adding stop solution (0.1 N sulfuric acid solution), the absorption at 450 nm relative to the reference absorption at 560 nm was determined with a spectrophotometer. The presence of Ab was defined as a signal/noise (S/N) ratio >3; L514S, L552S, and NY-ESO-1 served as reference antigens for each other.

Western blot analysis

Patient pleural effusions and sera antibody responses against L514S, L552S, and NY-ESO-1 were tested. Purified recombinant L514S, L552S, and NY-ESO-1 proteins (200 ng) were loaded in SDS-PAGE gel and electrophoresed. The gels were blotted onto nitrocellulose membranes. After blocking the membranes with 10% nonfat dry milk (BioRad)-containing PBS, the diluted sera (1:300 to 1:30,000 dilution) or pleural effusions (1:30 to 1:3,000 dilution) were added to the membranes and incubated for 3 h at room temperature or overnight at 4°C. The nitrocellulose membranes were then incubated with HRP-conjugated antihuman IgG (Jackson Laboratories, Bar Harbor, ME, USA) and visualized using a chemoluminescent substrate (ECL™, Amersham Pharmacia Biotech, Uppsala, Sweden). The presence of specific Abs was defined as distinct Western bands with the sample dilutions described. L514S, L552S, and NY-ESO-1 were used as reference antigens for each other.

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