Cancer Immunity, Vol. 4, p. 13 (17 November 2004)
Submitted: 3 June 2004. Accepted: 9 July 2004.
Contributed by: LJ Old

CANCER
IMMUNITY
Article

Identification of the gonad-specific anion transporter SLCO6A1 as a cancer/testis (CT) antigen expressed in human lung cancer

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Keywords: human, SEREX, cancer/testis, tumor antigens, SLCO6A1, mRNA, tissue distribution

Serological analysis of recombinant cDNA expression libraries (SEREX) has led to the identification of many of the antigens recognized by the immune system of cancer patients, which are collectively referred to as the *cancer immunome*. We used SEREX to screen a testicular cDNA expression library with sera obtained from non-small cell lung cancer patients and isolated cDNA clones for 82 antigens. These included a total of 31 antigens previously identified by SEREX, and 51 that did not match entries in the Cancer Immunome Database and were considered newly identified antigens. Overall, the antigens comprised 62 known proteins and 20 uncharacterized gene products. Six antigens (NY-TLU-6, -37, -39, -57, -70, -75) were identified as putative cell surface proteins that are potential targets for monoclonal antibody-based immunotherapy. Of these, the gonad-specific anion transport protein SLCO6A1 (NY-TLU-57) was shown to be tissue-restricted. RT-PCR showed it to be expressed strongly only in normal testis, and weakly in spleen, brain, fetal brain, and placenta. In addition, NY-TLU-57 mRNA was found in lung tumor samples (5/10) and lung cancer cell lines (6/11), as well as bladder (5/12) and esophageal (5/12) tumor samples. These data suggest that SLCO6A1 is a putative cancer/testis (CT) cell surface antigen of potential utility as a target for antibody-based therapy for a variety of tumor types. The analysis also permits us to estimate the eventual size of the SEREX-defined cancer immunome at around 4000 genes. This emphasizes the importance of continued SEREX screening to define the cancer immunome.

Introduction

Studies of the cellular and humoral immune responses to cancer have revealed an extensive repertoire of tumor antigens recognized by the immune system, which are collectively termed *the cancer immunome* (1, 2). SEREX was designed to combine serological analysis with antigenic cloning techniques to identify human tumor antigens eliciting high-titered IgG antibodies (3). SEREX has led to the identification of a multitude of new cancer antigens in many different tumor entities, and the international Cancer Immunome Database currently has more than 1200 different entries of cancer-related antigens identified in this manner.

The past decade has seen enormous strides in the structural definition of human tumor antigens. To date, six categories of human tumor antigens have been identified: (i) differentiation antigens expressed by cancers and a restricted subset of normal cells, such as Melan-A/MART-1 (4) and NY-BR-1 (5); (ii) mutational antigens (6); (iii) over-expressed or amplified gene products such as HER-2/neu (7) and NY-CO-58 (8); (iv) splice variant antigens (9); (v) viral antigens, including the HERV family (10); and (vi) CT antigens (11).

In the present study, a testis cDNA library was used as the antigen source for SEREX analysis, and sera derived from non-small cell lung cancer patients were used for the screening. We isolated 82 distinct antigen encoding cDNAs, designated NY-TLU-1 through NY-TLU-82. Of these, 31 antigens had been previously identified by SEREX analysis of other tumor types, whereas 51 did not match existing entries in the Cancer Immunome Database. We carefully analyzed the predicted subcellular localization of the new antigens and found 6 to be putative cell surface proteins. Based on expression in normal and tumor-derived specimens, we identified one of these, the gonad-specific anion transport protein NY-TLU-57, as exhibiting a CT-like expression profile and being of potential utility as a therapeutic antibody target.

Results

Identification of lung cancer antigens by SEREX

Four independent rounds of SEREX immunoscreening were performed, using both individual and pooled sera from lung

cancer patients. This resulted in the identification of cDNA clones for 82 distinct antigens designated NY-TLU-1 through NY-TLU-82 (Table 1). These antigens comprised 62 known proteins and 20 uncharacterized gene products, including sequences designated in the databases as expressed sequence tags (ESTs), KIAA series clones, FLJ series clones, MGC series clones, DKFZ series clones, and anonymous open reading frames (ORFs) (Table 1). Sixty-two antigens were identified with the serum from patient Lu 413, six antigens were identified with the Mix I sera, ten antigens were identified with the Mix II sera, and four antigens were identified with the Mix III sera. Only three antigens (NY-TLU-65, -71, and -79) were recognized by more than one serum preparation. When the cDNA sequences encoding the 82 lung cancer antigens were compared to those deposited in the Cancer Immunome Database (12), it was found that only 31 of the 82 lung cancer antigens identified (38%) had been previously identified by SEREX analyses with any cDNA/serum combination, whereas 51 (62%) had not been previously reported (Table 1).

Putative cell surface antigens

A preliminary *in silico* mRNA expression profile and characterization of the gene products identified in this study was undertaken based on the tissue distribution of expressed sequence tags (ESTs) (13) and serial analysis of gene expression (SAGE) tags (14) in the Cancer Genome Anatomy Project database (CGAP) (15), as well as on the information contained in the GeneCards database (16). Six antigens (NY-TLU-6, -37, -39, -57, -70, -75) were identified as putative cell surface proteins that could potentially serve as targets for monoclonal antibody-based immunotherapy (Table 2). Five of these antigens were previously characterized as surface membrane proteins, whereas

Table 1

Lung cancer antigens defined by serological analysis of testis cDNA expression libraries.

NY-TLU-Antigen	Gene (UniGene Cluster)	Serum	NY-TLU-Antigen	Gene (UniGene Cluster)	Serum
1	HOXB6 (Hs.98428)	LU413	42	PHIP (Hs.9927)	LU413
2 [a]	DR1 (Hs.348418)	LU413	43 [a]	MPHOSPH1 (Hs.240)	LU413
3 [a]	BRAP (Hs.122764)	LU413	44	NARF (Hs.256526)	LU413
4	ATF7IP (Hs.272210)	LU413	45	KNSL7 (Hs.315051)	LU413
5 [a]	KIAA1641 (Hs.458330)	LU413	46 [a]	S164 (Hs.197184)	LU413
6	CDH18 (Hs.57691)	LU413	47	ATP5F1 (Hs.816341)	LU413
7	I-4 (Hs.127689)	LU413	48	PELO (Hs.415308)	LU413
8	MTO1 (Hs.33979)	LU413	49	UMPK (Hs.458360)	LU413
9 [a]	BCAA (Hs.17428)	LU413	50	IK (Hs.421245)	LU413
10	TPM3 (Hs.178468)	LU413	51	CFL1 (Hs.170622)	LU413
11 [a]	HSPCB (Hs.74335)	LU413	52	MAP7 (Hs.254605)	LU413
12	HOMER-2B (Hs.93564)	LU413	53	NICE-4 (Hs.8127)	LU413
13 [a]	PABPC4 (Hs.169900)	LU413	54 [a]	ROCK2 (Hs.58617)	LU413
14	HTATSF1 (Hs.204475)	LU413	55	ODF1 (Hs.159274)	LU413
15	LOC134218 (Hs.131887)	LU413	56 [a]	STX4A (Hs.83734)	LU413
16	RDBP (Hs.423935)	LU413	57	SLCO6A1 (Hs.388874)	LU413
17	RANBP2 (Hs.179825)	LU413	58	RNF38 (Hs.333503)	LU413
18 [a]	WDR11 (Hs.16677)	LU413	59	C1orf33 (Hs.274201)	LU413
19	FLJ20542 (Hs.6449)	LU413	60	NKGK (Hs.7036)	LU413
20 [a]	C2orf3 (Hs.184175)	LU413	61 [a]	PMSCL1 (Hs.91728)	LU413
21	DKFZP564 (Hs.42954)	LU413	62	FLJ10980 (Hs.308019)	LU413
22 [a]	TBC1D4 (Hs.173802)	LU413	63 [a]	ADPRT (Hs.177766)	Mix I
23	DKFZP566 (Hs.202306)	LU413	64	NUP50 (Hs.362841)	Mix I
24 [a]	HSPCA (Hs.446579)	LU413	65 [a]	RBPJK (Hs.347340)	Mix I,II,III
25 [a]	MGC;27017 (Hs.313413)	LU413	66 [a]	KIAA0373 (Hs.150444)	Mix I
26	KIF9 (Hs.373617)	LU413	67	METTL3 (Hs.168799)	Mix I
27	COPS6 (Hs.15591)	LU413	68 [a]	HSF2 (Hs.158195)	Mix I
28	HOZFP (Hs.351839)	LU413	69	RTN4 (Hs.436349)	Mix II
29	FLJ10890 (Hs.17283)	LU413	70 [a]	RHAMM (Hs.72550)	Mix II
30	RPL31 (Hs.375921)	LU413	71	CCNL1 (Hs.4859)	Mix II,III
31	FLJ10980 (Hs.308019)	LU413	72 [a]	USP16 (Hs.99819)	Mix II
32	FLJ20635 (Hs.265018)	LU413	73	EST (Hs.449083)	Mix II
33	C20orf4 (Hs.11314)	LU413	74 [a]	RNASE3L (Hs.438951)	Mix II
34 [a]	MKRN1 (Hs.7838)	LU413	75	NCOA6IP (Hs.179909)	Mix II
35 [a]	U2AFIRS2 (Hs.171909)	LU413	76	THOC2 (Hs.81376)	Mix II
36	TDRKH (Hs.144439)	LU413	77 [a]	HCF-2 (Hs.55601)	Mix II,III
37	AKAP3 (Hs.98397)	LU413	78	FLJ10747 (Hs.189782)	Mix II
38	Noble (-)	LU413	79	C20orf19 (Hs.436632)	Mix III
39	KIAA1317 (Hs.272254)	LU413	80 [a]	FLJ20274 (Hs.268371)	Mix III
40 [a]	IGHG3 (Hs.300697)	LU413	81	AKP10 (Hs.372446)	Mix III
41 [a]	FLJ20274 (Hs.268371)	LU413	82 [a]	ORMDL2 (Hs.13144)	Mix III

[a] The antigens were previously identified by SEREX analysis of other tumor types, as determined by sequence comparison with the SEREX entries in the Cancer Immunome Database (12).

Table 2
Characterization of putative cell surface antigens

NY-TLU-Antigen (Gene)	Subcellular Location	mRNA Expression Pattern of Normal Tissues and Cancer Types in CGAP [a]
6 (CDH18)	Type I membrane protein	Normal: brain (E5, S7), cerebrum (E6), cerebellum (S4), placenta (S1), testis (E1) Cancer: bone (E1), brain (E, S13), cerebellum (S48), mammary gland (E2, S2), muscle (E2), ovary (E2, S2)
37 (AKP3)	Sperm tail	Normal: bone marrow (S1), brain (S3), lung (S1), lymph node (S33), mammary gland (S3), prostate (S1), retina (E2, S2), testis (E19) Cancer: brain (S15), cartilage (S4), cerebellum (S1), colon (S2), lymph node (E1), mammary gland (S1), ovary (E1), bone (S1), colon (S1), stomach (S1)
39 (KIAA1317)	Putative membrane	Normal: brain (E1, S3) Cancer: brain (S8), cerebellum (S2), liver (S1), mammary gland (S3)
57 (SLCO6A1)	Transport protein	Normal: brain (E2), cerebrum (E1), lymph node (S4), testis (E6)
70 (HMMR)	Cell surface and cytoplasmic	Normal: bone marrow (S1), brain (S3), cartilage (E1), cerebrum (E2), colon (S1), kidney (S1), liver (E2), lung (E1), lymph node (E5, S3), lymphoreticular (E2), muscle (E1, S1), ovary (S5), placenta (E3, S3), prostate (S3), skin (S1), stomach (S1), testis (E10) Cancer: ubiquitous
75 (NCOA6IP)	Sperm head	Normal: ubiquitous Cancer: ubiquitous

[a] The number in brackets refers to the number of EST (E) or SAGE (S) tags.

one was identified only on the basis of a predicted transmembrane domain. Two of these surface proteins, NY-TLU-50 and -75, exhibited widespread expression in both normal tissues and tumor, thus rendering them unlikely candidates as targets for cancer therapy. The remaining four antigens exhibited more tissue-restricted expression. Of particular interest was NY-TLU-57 (SLCO6A1, also known as OATPY), which was first identified as a gonad-specific anion transporter (17).

Experimental determination of the expression of NY-TLU-57 in normal and tumor tissues

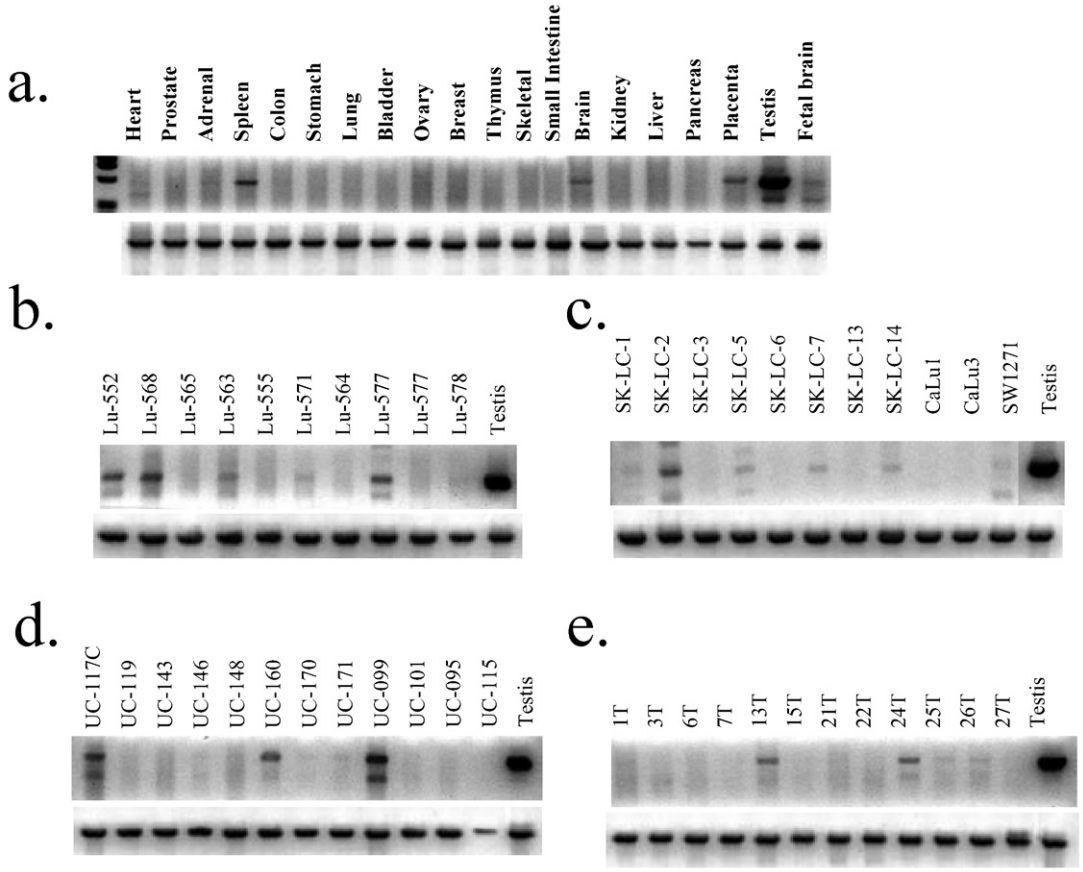
To examine the distribution of NY-TLU-57 gene expression in detail, RT-PCR was performed using mRNA from normal tissues, tumors, and cancer cell lines. Among normal tissues, NY-TLU-57 was expressed strongly only in normal testis. It was expressed weakly in spleen, brain, fetal brain, and placenta, and was absent from all other tissues (Figure 1A). In addition, NY-TLU-57 expression was detected in 5/10 lung tumors (Figure 1B), 6/11 lung cancer cell lines (Figure 1C), 3/12 bladder tumors (Figure 1D), and 4/12 esophageal tumors (Figure 1E). In many of the lanes in Figure 1, a second, apparently smaller, amplicon is visible. DNA sequencing is needed in order to determine whether it is an alternatively spliced transcript or an experimental artifact.

Discussion

A number of powerful methodologies are being utilized to define the complete repertoire of human cancer antigens, which we have termed the *human cancer immunome*. The development of immunological cloning methodologies, including T-cell epitope cloning and SEREX, have been of particular importance in this regard because they can identify tumor targets recognized by CD8+ T-cells and by antibodies, respectively. These approaches are now being supplemented by bioinformatics, transcriptomics, and proteomics-based assays to accelerate the definition of the cancer immunome (1).

In the present study, we employed a testis cDNA library as the antigen source for a SEREX analysis using sera from non-small cell lung cancer patients. This resulted in the isolation of 82 distinct cDNAs encoding tumor antigens, of which only 31 had been previously identified by SEREX with any tumor type, and of which only 3 were identified by more than 1 of the serum preparations used for the present analysis. Furthermore, 20 of the antigens comprised still-uncharacterized gene products. These results are consistent with our recent report (18) of the identification of 113 sarcoma antigens by SEREX, wherein 65% were identified as novel cancer antigens and 20% were uncharacterized genes. These data indicate that we are far from having defined all cancer antigens and their function. In fact, we can utilize the screening data to estimate an overall predicted size of the SEREX-defined cancer immunome and hence ascertain how far we are from an overall view of cancer immunogenicity. If we take the screening to represent a nonbiased sampling of all possible SEREX antigens, then the total predicted size of the SEREX-defined cancer immunome is equal to the number of SEREX entries in the Cancer Immunome Database multiplied by the inverse of the proportion of antigens identified in this

Figure 1



Expression profile of NY-TLU-57. (A) NY-TLU-57 was found by RT-PCR to be expressed strongly in normal testis and weakly in spleen, brain, fetal brain, and placenta. NY-TLU-57 mRNA was detected in a variety of tumor specimens and tumor lines, including in (B) 5/10 lung tumors, (C) 6/11 lung cancer cell lines, (D) 3/12 bladder tumors, and (E) 4/12 esophageal tumors. The cDNA templates used were normalized using G3PDH, as shown at the bottom of each panel.

study that were already in the database (1390 x 82/31). This yields an estimate of 3677 total antigen genes. A good test of the likely validity of this approach, and the assumption of effectively nonbiased sampling, can be gained by repeating the estimate using the earlier data from our laboratory, which was generated using a completely distinct cDNA library (sarcoma) and sera (two sarcoma patients) (18). In this case, 113 distinct antigens were identified, of which 39 were found to be in the Cancer Immunome Database. Since the size of the database has not altered substantially in the period between the two studies, the estimate of overall database complexity is 1390 x 113/39 = 4027. The two estimates thus vary by less than 10% and provide a consolidated estimate of around 4000 antigenic genes as the SEREX-defined cancer immunome, of which only one-third has been defined to date. SEREX thus remains a powerful tool for cancer antigen identification, and the repeated screening of existing cDNA expression libraries with sera from additional patients, as well as the construction and screening of a number of additional libraries, would be worthwhile. It should be borne in mind that many antigens may not display functional epitopes when expressed from cDNA clones in bacteria, thus the SEREX-defined antigens will comprise only a fraction of the overall protein-dependent cancer immunome. Nevertheless, it is clear that we are far from defining even that portion of the cancer immunome that can be approached in this reasonably high throughput fashion.

To date, relatively few surface-associated antigens have been identified using SEREX. Nevertheless, the increasingly detailed annotation of the human genome and the availability online of details of the function and subcellular distribution of gene products render the deliberate search for surface antigens by SEREX a practical proposition. By applying this approach, we identified 6 antigens (NY-TLU-6, -37, -39, -57, -70, -75) that are putatively exposed on the cell surface and might thus serve as targets for monoclonal antibody-based immunotherapy (Table 2). One of these antigens (NY-TLU-75) was found to have a ubiquitous expression pattern in normal tissues, as judged by the EST and SAGE tags in the CGAP databases (15). On this basis, we did not analyze this antigen in detail, although it should be remembered that at least one of the currently utilized targets of antibody therapy, Her2/neu, is widely expressed in normal tissues. Thus, this characteristic does not immediately disqualify the molecule from being utilized in this context. A second of these surface-associated antigens, NY-TLU-6/CDH18, a transmembrane cell adhesion molecule that is expressed specifically in the central nervous system and is

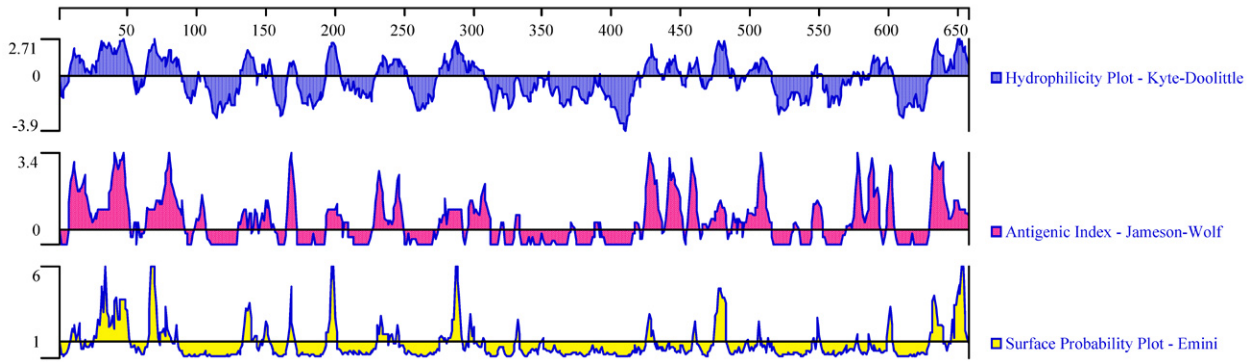
putatively involved in synaptic adhesion and axon outgrowth, was previously identified as being strongly upregulated in small-cell lung carcinoma (19). This is the first time that this molecule has been identified as a tumor antigen, however, and despite relatively high levels of expression in the human brain, further analysis might be warranted. The uncharacterized gene NYU-TLU-39/KIAA1317 is a putative membrane-associated protein and exhibits sequence similarity to a potassium ion transporter first isolated from the brain. NY-TLU-37/AKAP3 encodes a member of the AKAP family that reportedly is expressed only in testis and is localized to the ribs of the fibrous sheath of the sperm tail (20). From EST data, it appears to be present in several tumor types, which again suggests it is worth a closer examination in the future. NY-TLU-70/HMMR is a cell surface receptor of extracellular matrix components that was previously reported as being immunogenic in leukemia and in solid tumors (21, 22). Its presence among the antigens we identified validates the utility of the approach that we have adopted (18). However, we judged the most interesting of the antigens we identified to be NY-TLU-57, which we demonstrated by RT-PCR to be a CT-like antigen strongly expressed in normal testis and weakly expressed in spleen, brain, fetal brain, and placenta. The relevance of the expression in normal tissues, particularly circulating blood cells, will need to be carefully assessed in terms of possible limitations on the use of NY-TLU-57 as the target of monoclonal antibody therapy. Nevertheless, we were able to demonstrate the presence of NY-TLU-57 mRNA in a variety of tumor specimens and tumor lines, including in 5/10 lung tumors, 6/11 lung cancer cell lines, 3/12 bladder tumors, and 4/12 esophageal tumors (Figure 1). NY-TLU-57 (SLCO6A1) encodes an organic anion transport protein with 12 transmembrane domains that is thought to be responsible for transporting dehydroepiandrosterone sulfate and thyroid hormones involved in the regulation of spermatogenesis in the gonad (17). The human organic anion transporting polypeptides (OATPs) constitute a family of proteins with 12 predicted hydrophobic transmembrane domains that can mediate the sodium-independent membrane transport of numerous endogenous and xenobiotic amphipathic compounds such as bile acids, thyroid hormones, prostaglandins, conjugated steroids, antibiotics, and nonsteroidal anti-inflammatory drugs (17, 23). For some members of this family, the subcellular localization has been experimentally demonstrated. For example, OATP-B (SLC22A9) has been shown by immunohistochemistry to be exposed on the surface of both intestinal (24) and breast myoepithelial cells (25). In addition, OATP-B, when overexpressed in stably transfected CHO cells, was both immunolocalized to the cell surface and shown to impart novel transport capacities (25). Similarly, OATP2 (SLC21A6) has been shown immunochemically to be localized to the basolateral membrane of human hepatocytes (26, 27). Interestingly, however, neither NY-TLU-57 (SLCO6A1, OATPY), nor OATP-B, nor OATP2 have an identifiable signal peptide based on either of the prediction algorithms (28, 29). Others have also noted that many secreted and membrane proteins have no identifiable signal sequences (30, 31), and this lack of signal sequence in no way excludes NY-TLU-57 (SLCO6A1) from being a putative plasma membrane protein. Nevertheless, this remains to be demonstrated conclusively by immunohistochemical localization, and the immediate generation of antibodies against this molecule is certainly warranted in order to examine its availability and density of expression on tumor cells. The frequent expression of SLCO6A1 in tumors suggests that it might play a functional role in tumorigenesis, possibly by rendering the cells sensitive to hormonal growth stimuli. This also raises the possibility that it could be a target of small molecule therapy.

Although over 40 CT antigens have been described, few of these are likely to be exposed on the cell surface (32). The exceptions include the surface metaloprotease ADAM-2, which has CT-restricted expression, although the spontaneous antigenicity of this molecule has yet to be demonstrated (33, 34). In addition, structural analysis of both CTAGE and NY-SAR-35 suggests surface association, although, as in the case of SLCO6A1, this localization has yet to be conclusively demonstrated (18, 35).

The predicted structure of NY-TLU-57 (SLCO6A1) is shown in Figure 2, which reveals that approximately one-third of the protein is extracellular, with such regions being interspersed with transmembrane domains. Furthermore, it is predicted that many of these hydrophilic extracellular domains will be found to be antigenic. These data all support the candidacy of the antigen as a target for monoclonal antibody therapy. The cDNA clone identified by SEREX, and thus functionally demonstrated to contain at least one antibody-binding epitope, encompassed only the very C-terminal of the protein, comprising amino acids 572 to 657. This region contains three hydrophilic surface domains with a high antigenic index, all candidate targets for the antibody binding observed.

Monoclonal antibodies are becoming increasingly important to cancer therapy due to the advent of technologies that allow the bioengineering of immunoglobulins (36, 37). For example, chimeric human antibodies derived from murine monoclonal antibodies allow the immunogenicity of xenogenic antibodies in human patients to be overcome. In addition, strategies

Figure 2



Predicted structural features of NY-TLU-57

for improving the efficacy of therapeutic antibodies have been introduced, which include conjugating antibodies to novel cytoxic agents or radionucleotides. Nevertheless, there are relatively few antibodies to surface molecules in clinical trials, and most of these are directed at non-solid tumors. Thus, continued efforts to identify more of the cell surface molecules expressed predominantly in cancer are essential. The bioinformatics-enhanced SEREX approach utilized here was relatively successful at identifying potential cell surface targets, and both further rounds of screening and a careful retrospective analysis of the Cancer Immunome Database for other surface molecules are warranted. In the context of the latter, we searched a random sample of 500 genes from the database and identified 13 known surface-associated molecules, including transporters and receptors, several of which are represented by multiple independent clones. Thus, further potential targets are available, although the expression profile and surface availability of epitopes will have to be carefully examined to evaluate their potential as therapeutic targets.

In conclusion, we combined bioinformatics and SEREX-based screening for cancer antigens and identified at least one novel potential target for antibody-based cancer therapy, the gonad-specific organic anion transport protein, SLCO6A1. This gene is aberrantly expressed in a significant proportion of lung, esophageal, and bladder cancers, and is thus potentially of widespread utility. Given the continued high productivity of SEREX and the success of this approach in the present instance, we propose that continued SEREX-based searches for surface-associated cancer antigens should be undertaken to provide as wide an option as possible for the design of novel therapies.

Abbreviations

CT, cancer/testis; SEREX, serological analysis of recombinant cDNA expression

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Materials and methods

Cell lines, tissues, sera, and RNA

All cell lines used were obtained from the cell repository at the New York (USA) branch of the Memorial Sloan-Kettering Cancer Center of the Ludwig Institute for Cancer Research (LICR). Tumor tissues and sera were obtained from the Memorial Sloan-Kettering Cancer Center and from Weill Medical College of Cornell University (New York, USA). Normal tissue RNA preparations were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA) and Ambion, Inc. (Austin, Texas, USA). Total RNA was prepared from tumor tissues using guanidinium thiocyanate as described earlier (38).

SEREX analysis of cDNA, a testis expression library

Poly(A)+ RNA from normal testis was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA), and 5 μg was used to construct a cDNA library in the ZAP Express vector (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions. The library contained approximately 10^6 recombinants and was used for immunoscreening without prior amplification. Four preparations of sera from non-small cell lung cancer patients were used independently: (i) the serum from a 71-year-old female patient with non-small cell lung cancer (Lu413) diluted 1:200; (ii) Mix I, the pooled sera from 5 non-small cell lung cancer patients (Lu168, Lu179, Lu186, Lu219, and Lu232) diluted 1:200 (final dilution of 1:1000 for each serum); (iii) Mix II, the pooled sera from 5 non-small cell lung cancer patients (Lu322, Lu346, Lu374, Lu395, and Lu449) diluted 1:200 (final dilution of 1:1000 for each serum); and (iv) Mix III, the pooled sera from the same patients used in Mix II, 85 d after immunization with MAGE-3 protein and GM-CSF, diluted 1:200 (final dilution of 1:1000 for each serum). The removal of serum antibodies to bacterial antigens and subsequent library screening were undertaken exactly as described previously (18). Serum reactive phage clones were converted to plasmid forms and subjected to DNA sequencing using standard techniques (Cornell University DNA Services, Ithaca, NY, USA).

RT-PCR analysis

The cDNA preparations used as templates for RT-PCR reactions were prepared using 2.5 μg of total RNA in conjunction with the Superscript First Strand Synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR primers specific for amplifying NY-TLU-57 were: TLU-57-forward, 5'-TCATATGCCTAGCTCTGTCAAAAG-3'; and TLU-57 reverse, 5'-TCCCGGGTCTGGCATCAATAAAAT-3'. The cDNA templates used were normalized on the basis of amplification of G3PDH (BD Biosciences Clontech, Palo Alto, CA, USA). For PCR, 25-μl reaction mixtures were utilized, consisting of 2 μl cDNA, 0.2 mM dNTP, 1.5 mM MgCl2, 0.25 μM gene-specific forward and reverse primers, and 2.5 U Platinum® Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). Reaction mixes were heated to 94°C for 2 min, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 min (final cycle: 72°C for 5 min) using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplified products were analyzed on 1.5% Agarose/Tris-Acetate-EDTA gels stained with ethidium bromide.

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