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The humoral immune response to head and neck cancer antigens as defined by the serological analysis of tumor antigens by recombinant cDNA expression cloning

Hilary A. Vaughan[1], Fiona St Clair[1], Matthew J. Scanlan[2*], Yao-Tsen Chen[3], Eugene Maraskovsky[1], Andrew Sizeland[4], Lloyd J. Old[2], and Jonathan Cebon[1]

[1]Cancer Vaccine Laboratory, Ludwig Institute for Cancer Research, Austin Health, Studley Road, Heidelberg, 3084, Australia
[2]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY, USA
[3]Dept. of Pathology, Weill Medical College of Cornell University, New York, USA
[4]Peter MacCallum Cancer Institute, Melbourne, Australia
[*]Deceased

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Abstract

Learning to identify tumor and tumor-associated antigens in patients with squamous cell carcinoma of the head and neck (HNSCC) may bring about better diagnostic and prognostic evaluations of the disease, innovative therapies based on immunological approaches, and a better understanding of the biology of tumorigenesis. Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX) has been used to identify antigens in head and neck cancer to which patients have produced high-titered IgG antibodies. Four cDNA expression libraries have been screened with sera from 6 head and neck cancer patients. Thirty-seven individual gene products were identified. Thirty-one previously characterized proteins and 6 genes coding for molecules that are only partially characterized or novel were isolated. Tissue expression was evaluated by Northern blot analysis, RT-PCR, and in one case, quantitative real-time PCR (qPCR) using Taqman™ technology. Clone AU-HN-15 encoded a protein highly expressed in HNSCC tissues and cell lines. Tissue adjacent to the tumor had negligible expression. There was low or negligible expression in normal tissues, except for the brain and thymus. AU-HN-15 is identical to KIAA0530; it is an uncharacterized protein previously cloned from brain tissue and has a zinc finger domain. The cDNA encoding this protein has also been isolated in SEREX screens of testicular cancer, breast cancer, and colorectal cancer. Whether AU-HN-15 represents a tumor-antigen target suitable for prognostic or therapeutic purposes is still being analyzed.

Introduction

The incidence of HNSCC is increasing globally, and the survival rate of patients with this disease has not changed substantially over the past 2 decades (1). When advanced, these tumors are difficult to treat, and existing therapies often do not offer long-term disease control. The role of immunotherapy in this setting is problematic, since it is well recognized that patients with HNSCC have defective immune responses (2, 3). There

are therefore at least two major challenges in developing targeted immunotherapeutic regimes for these patients. First, it is necessary to understand and deal with defective immune function in these patients. Second, it is necessary to identify suitable tumor antigens to target, either by vaccination or with antibodies. Identifying the tumor antigens may also be an aid to diagnosing HNSCC and making a prognostic evaluation of the disease.

The first studies to successfully identify human tumor antigens that can be targeted by T lymphocytes used T-cell clones derived from cancer patients. As a result, MAGE-1 was identified (4), and in subsequent years a growing family of tumor antigens was found (5). These include molecules referred to as the cancer/testis antigens, whose expression is restricted to some cancers and testis (6, 7, 8), as well as molecules that are normally expressed in a very narrow range of cell types, such as melanocyte differentiation antigens (5). In other instances, genetic mutations causing aberrant expression or over-expression appeared to underlie the immunogenicity of molecules that are also commonly expressed in nonmalignant tissue (9, 10). Transcripts encoding cancer/testis antigens have been detected in HNSCC. For example, mRNAs encoding CT-7/MAGE-C1 and SSX antigens have been detected in 35% and 57% of HNSCC, respectively (11, 12). Other tumor antigens have also been identified in HNSCC by immunological techniques (12). Autologous CTLs that recognize a CASP-8 mutation on HNSCC tumor cells were found (13). In another study, CTLs established from an esophageal carcinoma recognized an HLA-A2601-restricted peptide encoded by the *SART-1* gene. Both *SART-1* alternative splicing products, the one localizing to the cytosol (14) and the 43 kDa protein localizing to the nucleus (15), are found in squamous cell carcinomas including HNSCC, and in adenocarcinomas (15, 16).

The analysis of cancer antigens advanced considerably with the introduction of the expression cloning technique known as SEREX (17, 18). SEREX utilizes the sera of cancer patients, which contain antibodies against a variety of tumor antigens, to screen for tumor antigens in cDNA expression libraries constructed from tumor tissue or cell lines. This technique has proven to be a powerful means of identifying antigens recognized by T cells as well as antibodies, and a number of molecules first discovered using T-cell-based methods have subsequently also been identified by SEREX. Cancer/testis antigens have been identified, including NY-ESO-1 (6), CT-7/MAGE-C1 (11), and members of the SSX family (12). The humoral and cellular immune responses against NY-ESO-1 found in melanoma patients is of clinical importance, and HLA class 1 and class 2 binding epitopes were defined (19, 20, 21, 22, 23, 24, 25). Therefore, NY-ESO-1, identified by SEREX, also elicits a specific CTL response that is essential for successful vaccine protocols, and tumor antigens such as NY-ESO-1 are proving to be promising candidate molecules for cancer vaccines (22, 26, 27).

The purpose of this study is to use SEREX in a detailed study of HNSCC in an attempt to identify potential new candidate molecules for vaccine therapy, and to broaden our understanding of the relationship between tumor antigens and the immune response in HNSCC. Four separate SEREX screens were undertaken, using different approaches. We describe the cloning and tissue distribution of antigens identified in these SEREX screens.

Results

Identity of cDNA clones isolated from HNSCC expression libraries

Fifty-four primary clones were reactive with cancer sera and did not secrete immunoglobulin. These clones were selected for further analysis and screened to clonality, *in vivo* excisions were performed, and plasmid DNA was isolated. Preliminary DNA sequencing data was obtained using T3 and T7 primers, and this sequence was used to search DNA databases to establish the identity of the proteins encoded by the cDNA clones and to compare

them with molecules isolated from other SEREX screens. The 54 clones encoded 37 individual proteins (Tables 1, 2 and 3), of which 48 clones (corresponding to 31 proteins) encoded known, characterized genes (Tables 1 and 2). The remaining 6 clones (corresponding to 6 proteins) encode molecules that are either novel or partially characterized (Table 3). A putative function is suggested for the proteins encoded by some of these genes, which fall into several groups; for example, regulatory molecules (AU-HN-18 and -23) and proteins with DNA binding properties (AU-HN-2, -4, -5, -10, -11, -16, -17, -27, and -37), RNA processing/translation (AU-HN-3, -13, -30, -31, -33, and -34); and apoptosis regulatory molecules (AU-HN-14 and -20). In addition, we isolated 6 proteins that had no known function (AU-HN-7, -8, -9, -15, -19, and -36). Some genes have been identified more than once in HNSCC SEREX screens and have also been identified in SEREX screens of other tumors (Table 2), confirming that the isolation of these genes in HNSCC is reliable.

Table 2. Correspondence of HNSCC clones with other SEREX clones.

Clone ID	SEREX Clones	Tumor Type
AU-HN-1	NGO-Lu-8	lung
AU-HN-4	P2B-25f, NGO-Br-5, NY-REN-63, MO-BR-100, RMNY-BR-52, MO-OVA-76, NY-CO-6, HOM-TS-GL1-16, TM-92, TE2-7	breast, renal, ovary, colon, normal testis
AU-HN-11	HOM-TSOv2.35, HOM-Ts-PMR2-12	normal testis
AU-HN-12	NGO-St-79, MO-REN-80, NY-REN-48, NGO-Br-47, NY-ESO-10, UL-AML-4, NGO-Co-8	stomach, renal, breast, esophagus, AML, colon
AU-HN-14	MO-BC-1128, se20-9, NGO-Lu-33	lung
AU-HN-16	MO-CO-135, MO-CO-2	colon
AU-HN-17	NGO-Pr-44	prostate
AU-HN-18	NGO-St-29, LONY-BR-81, MO-CO-103	stomach, breast, colon
AU-HN-24	NY-REN-14	renal
AU-HN-26	NY-CO-18	colon
AU-HN-27	NY-CO-2, NW-FW 22, NGO-Lu-27	colon, lung
AU-HN-28	NY-BR-101, NGO-Br-52	breast
AU-HN-35	NGO-Lu-17, 6-32, 6-25.1, 6-8.1, 6.26.1, 6.27	lung, liver

Table 3. HNSCC SEREX clones corresponding to novel or not fully characterized genes.

Function	Clone No.	Identity	Previously Identified SEREX[a] Clones	Tumor Type	Unigene Cluster
Regulatory/ DNA binding	AU-HN-9	unknown	no		Hs.95262
	AU-HN-15	KIAA0530, zinc finger domain	HOM-TSsemA-56, NY-CO-6, MOBr-17	colon, breast, normal testis	Hs.173081
TNF-inducible	AU-HN-36	rat stannin	no		Hs.76691
Unknown	AU-HN-7	hypothetical protein DKFZp4340032	no		Hs.114173
	AU-HN-8	human FLJ23089	no		Hs.130554
	AU-HN-19	PC326	HOM-TSsem4.95, P2A-89, HOM-TSMa4.9, MOVA95, HOM-HCC-27.8, HOM-Br3.40, Mz19-F-45	prostate, liver, melanoma, lung, ovary, normal testis	Hs.279882

[a]SEREX database (36).

Table 1. HNSCC clones corresponding to known genes.

Functional Group	Clone ID	No. of Times Isolated	Identity	Unigene Cluster	Previously Identified by SEREX
DNA binding/ Transcription	AU-HN-2	1	TREX1, 3' repair exonuclease	Hs.278408	no
	AU-HN-4	2	BRAP (BRCA1 associated): Nuclear localization protein	Hs.122764	yes
	AU-HN-5	1	Trim 26 zinc finger domains	Hs.1287	no
	AU-HN-10	1	GTF21 transcription factor. Murine homolog TF11-1	Hs.278589	no
	AU-HN-11	6	NSEP1 (YB-1) transcription factor	Hs.74497	yes
	AU-HN-16	1	MAZ transcription factor associated with *c-myc*	Hs.7647	yes
	AU-HN-17	1	SON (DBP-5; KIAA1019; NREBP DNA binding protein)	Hs.92909	yes
	AU-HN-27	1	NACA nascent polypeptide-associated complex	Hs.32916	yes
	AU-HN-37	1	NUBP2 nucleotide binding protein	Hs.256549	no
RNA processing/ Translation	AU-HN-3	1	EEF2 Translation elongation factor 2	Hs.75309	no
	AU-HN-13	2	GU2 Putative RNA helicase	Hs.7392	no
	AU-HN-30	1	RPLI3A ribosomal protein	Hs.119122	no
	AU-HN-31	1	SFRS21P (CASP11; SIP1; SRRP1290 splicing factor)	Hs.51957	no
	AU-HN-33	1	RPS12 ribosomal protein	Hs.339696	no
	AU-HN-34	1	MGC2835 RNA helicase	Hs.70582	no
Regulatory	AU-HN-18	1	TMF1, TATA modulatory factor	Hs.267632	yes
	AU-HN-23	1	PRC1 regulator of cytokinesis	Hs.344037	no
Structural	AU-HN-22	2	KRT14 keratin 14	Hs.355214	no
	AU-HN-28	1	Viniculin	Hs.75350	yes
	AU-HN-32	1	H2AFY histone family member	Hs.75258	no
Apoptosis repressor	AU-HN-14	2	SLK (KIAA02304) Ste related kinase	Hs.105751	no
	AU-HN-20	2	NOL3 (ARC) nuclear protein 3, apoptosis repressor	Hs.278439	no
Molecular chaperone	AU-HN-24	1	DNAJA2 member of Hsp40 family	Hs.21189	yes
	AU-HN-35	1	DNAJA1 member of HSP40 family	Hs.94	yes
Other	AU-HN-1	1	LINE-1 retrotransposon	No	yes
	AU-HN-6	1	MOG (HSPC 165) Homolog of yeast protein	Hs.13605	no
	AU-HN-12	4	LIMS1 (PINCH): LIM and senescent antigen-like domain	Hs.112378, Hs.193700	yes
	AU-HN-21	2	COPB2 coatomer protein complex subunit protein	Hs.75724	no
	AU-HN-25	1	FLJ22548 hypothetical protein	Hs.103267	no
	AU-HN-26	2	C21orf97; FLJ21324; MGC15873	Hs.4746	yes
	AU-HN-29	3	SSNA1 Sjogrens syndrome nuclear autoantigen 1	Hs.18528	no

Northern blot analysis

The normal tissue mRNA expression patterns of 11/36 clones identified in the current study were evaluated by Northern blot analysis. These clones were selected because they initially appeared to be novel. Of these 11 clones, only 5 showed differential or no expression in the normal tissues tested. AU-HN-4 and AU-HN-18 were only expressed in testis with bands of 2.0 kb and 3.9 kb, respectively (Figure 1, panels A and B). AU-HN-25 was expressed at a very high level in testis (2 kb) and at a much lower level in spleen, prostate, and colon (Figure 1C). AU-HN-6, obtained from the testis library, was also expressed strongly in testis (1 kb), with apparent low-level expression in the other tissues tested (Figure 1D). There was one clone, AU-HN-15, which was negative by Northern blot analysis on an extensive panel of normal tissues (Table 4) and was considered a potential target molecule for immunotherapy. Messenger RNA transcripts of all the remaining cDNA clones were expressed to a varying degree in normal tissues, as determined by Northern blot analyses (Table 4).

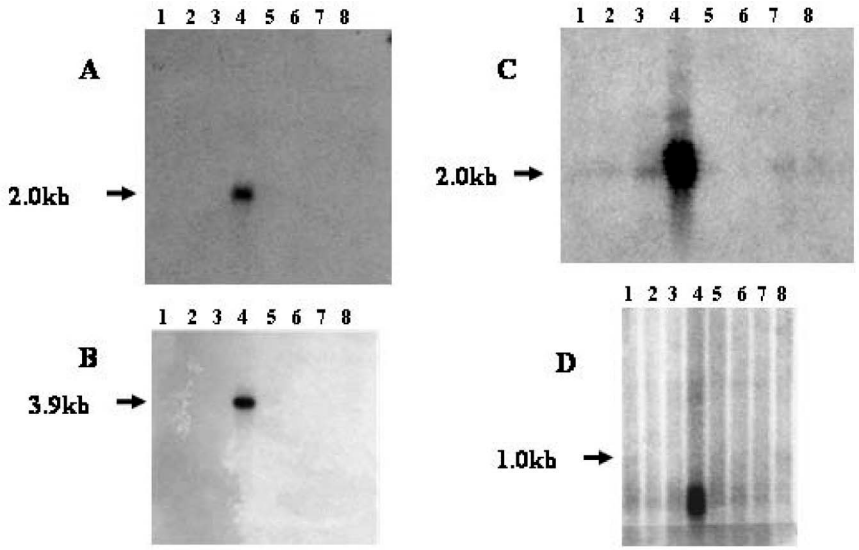

Figure 1. Northern blot analysis of HNSCC SEREX clones. The expression of clones AU-HN-4 (A), AU-HN-18 (B), AU-HN-25 (C), and AU-HN-6 (D) was assayed in the following normal human tissues: (1) spleen, (2) thymus, (3) prostate, (4) testis, (5) ovary, (6) small intestine, (7) colon, and (8) lymphocytes.

RT-PCR

The five transcripts showing differential expression by Northern blot analysis were tested further by performing RT-PCR on normal tissue mRNA. Although Northern blot analysis of normal tissues detected no transcripts encoding AU-HN-15, transcripts were detected in thymus, but not in testis (five samples), muscle, stomach (two samples), uterus, or colon (three samples) when analyzed by RT-PCR. AU-HN-15 was also detected in 5/8 HNSCC tissue samples and in 3/4 HNSCC cell lines (Figure 2). Normal tissues adjacent to AU-HN-15-expressing tumor samples were negative for AU-HN-15 mRNA expression. Conventional RT-PCR is not truly quantitative, and therefore AU-HN-15 expression was analyzed further in a larger panel of normal mRNA transcripts using qPCR. The remaining four transcripts that were differentially expressed by Northern blot analysis were found to be expressed in all tissues when tested by RT-PCR.

Table 4. Novel antigens identified in HNSCC by SEREX: summary of Northern blot analyses.

Tissue	Expression[a] of Clone AU-HN-										
	2	4	6	9	14	15	18	20	23	25	34
Spleen	++	-	-	+	+	-	-	+	nt	-	nt
Thymus	+	-	-	+	+	-	-	+	nt	(+)	nt
Prostate	++	-	-	+	+	-	-	+	nt	+	nt
Testis	+	++	+++	+	+	-	++	+++	nt	+++	nt
Lymphocytes	++	-	-	+	+	-	-	+	nt	(+)	nt
Ovary	+	-	-	+	+	-	-	+	nt	+	nt
Small intestine	+	-	-	+	+	-	-	+	nt	-	nt
Colon	+	-	-	+	+	-	-	+	-	+	-
Brain	nt	nt	nt	nt	nt	-	nt	nt	-	nt	+
Heart	nt	nt	nt	nt	nt	-	nt	nt	-	nt	+
Kidney	nt	nt	nt	nt	nt	-	nt	nt	++	nt	+
Liver	nt	nt	nt	nt	nt	-	nt	nt	++	nt	+
Lung	nt	nt	nt	nt	nt	-	nt	nt	-	nt	-

[a]Expression: +++, high expression; ++, medium expression; +, low expression; (+), very weak; nt, not tested.

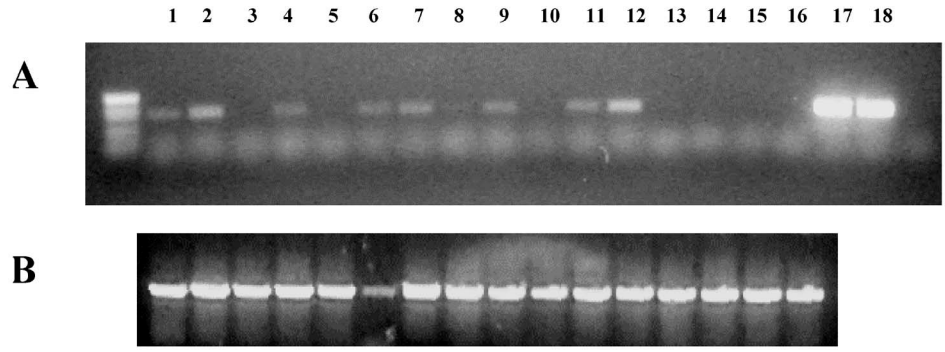

Figure 2. AU-HN-15 expression in HNSCC tumors. The expression of AU-HN-15 (A) and beta-actin (B) was determined by RT-PCR in the following samples: (1-8) HNSCC tumor tissue; (9-12) HNSCC cell lines: SCC-4 (9), HTB-43 (10), SCC-25 (11) and CCL-30 (12); (13-16) tissue adjacent to HNSCC tumor; (17-18) AU-HN-15 plasmid.

Real-time quantitative RT-PCR

Expression of AU-HN-15 was found in normal brain (305 copies), normal trachea (90 copies), and normal thymus tissues (190 copies) (Figure 3A). The expression levels in other normal tissues tested were negligible (0 to 40 copies). Five of 11 HNSCC tissues showed expression of 90 to 410 copies, and the remaining 6 ranged in expression levels from 10 to 80 copies. Two of the cell lines tested showed significant expression of AU-HN-15: SCC-25 had 260 copies and CCL-30 had 1,500 copies (Figure 3B). More importantly, there was up-regulation of AU-HN-15 in HNSCC tumors or HNSCC tumor-positive lymph nodes as compared to paired adjacent tissue (Figure 3C). The expression of AU-HN-15 in tumor tissue compared to paired adjacent tissue was 7 times higher in HNSCC tumor 1, 5 times higher in tumor 2, 7.5 times higher in tumor 3, and 10 times higher in lymph node with metastatic lesions. It should be noted that although tissue adjacent to these tumors may have appeared normal by microscopy, some undetectable changes may have occurred, so that these mucosae may not have been entirely normal.

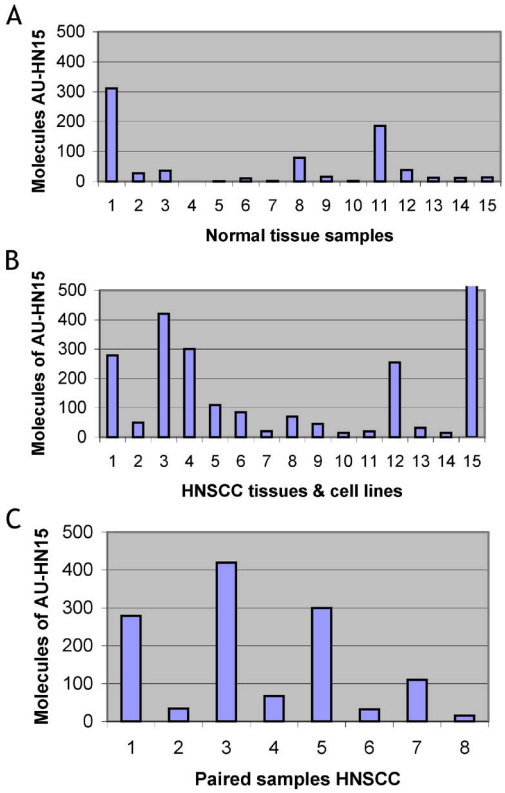

Figure 3. Quantification of clone AU-HN-15 mRNA in normal and tumor tissues by qRT-PCR. Messenger RNA copy number is expressed as the number of AU-HN-15 molecules in 100 ng of total RNA per 10^5 molecules of beta-glucoronidase. (A) Normal tissues: (1) brain, (2) testis-1, (3) colon, (4) liver, (5) testis-2, (6) kidney-1, (7) lung, (8) trachea, (9) small intestine, (10) stomach, (11) thymus, (12) heart, (13), spleen, (14) bone marrow, (15) kidney-2. (B) HNSCC tumor tissues (1-11) and HNSCC cell lines (12-15): SCC-25 (12), HTB-4B (13), SCC-4 (14), CCL-30 (15). (C) Paired samples: HNSCC (1, 3, 5) and adjacent mucosae (2, 4, 6) from three primary tumors; HNSCC metastatic to lymph node (7) and paired normal tissue (8).

Reactivity of sera from normal volunteers and HNSCC patients with cDNA clones isolated from HNSCC tumors

In order to further evaluate the immune response to these antigens in cancer patients, sera from healthy volunteers and from patients with HNSCC were tested for immune reactivity to the expressed clones AU-HN-15 and AU-HN-25 (data not shown). Initial testing had suggested that these clones were differentially expressed. In each case, the wild-type phagemid with no insert was negative, showing that the sera had no remaining cross-reactivity to bacterial proteins. AU-HN-15 showed no reactivity to any of the normal or tumor sera tested except for serum RL, which was used for screen 4. AU-HN-25 protein reacted with 2/9 normal sera and 3/15 HNSCC sera.

Since pooled sera were used in screen 5, we sought to determine the source of the immune responses in this screen by testing the serum from each source individually. Table 5 shows the results of testing the individual sera which made up the pool used for screen 5 with the isolated clones. Six of 19 clones reacted to one or both of the sera from HNSCC patients RM and DG, 6/19 clones reacted only to the non-HNSCC patient's serum BT, and 7/19 clones reacted to HNSCC sera and BT serum. Different clones from the same gene showed varying serological reactivity: that is, clones AU-HN-26 (a, b, c) and -29 (a, b, c). This probably reflects the different protein epitopes encoded by the same gene.

Table 5. Serological screening of the HNSCC screen 5 clones with the individual HNSCC sera making up the pool used for the screening.

Clone No.	Identity	Serum		
		BT	RM	DG
AU-HN-12	PINCH	+	+	+
AU-HN-22	keratin	-	-	+
AU-HN-23	human Hsp DNAj	(+)[a]	-	-
AU-HN-25	FLJ22548	-	-	+
AU-HN-26a	C21orf97, FDJ21324	(+)	-	-
AU-HN-26b		+	+	-
AU-HN-27	NACN, transcription activator	-	+	+
AU-HN-28	vinculin	-	+	+
AU-HN-29a	Sjogren's nuclear antigen NA14	+	-	+
AU-HN-29b		(+)	-	-
AU-HN-29c		+	+	+
AU-HN-30	L13a ribosomal protein	(+)	-	-
AU-HN-31	SFRS21 splicing factor	-	+	-
AU-HN-32	H2A.1 histone	+	-	+
AU-HN-33	S12 ribosomal protein	-	+	-
AU-HN-34	RNA helicase related	(+)	-	-
AU-HN-35	human Hsp DNAj	(+)	-	-
AU-HN-36	rat stannin related	+	+	-
AU-HN-97	laminin receptor	+	+	+

[a](+) HNSCC clones positive only with BT serum (adenocarcinoma).

Discussion

Four SEREX screens were carried out in order to evaluate antigens in HNSCC. The initial screen used autologous serum to screen an HNSCC cDNA expression library according to the original method described by Sahin (17). A normal testis cDNA library (Stratagene, La Jolla, CA) was then screened with serum from an HNSCC patient to preferentially identify cancer/testis antigens (6). The yield of clones from both these screens was low. In an attempt to improve yields, a cDNA library was constructed from a HNSCC cell line and screened with HNSCC serum from a patient with a high tumor burden. The final screen used a pool of HNSCC sera and a library made from HNSCC tumor tissue (Table 6). Fifty-four cDNA clones were isolated from these cDNA libraries. They consisted of 37 individual genes, of which 6 (16%) were partially characterized or novel. All of the genes identified are expressed in normal tissues as well as in cancer tissues. Of the novel gene products identified, AU-HN-15 showed increased expression in HNSCC tumors and cell lines, and low-level expression in most normal tissues. Some of the clones identified in HNSCC by expression cloning were previously reported to be associated with cancer. The proteins fell into distinct functional groups; that is, regulatory molecules, DNA binding proteins, molecules associated with apoptosis control, proteins involved in RNA processing and translation, and structural proteins. SEREX has shown that most cancer patients have an autologous, humoral response to proteins expressed in their tumors, and that many of the same molecules elicit an immune response in patients with tumors of different types. This study shows that this is also the case in HNSCC, despite the immune dysfunction reported in such patients (2, 3). The previous analysis of SEREX antigens showed that approximately 30% are novel antigens, that the majority are expressed on normal and tumor tissue, and that most SEREX antigens are intracellular (18). The antigens found in this study had similar characteristics.

Table 6. Description of SEREX screenings.

Screen No.	Tissue	Serum (dilution)	No. of pfu Screened	No. of Clones
1	JK[a] tumor	JK[a] (1:100)	5×10^5	3
2	normal testis	JK[a] (1:100)	1×10^6	9
4	SCC-25[b]	RL[c] (1:100)	1×10^6	19
5	RM[d] tumor	pool: RM[d] (1:100), DG[e] (1:150), and BT[f] (1:200)	9×10^5	19

[a]JK: Right neck dissection, LN with metastatic moderately differentiated keratinizing SCC.
[b]SCC-25: Cell line established from SCC of the tongue.
[c]RL: Neck dissection, metastatic moderately differentiated SCC from primary tongue lesion; large tumor burden.
[d]RM: Left cheek tumor, ulcerating moderately differentiated keratinizing SCC.
[e]DG: HNSCC Stage 4; large tumor burden.
[f]BT: Neck dissection, metastatic adenocarcinoma.

In previous analyses, SEREX identified genes known to be associated with cancer, such as oncogenes and tumor-suppressor genes (18). In the current study, cDNA clones that code for proteins that may be associated with cancer were also isolated, as shown in Tables 1, 2, and 3. For example, NSEP1 (YB-1) was identified in 6 clones from screens 2 and 4 (AU-HN-11) (Table 1). NSEP1 encodes a transcription factor, and the nuclear localization of this protein is known to be associated with the expression of the multidrug resistance gene *MDR-1*, which encodes P-glycoprotein (28, 29, 30). Another clone, AU-HN-16 (Tables 1 and 2), encodes the transcription factor MAZ, which regulates the expression of the oncogene *c-myc* and is expressed in many cancers (31, 32). Two AU-HN-4 clones (Tables 1 and 2) are homologous to BRAP2, a DNA binding protein that binds to nuclear localization motifs in the breast cancer-associated tumor-suppressor gene, *BRCA-1* (33). Interestingly, several SEREX antigens identified from other tumor expression libraries also have identity with *BRAP2* (Table 2) (34, 35, 36); however, the function of this gene is not yet clear, and antibodies to this molecule are found in normal sera.

Regulatory molecules and proteins associated with the cell cycle and proliferation have been isolated in SEREX screens of other tumors (18), as was the case in this SEREX analysis of HNSCC. AU-HN-20 (two clones) is homologous to *NOL3* (ARC), a gene situated on chromosome 16q21-q23 (Table 1). The protein encoded by this gene is implicated in the repression of apoptosis and has been shown to have a CARD domain and to inhibit caspase 2 and caspase 8 (37). Two other clones corresponding to AU-HN-14 (Table 1) encode a protein that is also involved in the regulation of apoptosis, an Ste2-related serine/threonine kinase (SLK) (38). The mouse homolog SMAK activates an apoptosis-inducing kinase domain and an actin-disassembling region (39). AU-HN-18 is homologous to another gene product involved in gene regulation that encodes a TATA element modulatory factor TMF1 (Table 1), located on chromosome 3p21-p12. This is a site of frequent rearrangements in lung and renal cancer (40), and gene products from this region have been detected in other SEREX screens (Table 2) (36). The FER nuclear tyrosine kinases phosphorylate a tyrosine residue of this molecule. AU-HN-17 (Table 1) has sequence homology with a gene encoding a negative regulatory element binding protein. The protein contains a leucine zipper motif (41) and has recently been shown to repress transcription of human hepatitis B virus genes (41). The DNA sequence of the negative regulatory element binding protein (GenBank Accession No. AP000046) is homologous to that of DBP-5, SONB, and SONA (GenBank Accession Nos. X63071, X63751, and X63753, respectively), which are thought to be either partial gene sequences or splice variants of the

negative regulatory element binding protein (41). One other SEREX clone similar to AU-HN-17 has been isolated from a prostate cancer library, NGO-Pr-44 (36). AU-HN-23 (Table 1) is homologous to PRC1, a human mitotic spindle-associated, cyclin-dependent kinase (CDK) substrate protein that is required for cytokinesis and is thus associated with the cell cycle (42). This molecule is a good substrate for several CDKs *in vitro* and is phosphorylated *in vivo* by CDK at sites that are phosphorylated by CDK *in vitro*. PRC1 levels are high during S and G2/M and drop dramatically after cells exit mitosis and enter the G1 phase. Injection of anti-PRC1 antibodies into HeLa cells blocked cellular cleavage but not nuclear division, thereby indicating a role for PRC1 in cytokinesis (42).

Of the uncharacterized gene products identified in this study, most are expressed in normal tissue, as demonstrated by Northern blot analysis and/or RT-PCR. Six of these do not fall into any known functional group. Clone number AU-HN-7 (Table 3), also isolated in screen 2 from the testis library with serum JK, had not been identified in previous SEREX screens. This gene, which maps to human chromosome 11, encodes a putative protein named DKFZp4340032, but nothing is known of its function. Another clone from the testis library AU-HN-8 (Table 3) mapped to chromosome 8 and is thought to encode a putative protein FLJ23089 (GenBank Accession No. AK026742). The cDNA sequence of AU-HN-19 (Table 3), is homologous to sequences located on chromosome 1 which encode a protein, PC326, originally cloned from human adrenal gland (GenBank Accession No. AF150734). The sequence of AU-HN-19 has a 60 base pair deletion when compared to the human PC326 sequence (bp 1034-1094). The gene encoding the putative murine homolog of PC326 (GenBank Accession No. M95564) was expressed in plasmacytomas, but not in normal plasma cells (43). This gene has also been identified in SEREX screens of testis (Hom-TSSem4.95), prostate cancer (P2A-89), hepatocarcinoma (HOM-HCC-27.8), melanoma cell line (Mz19-F-45), bronchial carcinoma (HOM-Br3-40), and ovarian cancer (MOVA 95) (36).

Finally, AU-HN-15 (Table 3) has a DNA sequence identical to that of KIAA0530, a sequence listed in the HUGE database for large uncharacterized proteins (44). This sequence encodes a putative protein of 1,563 amino acids that contains a zinc finger motif, suggesting it may be a DNA binding protein. DNA sequencing of the complete AU-HN-15 cDNA insert isolated from screen 4 showed 100% identity with the published sequence of KIAA0530 (data not shown). Unlike the other HNSCC clones, AU-HN-15 was not expressed in normal tissues when tested by Northern blot analysis. Further analysis by RT-PCR showed differential expression in normal and tumor tissue. The expression levels of AU-HN-15 appear to be up-regulated in some HNSCC tumors and cell lines. To study this further, mRNA expression was evaluated by qPCR, and expression levels were quantitated, showing that brain and thymus have the highest expression in normal tissue. Expression levels of AU-HN-15 were up-regulated in HNSCC tissues and cell lines when compared to most normal tissues and to tissue adjacent to tumor. Interestingly, sequences homologous to KIAA0530 have been isolated in other SEREX screens (34, 35, 36). The relevance of antibodies to KIAA0530, which have been previously reported in some normal sera (35), and to a cancer-specific immune response, is not known. However, it has been demonstrated that autoantibodies can be detected in sera several months before diagnosis of malignancy, for example in small cell lung cancer, and it has been suggested that the presence of these antibodies may be useful in early diagnosis (45).

Serological analysis of the products of two of the HNSCC cDNA clones, when tested with both normal and HNSCC sera, showed humoral responses directed against AU-HN-25 in 2/9 normal sera, as well as in 2/13 HNSCC sera (data not shown). There was no reactivity of the AU-HN-15 gene product with any of the sera tested except for serum RL (HNSCC), which was the serum used for the screen. Further testing of cancer sera for reactivity to AU-HN-15 may be of value. When using the individual sera from the screen 5 pool and testing the individual cDNA clones identified in screen 5 (Table 5), 3/19 reacted with all the cDNA clones, 6/19 with 2 of 3 sera, and 10/20 with only 1 serum. Six of the cDNA protein products reacted only with serum BT (non-HNSCC); the remainder reacted either with HNSCC sera alone or with both HNSCC and BT (non-HNSCC) sera. All of the cDNA clones isolated from screen 5 showed expression by Northern blot analysis in normal tissues and therefore will not be of use in immunotherapy trials. However, further testing of expression levels of these gene products in cancer patients may be of use in dissecting the humoral response of cancer patients to their tumors.

Although the four SEREX screens did not identify antigens that are strictly tumor specific or HNSCC specific, differential expression of AU-HN-15 was seen with over-expression in HNSCC tumor tissue. Antigens that are over-expressed in tumors, such as p53 or HER-2/*neu*, are being investigated as potential targets for immunotherapy, even though low-level expression is seen in normal tissues (10, 46). Over-expressed antigens have been isolated in previous SEREX screens and include carbonic anhydrase X11 in renal cancer (47); galectin-9/HOM-HD-21 in Hodgkin's disease (48); *KOC* family genes in melanoma (11), and e1F-4-gamma and aldose A in lung cancer (49, 50). The over-expression of these proteins may be the reason underlying their immunogenicity in cancer patients. In terms of CTL responses, it is not known what level of antigen expression/presentation is required to render cells targets for immune recognition. Clearly, the relationship between mRNA expression and antigen presentation is important and may vary from antigen to antigen. Nonetheless, quantitation of antigen expression by qPCR is proving to be a useful tool for identifying antigens that are over-expressed in tumors relative to normal tissue (51). Some such antigens may prove to be useful targets for immune recognition once the rules governing the threshold required for that recognition are understood. In addition, these over-expressed antigens may have potential as immunological markers for the diagnostic/prognostic evaluation of patients. Studies of the expression levels of these antigens in other cancers will also be useful.

Abbreviations

CDK, cyclin dependent kinase; HNSCC, squamous cell carcinoma of the head and neck; qPCR, quantitative real-time PCR; SEREX, serological analysis of tumor antigens by recombinant cDNA expression cloning

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Materials and methods

Cell lines and tissues

Cell line SCC-25 (squamous cell carcinoma of the tongue) was obtained from Dr. Nick Saunders (University of Queensland Department of Medicine, Woolloonggabba, Australia). The following cell lines were obtained from the American Tissue Type Collection (Rockville, MD): SCC-4 (ATCC CRL-1624), SCC-15 (ATCC CRL-1623, squamous cell carcinoma of the tongue), FaDu (ATCC HTB-43, squamous cell carcinoma of the pharynx), and RPMI 2650 (ATCC CCL-30, quasi diploid tumor). Cell lines were cultured in a 1:1 mix of HAMS F12 and Dulbecco's modified Eagles medium (Life Technologies, Rockville, MD) with 0.4 µg/ml hydrocortisone, 10% heat inactivated fetal bovine serum (Commonwealth Serum Laboratories, Melbourne, Australia). Samples of normal and tumor tissue were snap frozen in liquid nitrogen and stored at -70°C prior to processing for RNA. Institutional review board approval for the use of specimens from human subjects was obtained.

Construction and screening of cDNA expression libraries

Total RNA was extracted using TRI reagent (Sigma, St. Louis, MO). Messenger RNA was purified using the Fast Track 2.0 kit (Invitrogen, The Netherlands), and libraries were constructed in lambda-ZAP (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The libraries were constructed using mRNA isolated from testis, cancer tissue, or tumor cell lines and were screened with sera from HNSCC patients as outlined in Table 6. The sera were preabsorbed with lysates of *E. coli* immobilized on Sepharose 4B (5 Prime-3 Prime Inc., Boulder, CO) to eliminate cross-reactivity with bacterial proteins. Positive clones that did not secrete immunoglobulin were identified as described previously (6). Reactive clones were screened to clonality and the plasmid DNA was extracted and then sequenced using T3 and T7 primers (6). The results of DNA sequencing were compared with various nucleic acid databases (GenBank and EMBL) to identify known genes, and with the SEREX database (36) in order to identify antigens with previous histories of seroreactivity in cancer patients.

Northern blot analysis

Normal tissue RNA blots containing 2 µg of mRNA were obtained from either Clontech (Palo Alto, CA), or Origene (Rockville, MA) and hybridized at 65°C with purified cDNA from seropositive clones labeled with ^{32}P according to the manufacturer's instructions. After hybridization, the filters were washed at high stringency and analyzed by autoradiography.

RNA extraction and RT-PCR

Total RNA was isolated from normal or tumor tissue that had been snap frozen and stored at -70°C, or from cultured tumor cell lines, using a modified method of Chomczynski and Sacchi (52). Briefly, the tissue was homogenized in TRI reagent (Sigma), extracted with phenol, and the RNA was precipitated with isopropanol. RT-PCR was used to analyze the tissue distribution of gene expression of molecules identified in the SEREX screens. Two micrograms of total RNA was used to synthesize cDNA in a 20 µl reaction, using 400 ng random hexamers (Promega, Madison, WI), 1 mM dNTPs (Applied Biosystems, Foster City, CA), 2 units RNAse inhibitor (Promega), and 2 units M-MLV reverse transcriptase (Life Technologies) for 60 min at 37°C. One microliter of the resulting 20 µl cDNA (corresponding to 100 ng total RNA) was used in a PCR reaction. PCR primers were designed to amplify a product of approximately 300 bp from the 5' sequence. In order to maximize the yield of the PCR product, touchdown PCR was performed, where the annealing temperature of the primers was reduced half a degree per cycle. A 25 µl reaction was set up with a final concentration of 2 mM $MgCl_2$, 0.2 mM dNTPs (Applied Biosystems), 0.005 units Amplitaq Gold DNA polymerase (Applied Biosystems), and 2 ng of primers (Sigma). PCR products were analyzed on agarose gels.

Real-time qPCR

In order to quantify the copy number of mRNA of the AU-HN-15 gene in various tissues, real-time qPCR was performed using a Taqman 7700 (Applied Biosystems) (53). Complementary DNA was synthesized as described earlier. One microliter of cDNA (corresponding to 100 ng of total RNA) was used as template, and the PCR was set up according to the manufacturer's instructions. Reverse transcriptase negative controls were used to control for genomic contamination in the RNA preparations. A housekeeping gene (beta-glucuronidase) was used to normalize the cDNA samples. The primer and probe sequences for detecting AU-HN-15 were as follows:

Forward primer: 5'-CAGTATTCGGATGGTCTTTGTCAGT-3'
Reverse primer: 5'-TTAAGCCAATGGGATTTGAAGTATC-3'
Probe: 5'-FamCTTCACTGCAGACTCCTCAAGAAACTTCAGAAA-Tamra-3'

The copy number of AU-HN-15 was calculated from the standard curve obtained using known concentrations of the AU-HN-15 plasmid, taking into consideration controls for genomic contamination of RNA, and expressed per 10^5 copies of beta-glucuronidase in 100 ng of total RNA.

Serology

Sera from normal, healthy volunteers and from patients with HNSCC were tested for antibodies against two clones of potential interest, AU-HN-15 and AU-HN-25. In addition, cDNA clones isolated from screen 5 were tested against the three individual sera that made up the pool used for the screening. Sera were absorbed with *E. coli* and phage lysates as described previously (6), and tested against wild type lambda-ZAP to ensure that no cross-reactive antibodies remained. The absorbed sera was diluted at 1:200 and tested for reactivity by the plaque assay (6)

Contact

Address correspondence to:

Jonathan Cebon
Ludwig Institute for Cancer Research, Cancer Vaccine Laboratory
Austin Health
Studley Road, Heidelberg, VIC 3084
Australia
Tel.: + 61 3 9496-5462
Fax: + 61 3 9457-6698
E-mail: jonathan.cebon@ludwig.edu.au