Research Article

Identification of Human Aldehyde Dehydrogenase 1 Family Member A1 as a Novel CD8+ T-Cell–Defined Tumor Antigen in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Few epitopes are available for vaccination therapy of patients with squamous cell carcinoma of the head and neck (SCCHN). Using a tumor-specific CTL, aldehyde dehydrogenase 1 family member A1 (ALDH1A1) was identified as a novel tumor antigen in SCCHN. Mass spectral analysis of peptides in tumor-derived lysates was used to determine that the CTL line recognized the HLA-A*0201 (HLA-A2) binding ALDH1A1$_{88-96}$ peptide. Expression of ALDH1A1 in established SCCHN cell lines, normal mucosa, and primary keratinocytes was studied by quantitative reverse transcription-PCR and immunostaining. Protein expression was further defined by immunoblot analysis, whereas ALDH1A1 activity was measured using ALDEFLUOR. ALDH1A1$_{88-96}$ peptide was identified as an HLA-A2–restricted, naturally presented, CD8$^+$ T-cell–defined tumor peptide. ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells recognized only HLA-A2$^+$ SCCHN cell lines, which overexpressed ALDH1A1, as well as targets transfected with ALDH1A1 cDNA. Target recognition was blocked by anti-HLA class I and anti-HLA-A2 antibodies. SCCHN cell lines overexpressing ALDH1 had high enzymatic activity. ALDH1A1 protein was expressed in 12 of 17 SCCHN, and 30 of 40 dysplastic mucosa samples, but not in normal mucosa. ALDH1A1 expression levels in target cells correlated with their recognition by ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells. Our findings identify ALDH1A1, a metabolic antigen, as a potential target for vaccination therapy in the cohort of SCCHN subjects with tumors overexpressing this protein. A smaller cohort of subjects with SCCHN, whose tumors express little to no ALDH1A1, and thus are deficient in conversion of retinal to retinoic acid, could benefit from chemoprevention therapy. [Cancer Res 2007;67(21):10538–45]

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) occurs with a frequency of ~30,000 per year in the United States and is a major cause of morbidity and mortality (1). Because incidence and survival from SCCHN have only modestly improved over the past 30 years,[6] novel adjuvant therapies for this disease are urgently needed. Chemoprevention and immunotherapy represent two developing modalities for treatment of this disease.

Known risks for development of SCCHN as well as its progression and recurrence include excessive alcohol and tobacco use and decreased retinoic acid synthesis (2–4). These two factors link the human aldehyde dehydrogenases (ALDH) to development as well as chemoprevention of SCCHN. The ALDH1 family members, originally designated retinaldehyde dehydrogenase (RALDH) 1, RALDH2, and RALDH3, are involved in the synthesis of retinoic acids, which are critical for normal development and homeostasis of various tissues and organs (5–7). Retinoids, synthetic as well as naturally derived from vitamin A, alone or in combination with IFN-α and vitamin E, direct differentiation, modulate growth, and induce apoptosis of SCCHN and have been evaluated for chemoprevention of SCCHN (8–11). ALDH1 family member A1 (ALDH1A1) together with ALDH2 are involved in the conversion of acetaldehyde to acetic acid, the second step in alcohol metabolism. Consequently, decreased ALDH1A1 activity in tissues can contribute to elevated levels of acetaldehyde, a known carcinogen, and an increased risk for SCCHN development.

The identification of ALDH1A1 as a novel T-cell–defined target suggests that its epitopes could potentially be useful as immunogens. The key role of this enzyme in several biochemical pathways implicated in carcinogenesis further heightens its relevance for SCCHN development. In this article, we describe the immunologic properties of an HLA-A*0201 (HLA-A2)-restricted peptide, ALDH1A1$_{88-96}$, and its qualification as a candidate for use in developing vaccines for therapy of SCCHN that overexpress this antigen.

Materials and Methods

Cell lines, specimens, and antibodies. The SCCHN cell lines used in this study were either established at the University of Pittsburgh Cancer Institute (PCI series) or a gift of Dr. H. Bier (University of Düsseldorf, Düsseldorf, Germany). The T2 and MRC5 cell lines were obtained from the American Type Culture Collection (ATCC). Tumor cell lines were maintained in a complete medium consisting of RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 2 mmol/L L-glutamine, 50 μg/mL

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[6] U.S. Cancer Statistics Working Group. United States Cancer Statistics: 1999-2002 incidence and mortality Web-based report version. Atlanta: Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute; 2005. Available from: http://www.cdc.gov/cancer/npcr/uscs/.

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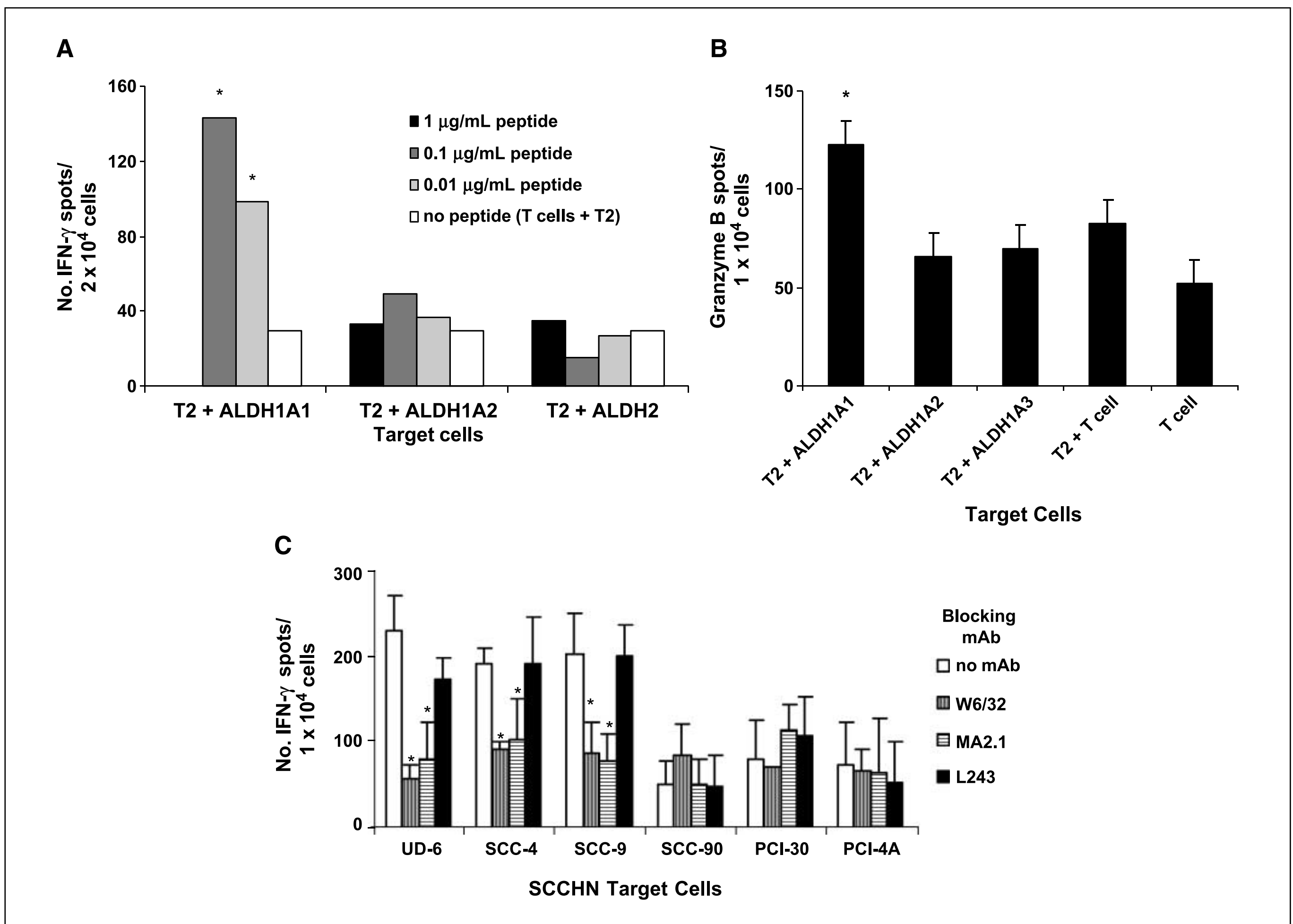

Figure 1. Reactivity of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells against peptide-pulsed target cells and SCCHN cell lines. *A* and *B,* ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T-cell line recognizes the ALDH1A1$_{88-96}$ peptide but not the sequence-related peptides derived from ALDH1A2 (LLDKLADLV) or ALDH2 (LLNRLADLI) in ELISPOT IFN-γ and granzyme B assays, respectively. *C,* ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells recognize three of five HLA-A2$^+$ SCCHN cell lines in ELISPOT IFN-γ assays, and this recognition was blocked by anti-HLA class I and anti-HLA-A2 mAb. These effectors did not recognize the HLA-A2$^+$ SCC-90 and PCI-30 cell lines nor the HLA-A2$^-$ SCCHN cell line PCI-4A. *, significant ($P < 0.05$) reactivity relative to other peptides and/or controls in *A* and *B* and significant anti-HLA class I and/or anti-HLA-A2 mAb blocking ($P < 0.05$) in *C*.

streptomycin, and 50 IU/mL penicillin (Life Technologies, Inc.). Human epidermal keratinocytes (HEKa) obtained from Cascade Biologics were maintained in Medium 154 supplemented with Human Keratinocyte Growth Supplement (Cascade Biologics).

Tumors, tissues, and venous blood specimens from consented subjects with SCCHN, including the PCI-13 patient (12–14), were obtained through the University of Pittsburgh Tissue Bank for Head and Neck Carcinoma under the Institutional Review Board (IRB)-approved protocol (IRB 991206). Blood samples were also obtained from HLA-A2$^+$ donors who signed informed consent (IRB 980633).

Polyclonal goat anti-ALDH1A1 NH$_2$-terminal peptide antibody was obtained from Santa Cruz Biotechnology. Purified anti-HLA class I (clone W6/32), anti-HLA-A2 (clone BB7.2), anti-HLA-A2 (clone MA2.1), and anti-HLA-DR (clone L243) monoclonal antibody (mAb) use in this study were from hybridomas obtained from ATCC. The anti-HLA-A mAb LGIII-147.4.1 was obtained from Dr. Soldano Ferrone (University of Pittsburgh Cancer Institute). PE-anti-CD34, ECD-anti-CD45RA, and PECy5-conjugated rabbit anti-mouse IgG antibodies were obtained from Beckman Coulter.

Isolation and analysis of PCI-13–derived peptides. HLA class I–associated peptides released from IFN-γ–treated PCI-13 cells by brief acid elution were resolved on reverse-phase high-performance liquid chromatography (HPLC) as described previously (15, 16). The mass of 2,905 species, which was present in the bioreactive fraction of HPLC-eluted PCI-13 peptides recognized by allogenic HLA-A2–restricted, anti-PCI-13–specific CD8$^+$ T cells, was fragmented for sequence analysis (17, 18).

***In vitro* stimulation of anti-ALDH1A1$_{88-96}$ CD8$^+$ T cells using peptide-pulsed autologous dendritic cells.** Autologous CD8$^+$ T cells were negatively selected from nonadherent peripheral blood mononuclear cells (PBMC) and cocultured with peptide-loaded dendritic cells in wells of a 96-well tissue culture plates for 6 days as described previously (13, 19, 20). On day 7, the responder cells were restimulated with peptide-pulsed autologous dendritic cells in AIM-V medium supplemented with 20 IU/mL interleukin (IL)-2 and 5 ng/mL IL-7. The responder cells were subsequently stimulated with irradiated peptide-pulsed autologous PBMC. After two or more rounds of stimulation, the reactivities of generated effectors were tested.

Transfection of PCI-30 cells with ALDH1A1 cDNA. The ALDH1A1$^-$ cell line PCI-30 was transfected with pN2a-Aldo (a retroviral vector encoding ALDH1A1 cDNA) or the control vector (both obtained from Dr. R. Dalla-Favera, Cancer Institute, Columbia University, New York, NY; ref. 21) by electroporation using a Nucleofector device according to the manufacturer's protocol. The nucleofection efficacy of vector and pN2a-Aldo–transfected PCI-30 cells was monitored by quantitative reverse transcription-PCR (qRT-PCR) of ALDH1A1 mRNA as detailed below.

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Enzyme-linked immunospot assays. Enzyme-linked immunospot (ELISPOT) IFN-γ and granzyme B assays were done in 96-well flat-bottomed nitrocellulose plates (Millipore) using, respectively, anti-IFN-γ capture mAb (1-D1K) and biotinylated anti-IFN-γ detection mAb (7-B6-1) or anti-granzyme B capture mAb (GB10) and the biotinylated anti-granzyme B detection mAb (GB-11 biotin; all mAbs obtained from Mabtech; refs. 19, 20). In ELISPOT, 1×10^4 effector and target cells each per well were plated in triplicate wells. Plates were developed with avidin peroxidase (Vectastain Elite kit, Vector Laboratories) followed by 3-amino-9-ethyl-carbazole (Sigma Chemical Co.). The spots were counted using the ELISPOT 4.14.3 analyzer (Zeiss). Experimental values were considered significantly different from control values based on the permutation test. Assay performance and reproducibility was monitored using aliquots of cryopreserved PBMC obtained from a single donor plated either without stimulation (negative control) or ionomycin (1 μmol/L; Sigma Chemical). The coefficient of variation for the assay was 15% (n = 50). In general, tumor cell lines were treated with IFN-γ (500 IU/mL) for 72 h before assays to enhance their sensitivity to antigen-specific CD8+ T-cell effectors (22). For mAb blocking experiments, target cells were preincubated with 10 μg/mL mAb for 30 min.

qRT-PCR analysis of ALDH1A1 mRNA. Expression of ALDH1A1 mRNA relative to that of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; an endogenous control gene) mRNA was determined using a previously validated qRT-PCR assay (22). The ALDH1A1 primers and probe (forward: 5′-cgcaagacaggcttttcag-3′; reverse: 5′-tgtataatagtcgcccccc-tctc-3′; probe: 5′-FAM-attggatccccgtggcgtactaactatggat-3′) were obtained from Integrated DNA Technologies, Inc. The GAPDH primer and probe set Hs 00266705 was obtained from Applied Biosystems. The Applied Biosystems 7700 Sequence Detection Instrument was used for conducting qRT-PCR.

Flow cytometry–based analysis for ALDH1 enzymatic activity in SCCHN cell lines. ALDEFLUOR (Stem Cell Technologies) was used to

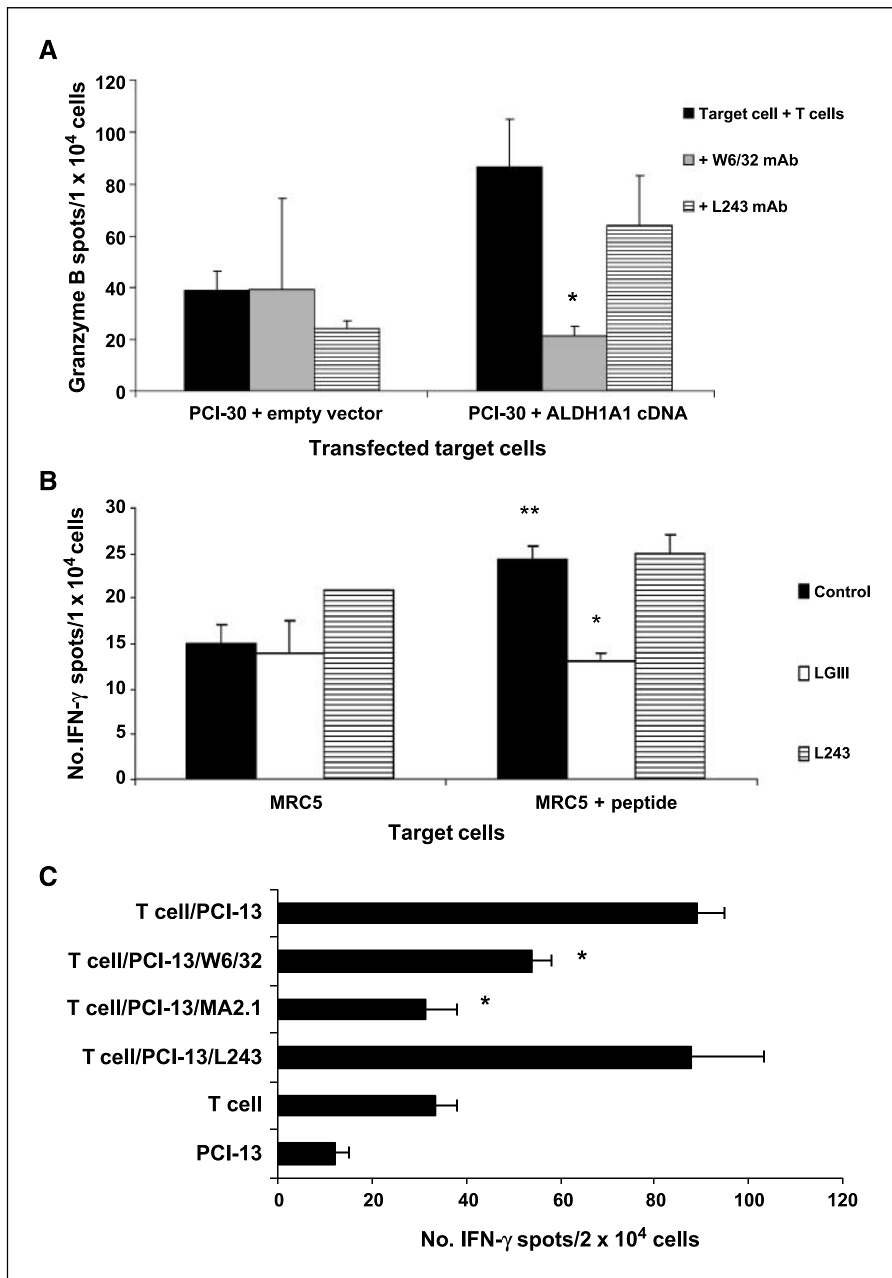

Figure 2. Reactivity of ALDH1A1$_{88-96}$ peptide-specific CD8+ T cells. *A,* ALDH1A1$_{88-96}$ peptide-specific CD8+ T cells recognized ALDH1A1 cDNA-transfected PCI-30 and this recognition was blocked by anti-HLA-A2 mAb. *B,* ALDH1A1$_{88-96}$ peptide-specific CD8+ T cells only recognize MRC5 human fibroblasts pulsed with ALDH1A1$_{88-96}$ with recognition blocked by the anti-HLA-A mAb LGIII-147.4.1 (*LGIII*). *C,* the ALDH1A1$_{88-96}$ peptide-specific CD8+ T cells induced from CD8+ T cells obtained from the PCI-13 subject recognize the autologous tumor cell line PCI-13. The anti-HLA class I mAb W6/32 and the anti-HLA-A2 mAb MA2.1 blocked recognition, but the anti-HLA-DR mAb L243 did not. *, significant anti-HLA class I mAb blocking (P < 0.05) in *A* and *C* as well as anti-HLA-A2 mAb blocking in *C* and anti-HLA-A mAb (LGIII-147.4.1) blocking in *B*; **, significant reactivity against peptide-pulsed MRC5 cell relative to control MRC5 cells in *B*.

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measure ALDH1 activity in cell lines by flow cytometry (23). Incubation of cells with ALDEFLUOR in the presence the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB), which results in a decreased in fluorescence, was used as a negative staining control for the assay (24).

Fluorescence-activated cell sorting of $CD45^+CD34^+$ hematopoietic bone marrow progenitor cells. $CD34^+$ hematopoietic progenitor cells were isolated from cryopreserved HLA-$A2^+$ bone marrow specimens under approval of IRB protocol 99-053. The cells were stained according to a protocol described previously (25) with CD34-PE, CD45-ECD, and propidium iodide. Sorting was done on a DakoCytomation MoFlo. A total of 5×10^6 to 10×10^6 cells per sample were sorted at rates not exceeding 1×10^4 events per second as described previously (26).

Immunohistochemical analysis of tumor and dysplastic mucosa specimens for ALDH1A1 expression. Formalin-fixed paraffin sections of SCCHN tumor and mucosa specimens were stained with goat anti-ALDH1A1 antibody (Santa Cruz Biotechnology) at a 1:100 dilution using standard procedures. The optimal staining dilution of the antibody was determined using PCI-13 cells. Controls included the use of the blocking peptide (Santa Cruz Biotechnology). The sections were analyzed microscopically by two pathologists to avoid bias, and the average of their scores was recorded. The sections were scored according to the following scheme of % cells staining: 0, no staining; 1+, <5%; 2+, 5% to 25%; 3+, 25% to 50%, and 4+, >50%.

Results

Isolation and identification of the $ALDH1A1_{76-99}$ peptide. Previously, an HLA-A2–restricted, CTL line was generated, which was reactive with the HLA-$A2^+$ allogenic SCCHN cell line PCI-13, using PCI-13 lysate-pulsed dendritic cells. This CTL line recognized T2 cells pulsed with 1 of 60 HPLC fractions of peptides derived from PCI-13 cells (16). Mass spectral analysis detected a multiply charged species, an average mass of 2,905 in the bioreactive fraction, which was identified by fragment and sequence tag analyses as a 24-residue peptide, PWRTMDASERGRLLYKLADLIERD, corresponding to $ALDH1A1_{76-99}$. This peptide could encode two overlapping HLA-A2 binding peptides, $ALDH1A1_{87-95}$ (RLLYKLADL) and $ALDH1A1_{88-96}$ (LLYKLADLI; ref. 27).

$ALDH1A1_{88-96}$, peptide identified as an HLA-A2–restricted T-cell–defined tumor peptide. *In vitro* stimulation with $CD8^+$ T cells obtained from HLA-$A2^+$ normal donors using autologous dendritic cells pulsed with either the $ALDH1A1_{87-95}$ or $ALDH1A1_{88-96}$ peptide as APC and subsequent testing in ELISPOT assays showed that only the $ALDH1A1_{88-96}$ peptide induced the expansion/generation of anti-ALDH1A1 peptide-specific $CD8^+$ T cells. These T-cell effectors released IFN-γ as well as granzyme B, an enzyme activity associated with cytolytic effectors. Furthermore, these $ALDH1A1_{88-96}$ peptide-specific $CD8^+$ T cells did not recognize T2 target cells pulsed with highly related peptides derived from either ALDH1A2 (LL*DK*LADLV), ALDH1A3 (LL*HQ*LADLV), or ALDH2 [LL*NR*LADLI; italicized amino acids indicate primary differences between these peptides and the $ALDH1A1_{88-96}$ peptide (LLYKLADLI); Fig. 1*A* and *B*].

The $ALDH1A1_{88-96}$ peptide-specific $CD8^+$ T cells also recognized three of five HLA-$A2^+$/$ALDH1A1^+$ cell lines, SCC-4, UD-6, and SCC-9, tested. Recognition was blocked by anti-HLA class I and anti-HLA-A2–specific mAb (Fig. 1*C*). The HLA-$A2^+$ SCC-90 and PCI-30 cell lines, as well as the HLA-$A2^-$/$ALDH1A1^+$ cell line PCI-4A, were not recognized. Furthermore, $ALDH1A1_{88-96}$ peptide-specific $CD8^+$ T cells recognized PCI-30 cells transfected with ALDH1A1 cDNA (Fig. 2*A*). Reactivity was blocked by anti-HLA class I mAb. Finally, these effectors failed to recognize HLA-$A2^+$ human lung fibroblast cells, MRC5, in an HLA class I–restricted manner, unless pulsed with exogenous peptide (Fig. 2*B*).

The immunogenicity of the $ALDH1A1_{88-96}$ peptide in subjects with SCCHN was shown in the autologous tumor system using PCI-13 cell line and PBMC obtained from the subject whose tumor was established as PCI-13 cell line (12–14). The $ALDH1A1_{88-96}$ peptide-specific $CD8^+$ T cells recognized the autologous PCI-13 tumor cells, which were blocked by anti-HLA-A2 mAb (Fig. 2*C*). In aggregate, our results confirm that the $ALDH1A1_{88-96}$ epitope is a naturally presented, HLA-A2–restricted, $CD8^+$ T-cell–defined tumor peptide.

ALDH mRNA expression in SCCHN cell lines and tissues. ALDH1A1 mRNA expression in normal mucosa, HEKa, PBMC, and SCCHN cell lines was analyzed by qRT-PCR. Several SCCHN cell lines expressed elevated levels of ALDH1A1 mRNA relative to normal cells and tissue, and these levels corresponded to their recognition by ALDH1A1-specific $CD8^+$ T-cell effector cells in ELISPOT assays (Table 1). The human breast cancer cell line MCF-7, previously shown to have little to no ALDH1A1 activity, was used as a negative control in these experiments.

Immunoblot analysis of ALDH1A1 protein levels in PCI-13 and normal mucosa. The expression of ALDH1A1 at the protein level in cell-free extracts of PCI-13 and normal mucosa was evaluated by immunoblot analysis using goat polyclonal anti-ALDH1A1 antibody (Santa Cruz Biotechnology; Fig. 3*A*). The apparent >10-fold increase in the level of ALDH1A1 protein detected in PCI-13 compared with normal mucosa is consistent with the results of qRT-PCR analysis of ALDH1A1 mRNA levels in these samples (Table 1).

Immunohistochemical analysis of ALDH1A1 expression in SCCHN and dysplastic mucosa specimens. Immunohistochemistry of 17 SCCHN specimens was done using the goat polyclonal anti-ALDH1A1 antibody. Expression of ALDH1A1 (2+ to 4+) was detected in 12 of 17 specimens, with the corresponding adjacent mucosa showing minimal to mild staining (0 to 1+; Fig. 3*B*). The five SCCHN specimens scoring negative for ALDH1A1 expression showed minimal staining (0 to 1+) in tumor and adjacent mucosa (data not shown).

In addition, 40 mucosa specimens obtained from subjects diagnosed with premalignant lesions were analyzed for ALDH1A1

Table 1. qRT-PCR analysis of ALDH1A1 mRNA in SCCHN cell lines and normal tissue/cells

Sample	ALDH1A1
Normal tissue/cells	
Normal mucosa*	1.48 ± 0.32
HEKa	1.62 ± 0.12
Tumor cell lines	
PCI-13	***17.6 ± 1.46***
PCI-30	0.72 ± 0.01
UD-6	***12.24 ± 0.35***
SCC-4	***8.34 ± 0.64***
MCF-7	<0.01

NOTE: Values in bold italics indicate levels of mRNA exceeding that detected in normal mucosa.
*Represents the values derived from analyses of three individual samples of normal mucosa.

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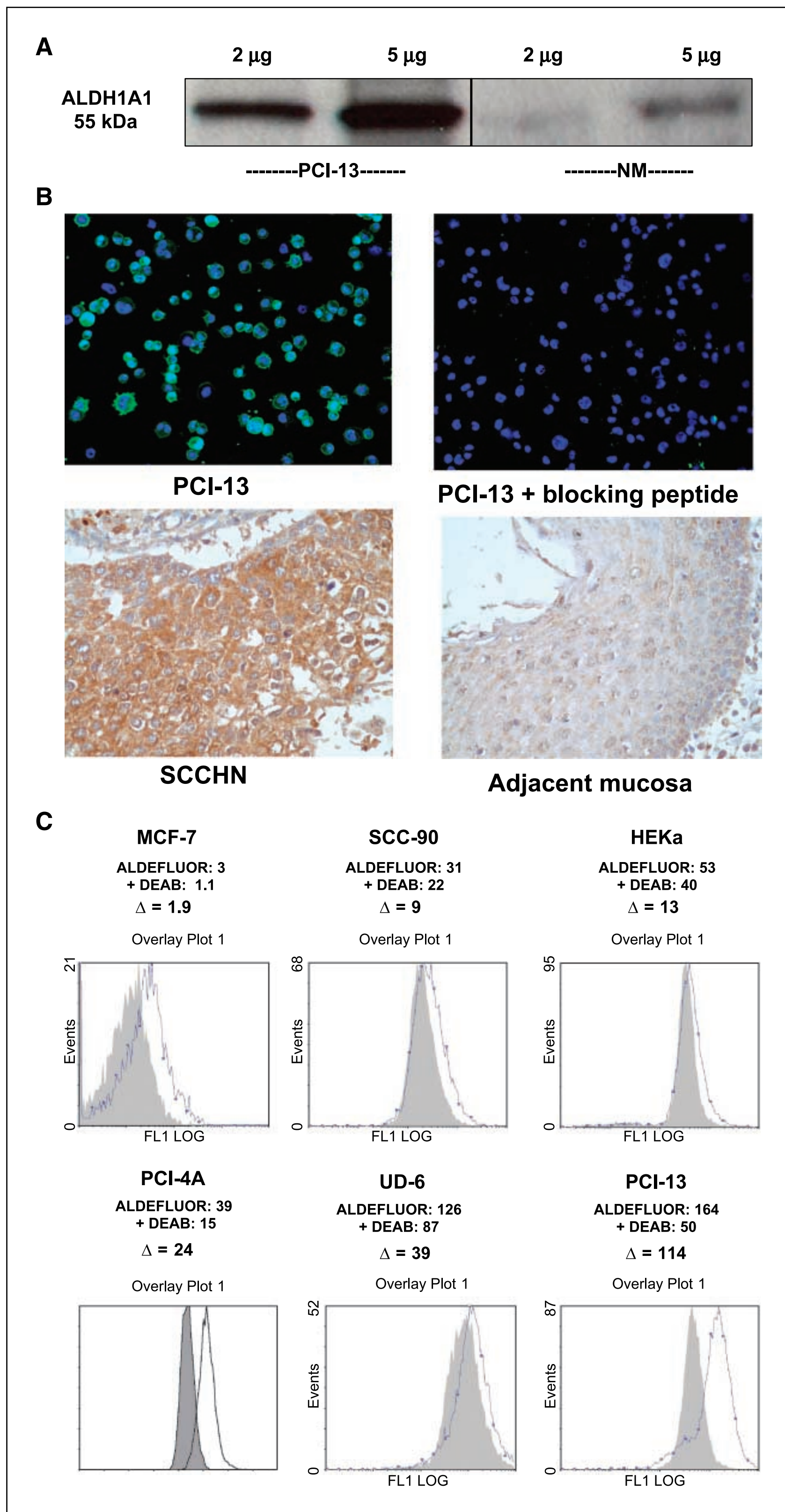

Figure 3. Analysis of ALDH1A1 expression and enzymatic activity in SCCHN cell lines, tumors, and normal cells. *A,* immunoblot analysis of ALDH1A1 in PCI-13 and normal mucosa (*NM*). Proteins present in cell-free extracts of PCI-13 and normal mucosa were separated by 4% to 15% SDS-PAGE under denatured conditions, transferred to a membrane, and probed with polyclonal goat anti-ALDH1A1 NH_2-terminal peptide antibody (1:1,000 dilution) and peroxidase-conjugated rabbit anti-goat IgG (1:2,000 dilution). *B,* immunohistochemistry of PCI-13 cells with and without blocking peptide (*top*) and a representative SCCHN and adjacent mucosa (*bottom*) stained for ALDH1A1 expression. Polyclonal goat anti-ALDH1A1 NH_2-terminal peptide antibody (1:100) was used to detect ALDH1A1 in formalin-fixed paraffin sections of SCCHN. Magnification, ×100. *C,* flow cytometry analysis of ALDH1 enzymatic activity in SCCHN cell lines using ALDEFLUOR, a fluorescent ALDH substrate. The amount of fluorescent product that accumulates in viable cells correlates to ALDH activity. The difference (*Δ*) between the mean fluorescence intensity of cells incubated with ALDEFLUOR alone and ALDEFLUOR and the ALDH inhibitor DEAB is equivalent of ALDH1 enzymatic activity in the cell line tested. PCI-13 cells show the highest activity for ALDH, whereas MCF-7 cells show the least. *Black line,* ALDEFLUOR; *shaded gray,* ALDEFLUOR + DEAB inhibitor.

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expression; 30 of 40 samples stained 1+ or greater for ALDH1A1 expression. In general, the intensity of staining increased with the severity of dysplasia of the specimens (Table 2) and shows the potential value of ALDH1A1 expression for discriminating subsets of SCCHN as well as distinguishing premalignant lesions from normal mucosa.

ALDH1 enzymatic activity in SCCHN cell lines. The ALDH1 enzymatic activity in tumor and normal cells was determined by

flow cytometry analysis using ALDEFLUOR (23, 24). The highest ALDH1 activity was detected in PCI-13 cells, UD-6 cells displayed an intermediate level of activity, whereas SCC-90 and HEKa showed little to no activity (Fig. 3*C*). The human MRC fibroblast cell line, which was not recognized by anti-ALDH1A1$_{88-96}$ CD8$^+$ T cells (Fig. 2*B*), expressed a level of ALDH1 activity comparable with that of HEKa cells (data not shown). The levels of ALDH1 enzyme activity in HEKa and tumor cell lines correlated with the levels of ALDH1A1 mRNA and protein in these cells as detailed above.

Reactivity of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells against CD34$^+$ hematopoietic cells. In addition to ALDH1A1, CD34$^+$ hematopoietic progenitor cells express two tumor-associated antigens (TAA), telomerase and PRAME, which are currently being targeted with cancer vaccines (28–31). CD8$^+$ T cells recognizing telomerase and PRAME-derived epitopes, however, did not recognize enriched populations of cord blood CD34$^+$ cells or PBMC, unless these cells were pulsed with exogenous TAA peptide. A concern in targeting ALDH1A1, therefore, is the potential toxicity of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T-cell recognition of CD34$^+$ hematopoietic progenitor cells. To this end, CD34$^+$ hematopoietic progenitor cells were isolated by fluorescence-activated cell sorting from HLA-A2$^+$ bone marrow cells (Fig. 4*A*). These cells expressed a low but distinct level of ALDH1A1 enzymatic activity. The ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells did not recognize these CD34$^+$ cells in ELISPOT IFN-γ assays, unless they were pulsed with exogenous ALDH1A1$_{88-96}$ peptide (Fig. 4*B*). Recognition of peptide-pulsed target cells was blocked by anti-HLA class I mAb.

Discussion

Our results show that the ALDH1A1$_{88-96}$ peptide is a naturally presented, HLA-A2–restricted, CD8$^+$ T-cell–defined antigen, which is overexpressed by most but not all SCCHN. ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells recognize HLA-A2$^+$ SCCHN cell lines overexpressing ALDH1A1 but not those with little to no expression of ALDH1A1. The sensitivity of SCCHN cell lines to ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells correlates well with their elevated levels of ALDH1A1 mRNA and protein as well as enzymatic activity. The overexpression of ALDH1A1 in many SCCHN relative to normal mucosa and the ability of these tumors to present ALDH1A1-derived epitopes for T-cell recognition make ALDH1A1 an attractive candidate for use in vaccines for this disease.

Most of the presently known T-cell–defined human tumor antigens are products of genes controlling the cell cycle or differentiation/tissue or cancer/testis antigens (32), and active immunization against some of these antigens can result in immunoediting/immunoselection of epitope loss variants. In contrast, we have identified a tumor peptide derived from a multifaceted and essential metabolic enzyme, a relatively rare class of tumor antigens. It will be of interest, therefore, to observe whether immunoediting/immunoselection of ALDH1A1 occurs in tumors following ALDH1A1-based immunization.

In addition to its role in alcohol metabolism, ALDH1A1 plays a critical role in defining resistance to a range of chemotherapeutic agents, which generate toxic aldehydes, such as cyclophosphamide and cisplatin (21, 33–36). Furthermore, elevated ALDH1A1 expression endows tumors with an enhanced capability to metabolize retinal to retinoic acid, which inhibits cell growth and induces apoptosis of tumor cells (6, 7) as well as down-regulates epidermal growth factor receptor expression (37). Counteracting this is PRAME, a cancer/testis antigen, which is expressed in $\sim$40% of SCCHN and acts as an oncogene by sequestering activated retinoic acid/retinoic acid receptor complexes (38). It would be of interest, therefore, to relate the effects that retinoic acid chemoprevention had on oral cancers and premalignant lesions to the molecular profile of ALDH and PRAME expression in these samples. The results may provide insights into the mixed outcomes of the use of retinoic acid for chemoprevention of SCCHN (39). Directing the delivery of chemoprevention therapy with retinoids to subjects with SCCHN based on ALDH1A1 expression in their tumors might well reverse the current lack of enthusiasm for retinoic acid chemoprevention.

The ability to predict based on ALDH1A1 expression, which subjects are likely to respond to a vaccine as opposed to chemoprevention therapy, would be a substantial achievement. Only a few immunogenic epitopes have been described for SCCHN, and in contrast to other solid cancers, therapeutic vaccination is only now being introduced for SCCHN. Therefore, development of ALDH1A1

Table 2. Immunohistochemical analysis of ALDH1A1 expression in dysplastic mucosa and SCCHN

Specimen classifications	No. specimens analyzed	Staining intensities*				
		0	1+	2+	3+	4+
Dysplasia						
Mild	4	2	2	0	0	0
Mild to moderate	8	4	4	0	0	0
Moderate	12	2	6	4	0	0
Moderate to severe	4	0	2	0	3	0
Severe	6	2	2	2	0	0
Severe/CIS	4	0	2	2	0	0
Dysplasia	2	0	0	2	0	0
SCCHN	13	5	0	4	3	1

Abbreviation: CIS, carcinoma *in situ*.
*The sections were analyzed microscopically and scored according to the following scheme of % cells staining: 0, no staining; 1+, <5%; 2+, 5% to 25%; 3+, 25% to 50%; and 4+, >50%.

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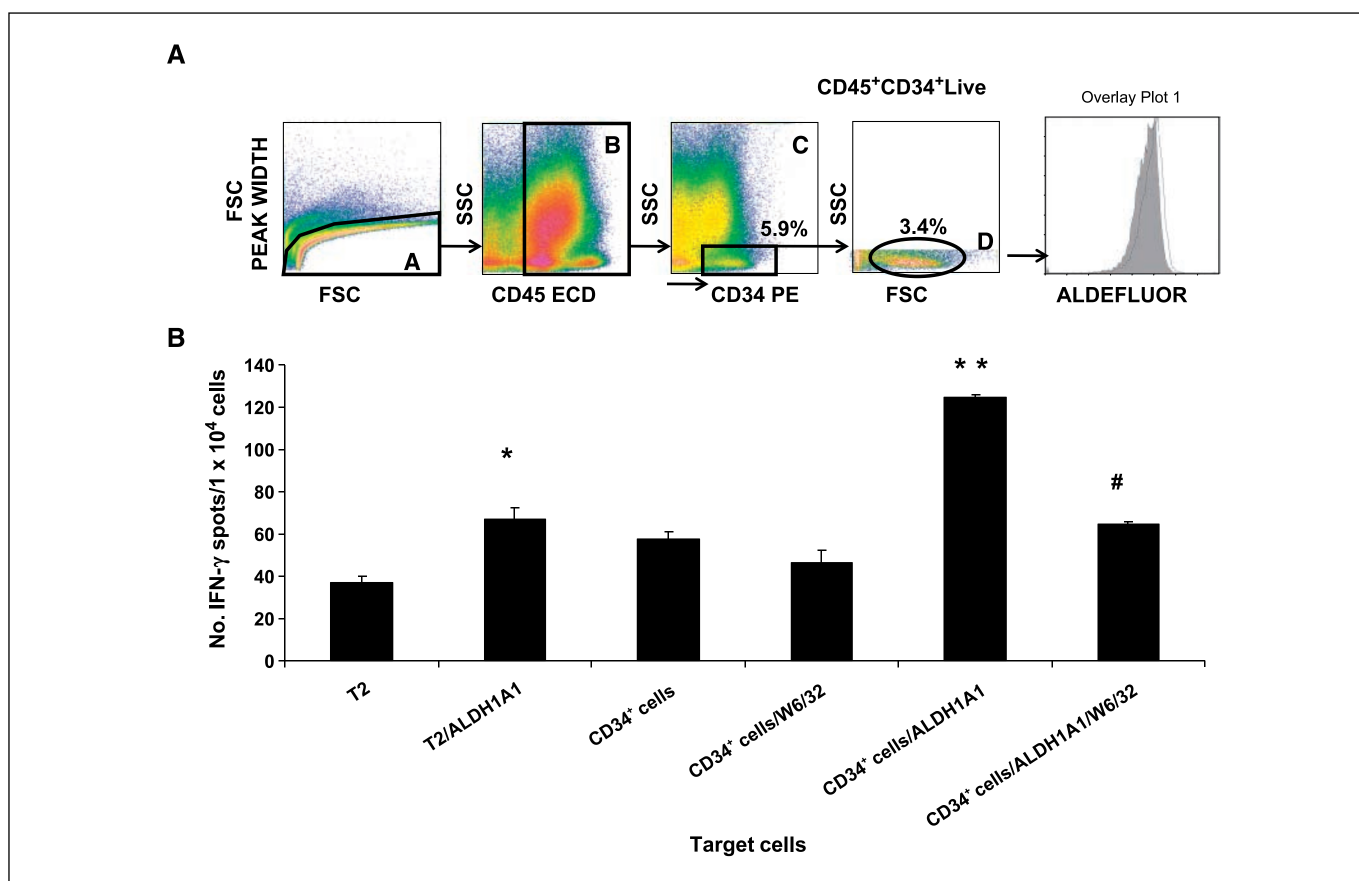

Figure 4. Reactivity of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells against CD34$^+$ hematopoietic bone marrow progenitors. Reactivity of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells against bone marrow–derived CD45$^+$CD34$^+$ hematopoietic bone marrow progenitors obtained from an HLA-A2$^+$ donor. *A,* identification and sorting strategy used for isolation of purified CD45$^+$CD34$^+$ hematopoietic progenitor cells used as target cells in the ELISPOT IFN-γ assays. Doublets and multicell clusters were identified (those cells not in *gate A*) by high peak width versus forward scatter (*FSC*) and eliminated from subset analysis. Total WBCs were identified by CD45 expression (*gate B*), and the WBC gate compounded with the singlet gate (*gate A*) was then passed to a histogram of CD34 versus side scatter (*SSC*), where CD34$^+$ events with low side scatter were identified (*gate C*). The compound gate of singlets/CD45$^+$/CD34$^+$/low side scatter was then passed to a histogram of forward scatter versus side scatter (*gate D*), which was used to eliminate low forward scatter events, which represent the debris/dying cells. The proportion of viable CD34$^+$ hematopoietic progenitors was then calculated as the ration of gate D events over the total CD45$^+$ events (*gate B*). The CD45$^+$/CD34$^+$ (*gate D*) events were sorted at $\sim 1 \times 10^4$ cells per second. The ALDH1A1 activity of these cells was then determined using ALDEFLUOR in the presence and absence of DEAB. The ALDH1A1 activity was 7.6. *B,* reactivity in ELISPOT IFN-γ assays of ALDH1A1$_{88-96}$ peptide-specific CD8$^+$ T cells against CD45$^+$CD34$^+$ hematopoietic bone marrow progenitors (CD34$^+$ cells) in the absence and presence of the ALDH1A1$_{88-96}$ peptide. Peptide-pulsed T2 target cells were the positive control for the assay. *, significant reactivity ($P < 0.05$) of effectors against ALDH1A1$_{88-96}$ peptide-pulsed T2 target cells compared with T2 target cells; **, significant reactivity ($P < 0.05$) of effectors against ALDH1A1$_{88-96}$ peptide-pulsed CD34$^+$ cells relative to CD34$^+$ cells; #, significant blocking of reactivity against ALDH1A1$_{88-96}$ peptide-pulsed CD34$^+$ target cells by anti-HLA class I mAb (W6/32).

peptide-based vaccines for therapy and, perhaps, prevention of SCCHN represents a novel area for future research in SCCHN.

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