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Identification of Hydroxysteroid (17 β) dehydrogenase type 12 (HSD17B12) as a CD8⁺ T-cell-defined human tumor antigen of human carcinomas

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Abstract

Hydroxysteroid (17 β) dehydrogenase type 12 (HSD17B12) is a multifunctional isoenzyme functional in the conversion of estrone to estradiol (E2), and elongation of long-chain fatty acids, in particular the conversion of palmitic to arachidonic (AA) acid, the precursor of sterols and the inflammatory mediator, prostaglandin E₂. Its overexpression together with that of COX-2 in breast carcinoma is associated with a poor prognosis. We have identified the HSD17B12_{114–122} peptide

(IYDKIKTGL) as a naturally presented HLA-A*0201 (HLA-A2)-restricted CD8⁺ T-cell-defined epitope. The HSD17B12_{114–122} peptide, however, is poorly immunogenic in its in vitro ability to induce peptide-specific CD8⁺ T cells. Acting as an “optimized peptide”, a peptide (TYDKIKTGL), which is identical to the HSD17B12_{114–122} peptide except for threonine at residue 1, was required for inducing in vitro the expansion of CD8⁺ T-cell effectors cross-reactive against the HSD17B12_{114–122} peptide. In IFN- γ ELISPOT assays, these effector cells recognize HSD17B12_{114–122} peptide-pulsed target cells, as well as HLA-A2⁺ squamous cell carcinoma of the head and neck (SCCHN) and breast carcinoma cell lines overexpressing HSD17B12 and naturally presenting the epitope. Whereas growth inhibition of a breast carcinoma cell line induced by HSD17B12 knockdown was only reversed by AA, in a similar manner, the growth inhibition of the SCCHN PCI-13 cell line by HSD17B12 knockdown was reversed by E2 and AA. Our findings provide the basis for future studies aimed at developing cancer vaccines for targeting HSD17B12, which apparently can be functional in critical metabolic pathways involved in inflammation and cancer.

Keywords

HSD17B12; CTL; Head and neck cancer; Tumor antigen

Introduction

Hydroxysteroid (17 β) dehydrogenase type 12 (HSD17B12) is one of 14 currently identified human HSD17B enzymes and is regulated by the sterol regulatory element binding protein 1 (SREBP-1) [1, 2]. It is considered a multifunctional enzyme that is potentially able to convert estrone (E1) to estradiol (E2) as well as elongate long-chain fatty acids, in particular, palmitic acid to arachidonic acid (AA) [1, 3, 4]. The latter is the precursor of eicosanoids, which have important biological functions and include the prostaglandins, in particular, the inflammatory mediator, prostaglandin E2 (PGE₂) [5].

Consistent with its potential multifunctional activities, elevated HSD17B12 expression has been identified in endocrine organs as well as those involved in lipid metabolism [6]. Its expression has also been shown to be associated with adipocyte differentiation [7], as well as mouse embryogenesis and differentiation [8, 9]. Relative to normal breast and ovarian tissues, HSD17B12 expression is elevated in breast, ovarian, and endometrial carcinomas [10–14]. Whether this reflects its role in estrogen metabolism or fatty acid elongation in these tumors is controversial. Fournier and Poirier indicated that selective inhibition of HSD17B12 did not block E2 synthesis in endometrial carcinoma cell lines [12], while Day et al. [13] demonstrated that HSD17B12 was not responsible for efficient E2 synthesis in human breast carcinoma cell lines. Nagasaki et al. [14] confirmed this finding and indirectly showed that more likely the metabolic function of HSD17B12 in breast cancer is fatty acid elongation. Furthermore, its overexpression with that of COX2 in breast carcinoma lesions was found to be associated with poor survival, thereby linking HSD17B12 expression to inflammation and cancer via its role in the biosynthesis of the inflammatory mediator PGE₂. In squamous cell carcinoma of the head and neck (SCCHN), elevated expression of HSD17B12 mRNA is predictive of metastasis [15]. Consequently, while it would appear that while fatty acid elongation is probably the predominant metabolic activity of HSD17B12, clearly its overexpression is associated with cancer.

The clinical implications attributed to HSD17B12 overexpression in human cancer prompted us to evaluate its potential as a human tumor antigen (TA) and candidate for T-cell-based immunotherapy. Interest in this effort was further prompted by our previous identification of a highly restricted tumor rejection antigen (TRA) of the BALB/c mouse

Meth A sarcoma as a CD8⁺ T-cell-defined peptide derived from a aberrantly expressed pseudogene related to *Hsd17b12*, the mouse homolog of human HSD17B12 [16]. This study was initiated using the algorithm predicted, HLA-A2-binding HSD17B12_{114–122} peptide (IYDKIKTGL), which is nearly identical to the Meth A TRA (TYDKIKTGL) peptide, designated Hsd17b12^{114T}, but differs from it in the amino acid exchange of threonine for isoleucine at position 1.

Materials and methods

Human carcinoma and normal cell lines

The HLA-A2⁺ PCI-13 and PCI-30, and HLA-A2^{neg} PCI-4A SCCHN cell lines used in this study were established at UPCI by Dr. Teresa Whiteside [17]. The HLA-A2⁺ UD-SCC 6 SCCHN cell line was obtained from Dr. H. Bier, Technical University of Munich, Munich, Germany. The HLA-A2⁺ SCCHN SCC-4, and MDA-MB-453, MDA-MB-231, and MCF-7 breast carcinoma cell lines, as well as the HLA-A2⁺ normal human fibroblast cell line, MCR-5, were obtained from American Type Culture Collection (Rockville, MD). The HLA-A2⁺AD10, OVCAR429, and OVCAR3 ovarian carcinoma cell lines were obtained from Dr. S. Khelif (Naval Hospital, Bethesda MD). The JL-BRL 6 cell line is a low-passage human breast tissue cell line established by Dr. Latimer from a reduction mammoplasty [18]. All the cell lines, with the exception of JL-BRL 6, were maintained in a complete medium (CM) consisting of RPMI-1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50 IU/ml penicillin (Life Technologies, Inc., Grand Island, NY). The JL-BRL 6 cell line was maintained in a defined culture medium developed and supplied by Dr. Latimer [18].

Antibodies and peptides

Immunoaffinity-purified mAb used in these studies were obtained from supernatants of hybridomas producing HLA-A,-B,-C antigen (Ag)-specific mAb (clone W6/32, IgG_{2a}), HLA-DR-specific mAb (clone L243, IgG_{2a}), and the HLA-A2,-A28 Ag-specific mAb (KS1, IgG₁). The W6/32 and L243 hybridomas were obtained from ATCC. The KS1 and an IgG1 isotype mAb were from Dr. Soldano Ferrone (University of Pittsburgh, Pittsburgh PA) [19]. The peptide-immunoaffinity-purified polyclonal rabbit anti-TY-DKIKTGL peptide antibody, which cross-reacts with the IYDKIKTGL (Hsd17b12/HSD17B12_{114–122}) peptide, has previously been described [16]. Peptides were synthesized using standard Fmoc chemistry and their sequences confirmed by tandem MS.

In vitro stimulation (IVS) of HLA-A2-restricted, anti-HSD17B12_{114–122} peptide human CD8⁺ T cells

HSD17B12_{114–122} peptide CD8⁺ T-cell effectors were generated following in vitro stimulation (IVS) of CD8⁺ T cells obtained from PBMC of HLA-A2⁺ healthy donors using peptide-pulsed autologous DC as antigen presenting cells (APC) as previously described [20].

INFγ ELISPOT assay

INFγ ELISPOT assays were performed in 96-well flat-bottomed nitrocellulose plates (MAHAS4510; Millipore, Bedford, MA) using, respectively, anti-INFγ capture mAb and biotinylated anti-INFγ detection mAb (Mabtech, Inc. Cincinnati, OH), as previously described [20]. The assays consisted of 1 × 10⁴ target cells and CD8⁺ T cells at an E-/T-cell ratio of 2:1. Blocking experiments were performed by incubating target cells with blocking and isotype control mAb (10 µg/ml) for 30 min at 4°C prior to addition of effectors.

Transfection of MCR-5 cells with HSD17B12 cDNA

MRC-5 cells were transfected with HSD17B12 cDNA (SC114479, OriGene Technologies, Inc. Rockville, MD) by electroporation using a Nucleofector device according to the manufacturer's protocol. The nucleofection efficacy of empty pCMV6-XL5 vector and pCMV6-XL5-HSD17B12-transfected MCR-5 cells was monitored by quantitative reverse transcription-PCR (qRT-PCR) of HSD17B12 mRNA using the following primers designed in this laboratory; *forward primer*: TTGCTGTTGACTT TGCATCAG; *reverse primer*: TTCACTAAGATGCCGA TTTCAA and *probe*: 5'-/56 FAM/TGATAAAATTAAAA CAGGCTTGGCTGGT/3BHQ-1/3'.

Immunoblot analyses of HSD17B12 expression in human normal and tumor cell lines

The expression of HSD17B12 in human normal and tumor cell lines was analyzed by immunoblot using the purified rabbit antibody at a concentration of 1 µg/ml and developed using horseradish peroxidase-conjugated goat anti-rabbit IgG Fc fragment-specific antibody (Jackson Immuno-Research Laboratories, Inc. West Grove, PA) at 1:10,000 dilution and Western Lightning Plus-ECL (Perkin Elmer, Inc., Waltham MA) [16].

Immunohistochemical analysis of human tumor cell lines and normal cells and tissues for HSD17B12 expression

The optimal staining dilution of the peptide immunoaffinity polyclonal rabbit anti-TYDKIKTGL antibody (1 µg/ml) for HSD17B12 was determined by immunofluorescence microscopy using formalin-fixed PCI-13 cells by Dr. Dhir (Director, Department of Pathology, UPMC Shadyside Hospital, Pittsburgh PA) using an Olympus BX-41 microscope. Controls included the use of the blocking peptide. Two formalin-fixed paraffin-embedded tissue microarrays (TMA) also were analyzed for HSD17B12 expression with the peptide-immunoaffinity-purified, polyclonal rabbit anti-TYDKIKTGL antibody using standard procedures. The stained sections were analyzed using an Olympus BX-41 microscope. One TMA consisting of 15 oral cavity SCCHN specimens, which included surrounding mucosa, was constructed in the laboratory of Dr. Dhir from IRB approved excess sections of paraffin blocks of specimens that were initially generated for clinical evaluation. The second TMA, the commercially available SCCHN TMA (cat # HN803, US Biomax Inc, Rockville, MD) was analyzed using a Nikon Eclipse microscope. All stained sections were analyzed and scored by two pathologists to avoid bias, and the average of their scores recorded. The sections were scored according to the % of cells staining (<25%: negative; 25–75%: heterogenous; and >75%: positive), staining intensity (weak, moderate, and strong) and cellular localization (nucleus or cytoplasm).

Small interfering RNA (siRNA) inhibition of HSD17B12 expression

HSD17B12 siRNA (sc-96987) and two control siRNA (sc-37007 and sc-36869) purchased from Santa Cruz Bio-technology, Inc. Santa Cruz, CA) were used to demonstrate siRNA inhibition of the expression of HSD17B12 in PCI-13 cells in a protocol suggested by the manufacturer. To accomplish maximum inhibition of HSD17B12 mRNA synthesis in PCI-13 cells, 5×10^4 cells/well/6-well plates were transfected with 8 µg/siRNA in complete medium without antibiotics. After a 24 h incubation, supernatant was removed and serum-free RPMI-1640 medium added with or without 1 µM arachidonic acid (MP Biomedicals, Solon, OH) (1:10 dilution of a 1:100 dilution in PBS of a 1 mM AA/DMSO stock solution) or 1nM estradiol (Sigma, St. Louis) (1:10 dilution of a 1:100 dilution in PBS of a 1 µM E2/ethanol stock solution). After 48 h incubation, cells were harvested for analysis. Fluorescein isothiocyanate-conjugated control siRNA-A (sc-36869) and control siRNA-A (sc-37007) were the controls. The knockdown of HSD17B12 expression in PCI-13 was monitored by qRT-PCR relative to reporter gene β -glucuronidase (β GUS) mRNA using primers listed

above for HSD17B12 and those previously described [20]. The effect of HSD17B12 knockdown on growth of transfected cells PCI-13 cells cultured in the absence or presence of 1 μ M AA or 1nM E2 for 48 h was determined by harvesting and determining the number of viable cells (cells excluding trypan blue).

Statistical analysis

The results of ELISPOT assays and siRNA experiments were analyzed using Students' *t*-tests and considered significant at $P < 0.05$. The significance of the results of staining the TMA relative to clinicopathological characteristics of the specimens was determined using Pearson Correlation Asymp Sig. (2-sided) analysis.

Results

Generation of HSD17B12 peptide-reactive CD8⁺ T cells

The HSD17B12_{114–122} peptide was tested for its ability to induce the expansion of peptide-specific CD8⁺ T cells following IVS of CD8⁺ T cells isolated from PBMC obtained from HLA-A2⁺ normal donors using peptide-pulsed autologous dendritic cells as the APC [20]. IVS of CD8⁺ T cells obtained from 2 of 3 HLA-A2⁺ donors yielded effector cells that essentially did not significantly recognize HSD17B12_{114–122} peptide-pulsed T2 cells compared with irrelevant HIV peptide-pulsed or unpulsed T2 target cells in ELISPOT IFN γ assays (Fig. 1). Further stimulation of these effector cells using peptide-pulsed PBMC as APC repeatedly resulted in their loss of activity, and attempts by single cell dilution to obtain enriched stable populations of HSD17B12_{114–122} peptide-specific CD8⁺ T cells from these IVS populations failed (data not shown). Therefore, using a technique we and other have used to enhance the immunogenicity of an epitope, we investigated the possibility that the Meth A Hsd17b12^{114T} peptide (TYDKIKTGL) might act as an optimized peptide in inducing CD8⁺ T cells cross-reactive against the HSD17B12_{114–122} peptide [21–23]. IVS of CD8⁺ T cells isolated from PBMC obtained from 3 of 4 HLA-A2⁺ normal donors using autologous dendritic cells pulsed with the Hsd17b12^{114T} peptide yielded effector cells that recognized the Hsd17b12^{114T} peptide-pulsed T2 target cells, but most importantly, also recognized HSD17B12_{114–122} peptide-pulsed T2 cells compared with irrelevant HIV peptide-pulsed or unpulsed T2 target cells in ELISPOT IFN γ assays (Fig. 1).

Recognition of naturally presented HSD17B12_{114–122} peptide by HLA-A2-restricted CD8⁺ T cells cross-reactive against the HSD17B12_{114–122} peptide. The ability of HLA-A2-restricted CD8⁺ T cells reactive against HSD17B12_{114–122} peptide to recognize HLA-A2⁺ human carcinoma cells naturally presenting the HSD17B12_{114–122} peptide was tested using panels of human SCCHN, breast and ovarian carcinoma cell lines as target cells in ELISPOT IFN γ assays. All the cell lines were pretreated with IFN γ to enhance their presentation of HLA class I antigen/tumor peptide complexes for recognition by the CD8⁺ T cells [24]. Five SCCHN cell lines were tested: the HLA-A2⁺ PCI-13, SCC-4, UD-SCC6, and PCI-30 and HLA-A2^{neg} PCI-4A (Fig. 2a). Of these five SCCHN cell lines, the HLA-A2⁺ PCI-13, SCC-4, UD-SCC6 were recognized, while PCI-30 and HLA-A2^{neg} PCI-4A were not (Fig. 2b). The recognition of the HLA-A2⁺ PCI-13, SCC-4, UD-SCC6 cell lines was blocked by the HLA A2,-A28-specific mAb KS1, but not the HLA-DR-specific mAb L243, confirming the HLA-A2 restriction of the effectors. The epitope specificity of the effector cells used for these experiments was indicated by their recognition of HSD17B12_{114–122} peptide-pulsed T2 target cells but not the irrelevant HIV-pulsed T2 target cells (Fig. 2b). Three HLA-A2⁺ breast carcinoma cell lines, MDA-MB-231, MDA-MB-453 and MCF-7 cell lines, and three HLA-A2⁺ ovarian carcinoma cell lines, AD10, OVCAR429 and OVCAR3, were also tested for recognition by these effector cells. The CD8⁺ T cells recognized all three breast carcinoma cell lines in ELISPOT IFN γ assays (Fig. 2b). The

recognition of the HLA-A2⁺ breast carcinoma cell lines was blocked by the HLA A2,-A28-specific mAb KS1, but not the HLA-DR-specific mAb L243. In contrast to these findings, the effector cells recognized none of the ovarian carcinoma cell lines (data not shown). Further analysis indicated that HLA class I expression was not upregulated in the ovarian carcinoma cell lines by IFN γ -pretreatment (data not shown). This finding is consistent with reports of severe defects in antigen processing and presentation in ovarian carcinomas [25–27].

Critical to the potential use of “self” tumor antigens in cancer vaccines, the HSD17B12-reactive CD8⁺ T cells did not recognize JL-BRL 6 cells, a low-passage cell line established from the HLA-A2⁺ normal breast reduction tissue, even when pretreated with IFN γ , which upregulates HLA class I antigen expression [24] (Fig. 2b). In addition, the CD8⁺ T cell effectors did not recognize HLA-A2⁺ human lung fibroblast cells, MRC-5, unless the cells were transfected with HSD17B12 cDNA (Fig. 2c). Reactivity of the effector cells against the transfected fibroblasts was blocked by the HLA-A2,-A28-specific mAb KS1, but not the HLA-DR-specific mAb L243. Together with the results obtained using normal human fibroblasts, the results strongly support the ability of HSD17B12-specific CD8⁺ T cells to recognize most types of tumor cells overexpressing HSD17B12, but not normal cells.

Analysis of HSD17B12 expression in human carcinoma cell lines and normal cells

The expression of HSD17B12 at the protein level in cell-free extracts of the human SCCHN, breast, and ovarian carcinoma cell lines, as well as the JL-BRL 6 cell line used in this study was evaluated by immunoblot analysis using the rabbit anti-TYDKIKTGL antibody (Fig. 3). Based on a sequence of 312 amino acids, HSD17B12 should be detected as M_r35 kDa species in immunoblot assays. In most of tumor cell-derived extracts analyzed, however, a doublet consisting of a M_r35 kDa and a M_r40 kDa species was detected. The higher molecular weight species was prominent in the most of the breast, ovarian, and SCCHN cell lines analyzed. In the case of the SCCHN SCC-4 cell, the higher M_r species, itself, appeared to be a doublet. The exceptions were the UD-SCC 6 and OVCAR-3 cell lines; the latter expressing the lowest level of HSD17B12 of any of the tumor cell lines analyzed. Only the lower M_r HSD17B12 species was detected in the cell-free extract of the non-transformed, low-passage breast reduction JL-BRL 6 cell line. The significance of these findings requires further study, but they suggest that processing and post-translational modifications of HSD17B12, namely glycosylation, are probably different in the tumor cells compared with the non-transformed “normal” cells studied. Based on the results of this immunoblot analysis, with the exception of the UD-SCC 6 cell line, recognition of a tumor cells overexpressing HSD17B12 correlated with expression of high levels of the higher rather than lower M_r HSD17B12 species (Figs. 2 and 3).

Immunohistochemistry analysis of HSD17B12 expression in SCCHN

HSD17B12 expression in an SCCHN cell line and two SCCHN TMA was analyzed by immunohistochemistry using the rabbit anti-TYDKIKTGL antiserum. The specificity of the antiserum for HSD17B12 was established using paraffin-embedded sections of formalin-fixed PCI-13 cells (Fig. 4). It showed predominate cytoplasmic staining with some nuclear staining of PCI-13 cells, which were derived from a poorly differentiated SCCHN lesion [17]. Comparable staining was also obtained with an SCCHN lesion. In the presence of a fixed concentration of blocking peptide, staining of PCI-13 was completely blocked and significant reduced in the lesion; indicative of the specificity of this antiserum. The first SCCHN TMA analyzed consisted exclusively of 15 oral cavity lesions with a range of tumor grades; nine well-differentiated, four moderately differentiated, and two poorly differentiated, all with adjacent normal mucosa tissue. HSD17B12 expression was detected in all the lesions, whereas the non-neoplastic squamous mucosa showed absence of

expression (Fig. 5a). HSD17B12 expression was detected in the cytoplasm of all the lesions in this TMA. All of the well-differentiated carcinomas also showed some degree of nuclear localization (Fig. 5b). Of the four moderately differentiated SCCHN, two had nuclear/cytoplasmic staining (Fig. 5c), the other two had cytoplasmic expression only. Of the two poorly differentiated SCCHN, one showed cytoplasmic staining, the other cytoplasmic/nuclear staining (Fig. 5d).

The IHC analysis of a second SCCHN TMA, a commercially available TMA consisting of specimens of 48 lesions of various sites, only 25% of which were oral cavity tumors. The clinicopathological characteristics of the specimens in this TMA, which consisted of sex and age of the subjects, tumor site, grade, and stage, as well as nodal involvement and metastasis, are listed in Table 1. Varied levels of HSD17B12 expression were detected in 44/48 (91.7%) specimens in the TMA. Moderate staining was detected in 13/44 (29.5%) of specimens and strong staining in 19/44 (43.2%). As noted with the previous oral cavity TMA, the staining of the multisite TMA also showed varied cellular localization of HSD17B12 expression among the specimens. A mixed cellular localization was evident in 34/44 specimens (77.3%). HSD17B12 expression in laryngeal tumors was significant compared with other sites, and in poorly differentiated tumors compared with moderate- and well-differentiated lesions. The results of staining of both TMA, therefore, are consistent with HSD17B12 overexpression being (1) associated with neoplasia, (2) present in nucleus and cytoplasm, and (3) predominate detected in poorly differentiated SCCHN lesions. These findings regarding nuclear as well as cytoplasmic cellular localization of HSD17B12 expression in SCCHN cell lines and lesions are in contrast to those of other laboratories involving breast carcinoma cell lines and lesions [11, 13, 14] and warrant further consideration.

Multifunctional role of HSD17B12 in PCI-13 cells

The role of HSD17B12 activity in E1 to E2 conversion and elongation of long-chain fatty acids in PCI-13 cells was investigated using the approach detailed by Nagasaki et al. [14]. In a series of experiments, we determined that inhibition of HSD17B12 mRNA in PCI-13 cells required transfection of the minimum number of cells needed for subsequent analysis at the maximum recommended, non-toxic concentration of 8 µg/ml of HSD17B12 siRNA. At this concentration, a 50% decrease in HSD17B12 mRNA was associated with a 38% decrease in cell growth (Fig. 6). The inhibition of cell growth was reversed by either AA or E2. Presumably, if functional in both pathways, one would have expected that both agents be required to reverse the growth inhibition due to HSD17B12 knockdown. Since only a maximum of a 50% decrease in HSD17B12 mRNA could be achieved using this approach; however, it is plausible that there remained sufficient residual HSD17B12 activity not to require this. Although the participation of HSD17B12 in these pathways was not directly studied, the result does support HSD17B12 functional activity in E1/E2 conversion as well as fatty acid elongation in the PCI-13 cell line and, presumably, SCCHN.

Discussion

Our results show that the HSD17B12_{114–122} epitope is naturally presented by HLA-A2⁺ breast carcinoma and SCCHN cell lines for recognition by HLA-A2-restricted, CD8⁺ T cells. Recognition of human carcinoma cell lines by the HSD17B12_{114–122} peptide-reactive CD8⁺ T cells correlates with HSD17B12 overexpression and identifies it as an attractive novel candidate for use in vaccines targeting a range of human carcinomas. However, the HSD17B12_{114–122} (IYDKIKTGL) peptide is relatively weakly immunogenic and an optimized peptide (TY-DKIKTGL), previously identified as a CTL-defined murine tumor antigen, was required to induce cross-reactive HLA-A2⁺ CD8⁺ T cells recognizing tumor cells naturally presenting the epitope.

In this report, we have expanded on the finding that HSD17B12 mRNA expression is one of a four-gene model predictive of metastasis in SCCHN [15] and demonstrate its overexpression at the protein level in SCCHN cell lines and lesions, with minimal expression in normal cells. The immunohistochemical analysis of HSD17B12 expression in SCCHN lesions, using a rabbit antibody prepared against the TYDKIKTGL peptide, which cross-reacts with the HSD17B12_{114–122} peptide, showed that staining was primarily cytoplasmic, but some nuclear staining was observed. In general, the intensity of HSD17B12 staining was more pronounced in poorly differentiated lesions than in more differentiated ones. Cytoplasmic and nuclear HSD17B12 staining in breast carcinoma lesions were noted by Song et al. [11] using a rabbit antiserum raised against the recombinant HSD17B12_{217–312} polypeptide. In contrast, the studies by Sakurai et al. [6] and Nagasaki et al. [14], both of which used the same rabbit anti-HSD17B12_{302–312} C-terminal peptide antibody indicate only cytoplasmic localization in breast carcinoma. The study by Moon and Horton [4] analyzed HSD17B12 expression in Chinese hamster cells transfected with a vector encoding HSD17B12 cDNA tagged with the N-terminal hemagglutinin (HA) peptide using anti-HA antibody and further localized HSD17B12 expression to the endoplasmic reticulum. At this point, with no obvious reason to place the metabolic activity of HSD17B12 requiring it to be found in the nucleus, one can only conjecture that the differences in these results might reflect differences in (1) presence of contaminating antibodies in the polyclonal rabbit antisera, (2) the regions of the protein targeted by the three rabbit antisera used, namely C-terminal peptide vs internal peptide/polypeptide or tag peptide sequences, and (3) types of carcinomas analyzed in these studies (breast, ovarian, and head and neck carcinomas). This dichotomy will probably only be resolved by the analysis of subcellular fractions of cells with one or more of the anti-HSD17B12 antibodies used in these studies. A related issue is the anti-HSD17B12_{302–312} C-terminal peptide antibody detection of a single M_r 35 kDa HSD17B12 species in the MCF-7 and MDA-MB-231 breast carcinoma cell lines, as reported by Nagasaki et al. [14], and the detection in this study of M_r 35 kDa and a higher M_r HSD17B12 species in these same breast carcinoma cell lines. This difference is probably reflective of the ability or inability of these probes to detect post-translational modifications and/or variants of HSD17B12 expressed in normal and transformed cells.

Our findings using siRNA to inhibit HSD17B12 mRNA synthesis in PCI-13 cells indicate that HSD17B12 in SCCHN appears to be multifunctional in E1/E2 conversion as well as fatty acid elongation. The finding is somewhat surprising since normally one does not consider SCCHN to be a hormone-related disease. However, SCCHN cell lines have been shown to express functional estrogen receptors ($ER\alpha/\beta$), which can cross-talk with epidermal growth factor receptors (EGFR), and that EGFR and nuclear $ER\alpha$ but not $ER\beta$ expression were significantly increased in SCCHN tumors compared with adjacent mucosa [28]. Furthermore, progression-free survival of patients with tumors expressing high $ER\alpha$ and EGFR levels was significantly reduced compared with that of patients with tumors expressing low levels of these receptors. The multifunctional results using the SCCHN PCI-13 cell line are in contrast to the results involving the SKBR3 breast carcinoma cell line, which strongly suggest that HSD17B12 only functions in fatty acid elongation (14). Again, it is also plausible that the differences in the functions attributed to HSD17B12 in cDNA-transfected and tumor cell lines might be more attributed to the tissue origins of the tumor cell lines used in these studies, rather than the HSD17B12 gene product expressed in the different cell lines.

Identifying HSD17B12 activity in fatty acid elongation in SCCHN links it to PGE_2 synthesis and inflammation and immunosuppression; conditions associated with this disease. Although SCCHN lesions and derived cell lines, such as PCI-13, were shown to produce high levels of PGE_2 , these levels are not predictive of the course of the disease [29].

However, a recent study related PGE₂ and COX-2 expression to VEGF production and metastases in SCCN [30]. It should also be noted that the recent attention paid to fatty acid synthase and fatty acid synthesis in relationship to oncogenesis by necessity also includes HSD17B12 via its activity in fatty acid elongation [31]. The present findings, therefore, strongly support HSD17B12 functional involvement in critical pathways linking inflammation and cancer and provide the basis for developing an immunotherapy strategy, perhaps used in combination with other modalities, to target HSD17B12 in human carcinomas.

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Fig. 1.

The Hsd17b12^{114T} peptide induces CD8⁺ T cells that recognize the HSD17B12₁₁₄₋₁₂₂ peptide. Following IVS of CD8⁺ T cells with the HSD17B12₁₁₄₋₁₂₂ peptide (IYDKIKTGL) or “optimized” Hsd17b12^{114T} peptide (TYDKIKTGL), the induced effector cells were tested for reactivity against HSD17B12₁₁₄₋₁₂₂ peptide, Hsd17b12^{114T} peptide or irrelevant HIV peptide-pulsed T2 cells in ELISPOT IFN γ assays. *Asterisk* denotes significant ($P < 0.05$) recognition relative to irrelevant peptide-pulsed T2 cells

Fig. 2.

HSD17B12_{114–122} peptide-reactive CD8⁺ T cells recognize human SCCHN and breast carcinoma cell lines in ELISPOT IFN γ assays. **a** HSD17B12_{114–122} reactive CD8⁺ T cells recognize three of four HLA-A2⁺ SCCHN cell lines in the presence of control mouse isotype mAb (IgG1 and IgG2a). Recognition was blocked by HLA-A2,-A28-specific mAb KS1, but not HLA-DR-specific mAb L243. These effector cells did not recognize the HLA-A2⁺ SCC-30 and HLA-A2^{neg} PCI-4A SCCHN cell lines. **b** HSD17B12_{114–122} peptide-reactive CD8⁺ T cells recognize HLA-A2⁺ breast carcinoma cell lines, but not the normal breast epithelial JL-BRL 6 cell line. The specificity of the effector cells used in this experiment is indicated by recognition of PCI-13 and HSD17B12 peptide-pulsed T2 target cells, but not PCI-4A or irrelevant peptide-pulsed T2 target cells. *Double Asterisk* denotes significant ($P < 0.05$) recognition compared with T cells only. *Asterisk* denotes significant ($P < 0.05$) blocking of recognition of target cells by HLA-A2,-A28-specific mAb KS1. **c** HSD17B12_{114–122} peptide-reactive CD8⁺ T cells recognized HSD17B12 cDNA-transfected MCR-5 fibroblasts, but not empty vector-transfected MCR-5 cells. *Double Asterisk* denotes significant ($P < 0.05$) recognition compared with empty vector-transfected MCR-5 cells. *Asterisk* denotes significant ($P < 0.05$) blocking of recognition of HSD17B12 cDNA-transfected MCR-5 cells by HLA-A2,-A28-specific mAb KS1.

Fig. 3.

HSD17B12 protein expression in human carcinoma cell lines and normal cells. Immunoblot analysis of HSD17B12 protein expression in SCCHN, breast and ovarian carcinoma cell lines, the breast reduction JL-BRL 6 cell line and mock and HSD17B12 cDNA-transfected human MCR-5 fibroblasts. Proteins present in cell-free extracts were separated by 4–15% SDS–PAGE under denatured conditions, transferred to a membrane, and probed with rabbit anti-TYDKIKTGL peptide antibody (1:1,000 dilution) and peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution). *Arrows* denote the M_r 35 and 40 kDa species

Fig. 4. Immunohistochemistry analysis of HSD17B12 expression in PCI-13 cell line and SCCHN lesion. The specificity of the rabbit anti-HSD17B12 (TYDKIKTGL peptide) antibody was established by staining sections of formalin-fixed paraffin-embedded PCI-13 cells in the presence and absence of blocking peptide and indicated nuclear as well as cytoplasmic localization of HSD17B12. Staining of an SCCHN lesion also showed a similar staining pattern. All sections were stained with hematoxylin and eosin followed by either normal rabbit antibody; HSD17B12 antibody or HSD17B12 antibody and blocking TYDKIKTGL peptide (10 μ g/ml), as indicated. Magnification $\times 400$ using a Nikon Eclipse microscope, Insets: $\times 1000$ magnification

Fig. 5. Immunohistochemistry analysis of HSD17B12 expression in a SCCHN TMA. SCCHN lesions were analyzed by immunohistochemistry for HSD17B12 expression, as detailed in “Materials and methods”. **a** Minimal staining in normal mucosa. Occasional basal cells show nuclear expression. **b** Nuclear staining in well-differentiated SCCHN. **c** Predominantly weak cytoplasmic and occasional nuclear staining in a moderately differentiated SCCHN. **d** Cytoplasmic and nuclear staining in a poorly differentiated SCCHN. Magnification $\times 200$ using an Olympus BX-41 microscope

Fig. 6.

HSD17B12 knockdown in PCI-13 induces growth inhibition. PCI-13 cells were treated with control or HSD17B12 siRNA in the presence of 1 μ M AA, 1 nM E2, or the appropriate PBS-based vehicle in which the agent was dissolved. **a** HSD17B12 mRNA levels relative to β GUS mRNA levels in the indicated cell populations and **b** % change in cell numbers in indicated cell populations

Table 1

Immunohistochemical analysis of HSD17B12 expression in a SCCHN TMA

<i>Clinicopathologic Parameters</i>		
Sex		
Female	8	(16.7%)
Male	40	(83.3%)
Age		
Min	38	
Max	77	
Mean	58.56	
Organ		
Oral cavity	12	(25%)
Larynx	19	(39.6%)
Paranasal sinuses	8	(16.7%)
Pharynx	9	(18.8%)
Tumor differentiation		
Well	10	(20.8%)
Moderate	31	(64.6%)
Poor	7	(14.6%)
Stage		
I	4	(8.3%)
II	20	(41.7%)
III	14	(29.2%)
IV	10	(20.8%)
Nodal involvement		
Negative	35	(72.9%)
Positive	13	(27.1%)
Metastasis		
None	46	(95.8%)
Distant	2	(4.2%)
<i>HSD17B12 expression</i>		
Staining		
Negative	4	(8.3%)
Heterogenous	9	(18.8%)
Positive	35	(72.9%)
Intensity		
Weak	12	(27.3%)
Moderate	13	(29.5%)
Strong	19	(43.2%)
Cellular localization		
Nucleus	10	(22.7%)
Nucleus + cytoplasm	34	(77.3%)

HSD17B12 expression correlates significantly with

Larynx

Positive

Moderate $P = 0.012$ Nucleus + cytoplasm $P = 0.045$

Poor differentiation

Positive $P = 0.005$ Moderate $P = 0.021$ Nucleus + cytoplasm

The clinicopathological parameters of specimens in the commercially available SCCHN TMA HN803 (US Biomax Inc) and the results of IHC staining with rabbit anti-TYDKIKTGL antiserum are listed. The number of specimens in each clinicopathologic and HSD17B12 staining parameter is listed. HSD17B12 was detected in 44/48 specimens in the TMA. The significance of the results of staining the TMA relative to clinicopathological characteristics of the specimens was determined using Pearson Correlation Asymp Sig. (2-sided) analysis