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EDDR1 is a Potential Immunotherapeutic Antigen in Ovarian, Breast, and Prostate Cancer

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Abstract

Selection of suitable antigens, preferably targets for cell mediated and humoral immune response is a critical step in the development of cancer vaccines. Cell surface proteins that are over-expressed in cancer cells thus constitute a very attractive class of antigens that can be targeted for effective cancer immunotherapy. Toward this goal, we characterized the relevance of Epithelial Discoidin Domain Receptor 1 (EDDR1) for such targeted therapeutics. EDDR1, a membrane expressed protein associated with adhesion, has recently emerged as a new therapeutic target in several tumor types. In the present study, we analyzed the expression profile of EDDR1 in a variety of normal and cancer cells of human origin by flow cytometry as well as immunohistochemistry. EDDR1 was found to be abundantly expressed on the surface of ovarian, prostate and breast cancer cells but not on the normal counterparts, making it a suitable candidate for antibody mediated therapy. Furthermore, a Human Leukocyte Antigen (HLA) A2-restricted epitope derived from EDDR1 was efficiently presented by various cancer cells to EDDR1 epitope-specific T cells. Collectively, our data present evidence that EDDR1 could be a potential target antigen for immunotherapy.

Keywords: EDDR1; Epitope; Antigen; Immunotherapy; MHC; CTL; Immunohistochemistry; Flow cytometry; qRT-PCR

Abbreviations: EDDR1: Epithelial Discoidin Domain Receptor 1; MHC: Major Histocompatibility Complex; HLA: Human Leukocyte Antigen; CTL: Cytotoxic T Lymphocytes; qRT-PCR: Quantitative Reverse Transcribed Polymerase Chain Reaction; PBMC: Peripheral blood mononuclear cells; GM-CSF: Granulocyte Macrophage Colony Stimulating Factor; KLH: Keyhole Limpet Hemocyanin; ELISpot: Enzyme Linked ImmunoSpot; HRP: Horse Radish Peroxidase; DAB: 3,3´-diaminodbenzidine; EOC: Epithelial Ovarian Cancer; CNS: Central Nervous System; PCA-1: Prostate Cancer Antigen-1

Introduction

Cancer vaccines, activating the immune system against specific antigens, have demonstrated clinical benefit and an increasing number are now in development; however, one ongoing challenge is to identify the most appropriate antigenic targets. Although tumor antigens were originally identified by cloning T cells that exhibited anti-tumor activity and then identifying the antigen to which the clones responded through genetic approaches, we have utilized a novel approach which exploits the differential expression of peptides displayed within the Major Histocompatibility Complex (MHC) [1] in tumors compared to normal cells (reviewed in [2]). We hypothesized that these MHC-presented peptides, relatively restricted to tumor cells, could be used to activate anti-tumor T cells. In addition, when we used this approach to search for ovarian cancer antigens [3], we observed that many of these peptide epitopes were derived from proteins with critical functions in tumor growth, survival and metastasis [4], suggesting that this method identifies proteins that may be indispensible to the tumor and might not be readily downregulated during attempted immune escape.

One peptide identified by our search for differentially MHC-expressed epitopes is derived from Epithelial Discoidin Domain Receptor 1 (EDDR1 or DDR1) protein [3,5,6]. Discoidin Domain Receptor 1 and -2, a subfamily of tyrosine kinase receptors, regulate cell adhesion, proliferation and extracellular matrix remodeling (reviewed in [7]). In this study, in order to utilize EDDR1 as part of a cancer vaccine, we characterized the T cell epitope for anti-tumor CTL activity and the range of protein expression across several malignancies and demonstrate that it can act as a tumor associated antigen for immunotherapeutic applications.

Materials and Methods

Cell lines and primary cells from human tissues

Human ovarian cancer cell lines SKOV3-A2 and OVCAR3, human breast cancer cell lines MDA-MB-231 and MCF7, and human prostate cancer cell line LNCaP were originally obtained from ATCC, Manassas, VA). SKOV3-A2, OVCAR3 and LNCaP were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), L-glutamine (300mg/ml), non-essential amino acids (1× concentration), penicillin and streptomycin (1× concentration, supplements were purchased from Mediatech). MDA-MB-231 and MCF7 were maintained in DMEM medium supplemented with 10% fetal bovine serum and other supplements listed above. All cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. Kidney and liver tissues from HLA-A2$^+$ human donors were obtained from National Disease Research Interchange, Philadelphia, PA. Tissues were enzymatically digested and cell suspensions were generated as per standard methods [3,5,6]. Briefly, tissue samples were minced and digested with 2mg/ml collagenase, 0.1mg/ml hyaluronidase and 0.15mg/ml DNAse in DMEM supplemented with 2 × concentration of antibiotics and antimycotics (all reagents were obtained from Sigma-Aldrich, St Louis, MO) at 37°C

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for 3 to 6 hours. Cell suspensions were pelleted and washed several times with PBS and DMEM supplemented with 10% FBS. Cell viability was assessed by trypan blue exclusion and cells were frozen in 90% FBS and 10% DMSO (Sigma-Aldrich) for future use.

Synthetic peptides

Synthetic peptides corresponding to the HLA-A2 presented EDDR1 epitope (p15-FLAEDALNTV) and an influenza A virus epitope derived from the matrix protein (GILGVFTL) were supplied by GenScript Corporation (Piscataway, NJ).

In vitro generation of peptide specific CTLs

Heparinized blood from healthy HLA-A2$^+$ donors was purchased from Research Blood Components, LLC (Brighton, MA). Blood samples from women with ovarian cancer undergoing surgery at Duke University Medical Center were obtained under IRB approved protocols. Peripheral blood mononuclear cells (PBMC), collected by density gradient separation over lymphocyte separation medium (Mediatech), were plated at 20×10^6 cells per well in 2 mL RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (300 mg/mL), non-essential amino acids, sodium pyruvate, penicillin and streptomycin (complete medium) in 6-well tissue culture plates (BD, Franklin Lakes, NJ) overnight. Non-adherent cells were removed and saved. Plastic adherent cells were pulsed with 50 μg/mL synthetic peptide and 1.5 μg/mL human β2-microglobulin (Sigma-Aldrich) in complete medium for 2 hours. Non-adherent cells were added back in 5 mL complete medium supplemented with IL-7 at 5 ng/mL, Keyhole Limpet Hemocyanin (KLH, Sigma-Aldrich) at 5 μg/mL, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) at 25 ng/mL and IL-4 at 50 ng/mL (all cytokines and growth factors were purchased from Peprotech, Rocky Hill, NJ). Plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 12 days. Medium (2.0 mL) was removed from each well and fresh complete medium supplemented with 10U/mL IL-2 was added for two days. T cells were restimulated with CD4/CD8 T cell depleted autologous monocytes pulsed with synthetic peptide at 10 μg/mL and 1.5 μg/mL human β2-microglobulin in complete medium containing 5 ng/mL IL-7 and 5 μg/mL KLH for 5 days. IL-2 treatment and *in vitro* restimulation were repeated thrice at the indicated time intervals prior to use of *in vitro* expanded T cells in Enzyme Linked ImmunoSpot (ELISpot) assays.

ELISpot assays

In vitro expanded T cells were used as effectors in ELISpot assays to assess antigen stimulated interferon-γ (IFN-γ) or Granzyme B release using human IFN-γ and Granzyme B assay kits (BD-Pharmingen, San Jose, CA) (BD-Pharmingen, San Jose, CA) according to the manufacturer's instructions. Typically, a fixed number of various target cells (5×10^3 cells per well) and effector cells (2×10^5 per well), at effector to target ratios of 40:1, were used in ELISpot assays. T2 cells were pulsed with 20 μg/mL synthetic peptides and 1.5 μg/mL human β2-microglobulin in RPMI 1640 medium supplemented with 1% FBS and other additives (as above) overnight for use as targets in ELISpot assays. For antibody blocking experiments, target cells were pretreated with purified W6/32 or BB7.2 antibodies (from BD-Pharmingen) at 1:50 dilution for 1hr prior to addition to T cell cultures. ELISpot assays were performed in replicate wells. Spots were quantitated using an Immunospot reader (Cellular Technologies Limited, Shaker Heights, OH). Results are presented as the number of IFN-γ-producing cells per 1×10^6 effector cells. Error bars represent SEM of experimental replicates.

Flow cytometry

Primary cells from human liver and kidney and cancer cell lines were treated with a blocking solution (1% normal mouse serum from Sigma and 2% BSA from Sigma in PBS) on ice for 1 hr. Cells were then washed once with PBS and treated with either FITC-labeled isotype matched control antibody (Invitrogen, Carlsbad, CA) or anti-human EDDR1-specific mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution in PBS containing 2% BSA for 1 hr on ice. Cells were washed three times with PBS and subjected to flow cytometry by Guava flow cytometer (Millipore). Samples were analyzed using GuavaSoft software (Millipore).

Immunohistochemistry

Paraffin coated tissue arrays comprising cancer and matched control tissue sections were purchased from Imgenex, San Diego, CA. All chemical reagents used in this protocol are purchased from Sigma unless noted otherwise. Tissue arrays were stained with anti-EDDR1 antibody and HRP-conjugated secondary reagent according to manufacturer's established procedure (www.imgenex.com). Briefly, tissue arrays were first de-paraffinized by incubating in a dry oven at 62°C for 1h. Subsequently slides were dewaxed using xylene and hydrated using ethanol and washed in tap water. Antigen retrieval was achieved by treating slides in citrate buffer (0.01M, pH 6.0) and microwaving as suggested by the protocol. Endogenous peroxidase was quenched by treating slides with 3% hydrogen peroxide solution for 6 minutes. Slides were then blocked with blocking serum (normal mouse serum from Sigma at 1:50) for 30 minutes and treated with anti-EDDR1 antibody (1:100) for 2h at room temperature. Slides were washed with PBS and incubated with biotinylated anti-mouse secondary antibody for 30 minutes at room temperature. Subsequently, slides were washed and incubated with Avidin-conjugated HRP (1:100) for 30 minutes at room temperature. Slides were washed with PBS and developed using DAB solution for 2 minutes. Reaction was stopped using tap water. Slides were finally counter-stained using Meyer's hematoxylin for 10 seconds and dehydrated in ethanol. Slides were then cleared using xylene and cover slides were mounted with Permount (Vectastain staining reagents were purchased from Vector Labs, Burlingame, CA). Slides were viewed under a fluorescence microscope equipped with a digital camera and micrographs were captured (Nikon Eclipse, TE 800-U).

Realtime qRT-PCR

Realtime-qRT-PCR analysis to determine the expression level of EDDR1 was carried out by SABiosciences, Frederick, MD. RNA isolated from normal liver and kidney tissues (obtained from NDRI, Philadelphia, PA) or cancer cell lines (SKOV3-A2, MDA-MB231 and LNCaP) were used for the analysis. GAPDH was used as internal control. Following first strand synthesis, Realtime RT-PCR was carried out using reagents generated by SABiosciences. Data analysis was carried out employing $\Delta\Delta C_t$ method. Results are presented as fold difference of EDDR1 mRNA expressed in cancer cell lines over the control cells obtained from a normal liver tissue.

Results

Ovarian, breast and prostate cancer cell lines endogenously express EDDR1 epitope, p15

HLA A2-restricted peptide epitope derived from EDDR1 (called p15 (FLAEDALNTV)) was identified in a MHC class I associated epitope screen from ovarian cancer cells [3,5,6]. Here we studied whether T cells generated against p15 could recognize targets of various tumor histologies. We generated T cells specific for p15 from

healthy HLA-A2+ donors in vitro. These p15 specific T cells were used as effectors and various cancer cell lines (ovarian, breast and prostate cancer cell lines) were used as targets in an overnight ELISpot assay to quantify IFNγ release. We first demonstrated that the CTLs were p15-specific since they could recognize T2 cells pulsed with p15 peptide. Next, we observed, as expected, that p15 specific T cells recognized ovarian cancer cells (SKOV3-A2 and OVCAR3) from which p15 peptide was originally identified [3]. In addition, two HLA-A2+ breast cancer cell lines (MDA-MB231 and MCF7) were also recognized by p15 specific T cells. Interestingly, HLA-A2+ prostate cancer cell line LNCaP were also recognized by p15 specific T cells albeit at a lower level. Other cancer cell lines we tested in culture including colon cancer cell lines did not activate p15 specific T cells (data not shown). Importantly, non-cancerous liver cells and lung and kidney cells (data not shown) were not recognized by p15-specific T cells, suggesting that p15 is not endogenously presented by normal cells. However, when pulsed with the synthetic p15 peptide, these cells readily activated T cells, thus demonstrating that these cells were capable of presenting exogenously provided peptide antigen. Thus, these results demonstrated that EDDR1 derived p15 epitope was presented by ovarian, breast and prostate cancer cells but not cells derived from normal healthy tissue and was not necessarily universally expressed across other cancer cell lines.

HLA-A2 restricted nature of EDDR1 epitope p15 presentation to specific T cells

In order to demonstrate the HLA restricted nature of p15 presentation to specific T cells, we included antibodies specific for pan HLA-A, B, C (W6/32) or HLA-A2 (BB7.2) in the ELISpot assays. Our results indicate that the presentation of p15 from various cancer cell lines was indeed HLA-A2 restricted as evident from the effective inhibition of IFNγ release by BB7.2 antibody (Figure 2, top panel). As expected, W6/32 also blocked T cell activation. T2 cells or normal liver cells from an HLA-A2+ donor pulsed with an irrelevant peptide (influenza A matrix protein derived peptide) did not activate p15 specific T cells appreciably. When pulsed with the synthetic p15 peptide, these cells readily activated p15 specific T cells. Thus, our results demonstrated that the presentation of p15 to T cells is highly specific and HLA-A2 restricted.

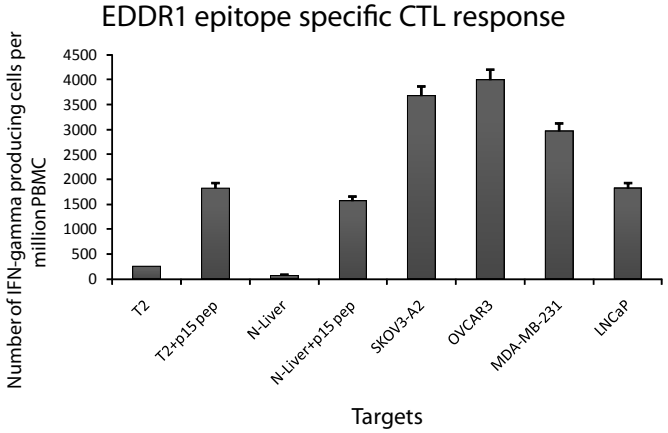

Figure 1: Characterization of T cells specific for the EDDR1 derived HLA-A2 restricted p15 epitope from healthy donors: PBMC from two healthy HLA-A2$^+$ donors were *in vitro* stimulated with synthetic peptides corresponding to the EDDR1 derived HLA-A2 restricted p15 epitope (FLAEDALNTV). These cells were tested in an overnight ELISpot assay using T2 cells loaded with the p15 synthetic peptide, normal liver cell suspensions obtained from a HLA-A2$^+$ healthy donor tissue (with or without exogenously provided p15 peptide) and HLA-A2$^+$ cancer cells lines [ovarian cancer cell lines SKOV3-A2 and OVCAR3, breast cancer cell lines MDA-MB231 and MCF7 and a prostate cancer cell line LNCaP]. Interferon-γ producing cells were quantitated using Immunospot reader. Error bars represent SEM of experimental replicates.

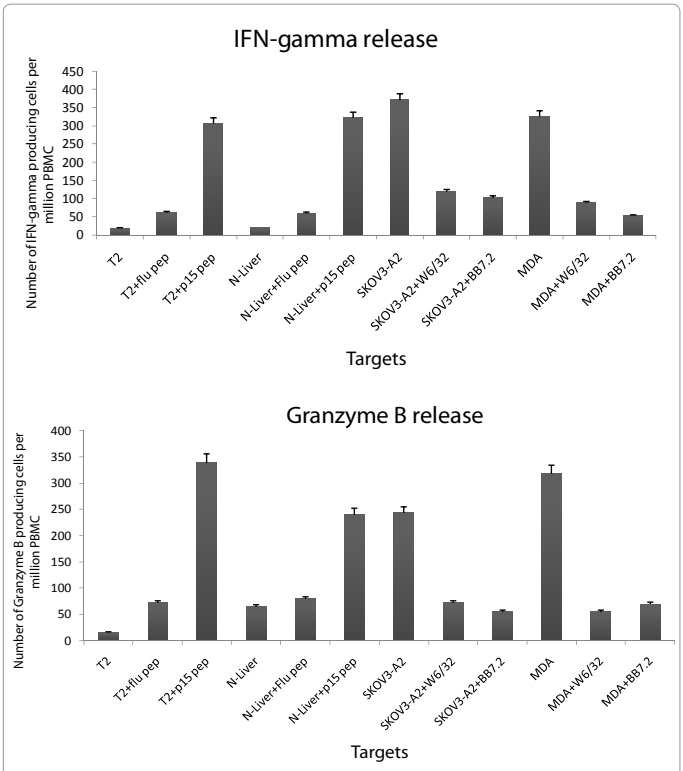

Figure 2: HLA-A2 restricted T cell response and cytolytic potential of T cells specific for the EDDR1 derived p15 epitope: PBMC from a healthy HLA-A2$^+$ donor were *in vitro* stimulated with the synthetic peptide corresponding to the EDDR1 derived p15 epitope. (A) These cells were tested in an ELISpot assay using T2 cells or normal liver cells (N-Liver) obtained from an HLA-A2$^+$ healthy donor tissue loaded with an irrelevant synthetic peptide (flu) or p15 peptide. HLA-A2$^+$ cancer cells lines [ovarian cancer cell line SKOV3-A2 and breast cancer cell line MDA-MB231] were pretreated with W6/32 or BB7.2 antibody prior to incubation with p15 specific T cells overnight. Interferon-γ producing cells were quantitated using Immunospot reader. (B) p15 specific T cells were used in an ELISpot assay with the above mentioned targets and the release of Granzyme B was measured as described above. Error bars represent SEM of experimental replicates.

EDDR1 epitope p15 specific T cells are cytolytic

In order to determine whether p15 activated T cells are cytolytic, we measured the release of Granzyme B in an ELISpot assay using various target cells. Results presented in Figure 2 (bottom panel) demonstrate that a significant portion of p15 activated T cells indeed secreted Granzyme B in response to p15 presentation by peptide loaded cells and cancer targets. This secretion could be blocked significantly by W6/32 or BB7.2 antibodies, demonstrating that the cytolytic activity was also dependent on HLA-A2 restricted peptide presentation.

EDDR1 derived epitope p15-specific T cells can be generated from ovarian cancer patients

Although T cells from healthy individuals, who may not have developed tolerance against p15, could be generated against p15, we wished to determine if T cells could be activated from PBMC of HLA-A2+ ovarian cancer patients using p15 synthetic peptide. We performed IFNγ ELISpot assay using T cells stimulated in vitro with p15 peptide and various targets. Our results shown in Figure 3 demonstrated that p15 specific T cells activated from three ovarian

cancer patients recognized peptide pulsed T2 cells as well as ovarian (SKOV3-A2 and OVCAR3) and breast cancer (MDA-MB-231) cell lines (Figure 3). Thus, these results demonstrated that p15 specific T cells can be generated from ovarian cancer patients.

Expression of EDDR1 on the surface of a variety of cancer cells

In order to evaluate the expression levels of EDDR1 on the surface of normal and cancer cells, we performed flow cytometry employing an antibody that recognizes the ectodomain of EDDR1 protein. EDDR1 expression levels were high on the surface of ovarian (SKOV3-A2 and OVCAR3), breast (MDA-MB-231 and MCF7) and prostate (LNCaP) cancer cell lines (Figure 4). Notably, SKOV3-A2 and LNCaP expressed very high levels of EDDR1 although the CTL activity (described above) was greater against SKOV3-A2 than LNCaP. In contrast, primary cells prepared from healthy human liver (Figure 4) and lung (data not shown) tissue did not express detectable levels of EDDR1 and primary cells from healthy human, kidney tissue expressed very low levels of EDDR1 on their surface. These results demonstrated that EDDR1 was highly expressed on the surface of a variety of cancer cells (ovary, breast and prostate – Figure 4; colon and lung – data not shown) but not on normal cells. Furthermore, the amount of protein expressed by flow cytometry did not necessarily correlate with CTL activity which was dependent on MHC-peptide expression.

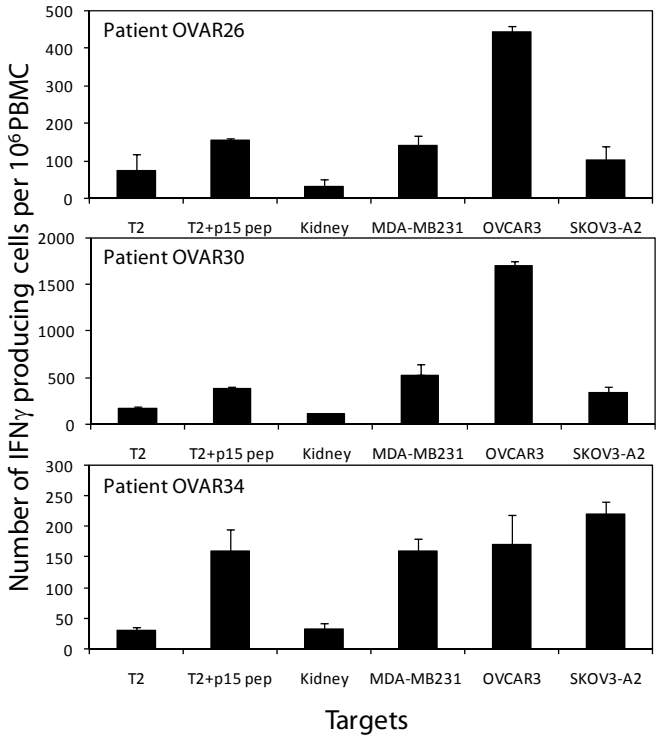

Figure 3: T cells specific for the EDDR1 derived HLA-A2 restricted p15 epitope can be generated from ovarian cancer patients: PBMC from three HLA-A2$^+$ ovarian cancer patients were *in vitro* stimulated with the p15 synthetic peptide. These cells were tested in an ELISpot assay using T2 cells loaded with the synthetic peptide, normal kidney cell suspensions obtained from a HLA-A2$^+$ healthy donor tissue (with or without exogenously provided p15 peptide) and HLA-A2$^+$ cancer cells lines [breast cancer cell line MDA-MB231 and ovarian cancer cell lines Ovcar3 and SKOV3-A2]. Interferon-γ producing cells were quantitated using Immunospot reader. Error bars represent SEM of experimental replicates.

Immunohistochemical analysis of EDDR1 expression in normal and cancer tissues

In order to confirm the EDDR1 expression levels in tissues form cancer patients, we performed immunohistochemical analysis on tissue sections from ovarian, breast and prostate cancers and tissue matched normal controls. EDDR1 was found to be expressed at very high levels in cancer tissues but its expression was low in normal tissues (Figure 5). In addition, we have tested a variety of normal tissue types (duodenum mucosa, spleen, pancreas, liver, lymph node, uterus myometrium) for EDDR1 expression (Figure S1) and found low expression profiles similar to normal ovary, breast and prostate. These results suggested that EDDR1 was not only over-expressed in cancer cell lines but also cancer tissues isolated from patients.

EDDR1 mRNA expression does not correlate with EDDR1 protein expression

In order to determine if there is a correlation between increased expression of EDDR1 protein and its transcript, we performed Realtime-qRT-PCR analysis, a powerful tool to determine the transcript levels of a given gene. We isolated RNA from normal liver and kidney cells to use as controls. Our analysis, shown in Figure 6, demonstrated that the EDDR1 mRNA level was elevated in SKOV3-A2 and LNCaP cells compared to normal liver and kidney cells. However, the levels were slightly reduced in MDA-MB231. These data supported the notion that elevated EDDR1 expression in cancer cells was regulated at the translational/post-translational but not at transcriptional level.

Discussion

Human cancers express antigenic targets of T cells and antibodies [8-11]; however, which antigens contain the epitopes leading to the most effective T cell rejection targets is largely unknown. Starting with the MHC class I-associated peptide repertoire of ovarian cancer cells, we identified a number of differentially expressed peptides including EDDR1 [3]. In our current work, we were able to generate EDDR1-epitope specific CTL from the PBMC of healthy individuals and cancer patients suggesting the presence of EDDR1-specific precursor T cells. Furthermore, flow cytometry and immunohistochemistry analyses demonstrated that this protein is abundantly expressed on the surface of several types of cancer cells including ovarian, prostate and breast cancer cells but not on their normal counterparts (Figs 5), making it a suitable candidate for immunotherapy applications.

Our observations on EDDR protein expression mirror those of others. For example EDDR1 protein has been shown to be restricted to epithelial cells, and is significantly over-expressed in several human tumors [12] including breast, ovarian [13], hepatocellular carcinoma [14] esophageal, and high grade pediatric brain tumor [15]. The DDR1 gene is highly over expressed in all histological subtypes of epithelial ovarian cancer (EOC) compared with normal ovarian surface epithelium, identifying EDDR1 as a new biomarker of EOC [13]. DDR1 is also expressed in ovarian epithelial inclusion cysts, a site of metaplastic changes within the normal ovary, in borderline tumors and in low-grade and stage cancers suggesting the up-regulation of EDDR1, is an early event in the development of EOC and have potential application in the early detection of disease [13]. In addition, EDDR1 has been shown to be consistently expressed in all high-grade Central Nervous System (CNS) neoplasms and by primitive cells of the embryonic ventricular zone suggests that EDDR1 is a potentially useful marker of tumor cells within the CNS [15]. Shimada et al [16] by immunohistochemical analysis of prostate carcinomas demonstrated that prostate cancer antigen 1 (PCA-1) and EDDR1 were strongly

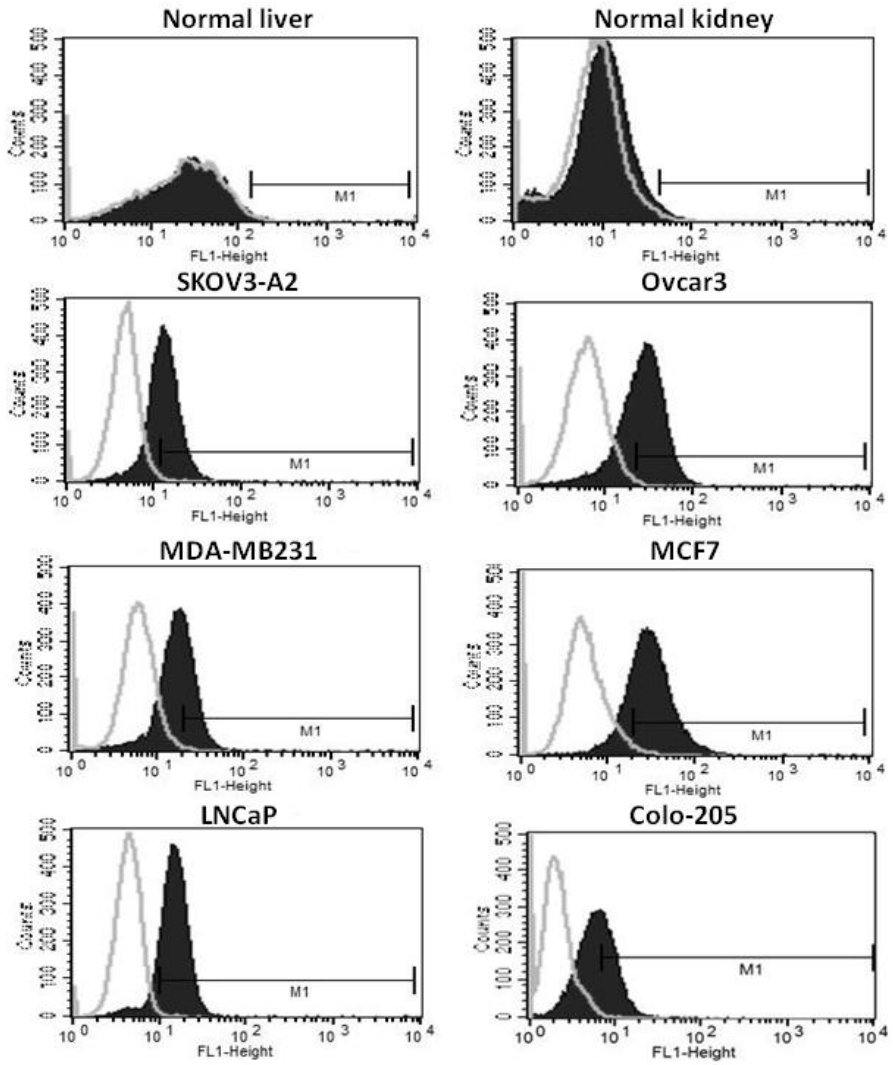

Figure 4: Analysis of surface expression of EDDR1 in normal and cancer cells: Normal liver and kidney cell suspensions obtained from healthy HLA-A2+ donors or cancer cell lines [ovarian cancer cell lines SKOV3-A2 and OVCAR3, breast cancer cell lines MDA-MB231 and MCF7, a prostate cancer cell line LNCaP and a colon cancer cell line Colo-205] were treated with either FITC-labeled isotype matched control antibody or FITC-labeled EDDR1 specific antibody and subjected to flow cytometry by Guava flow cytometer. Samples were analyzed using GuavaSoft software.

expressed in prostate cancer cells, including preneoplastic lesions with little or no expression in normal epithelium. Moreover, the expression of PCA-1 and EDDR-1 was associated with a hormone-independent state of prostate cancer suggesting PCA-1-EDDR1 signaling is a new important axis involved in malignant potential of prostate cancer associated with hormone-refractory status [16]. Furthermore, over expression of EDDR1 has been shown to increase the migration and invasion of hepatocellular carcinoma cells in association with matrix metalloproteinase [14]. Our work extends upon these other observations in that we have demonstrated not only protein expression, but also peptide epitope expression of EDDR1 in ovarian and breast cancer. Since EDDR1 is a membrane protein but is also processed and presented as peptide epitopes to T cells by MHC class I, this antigen qualifies for both cancer vaccine and antibody therapeutic applications.

One important observation is that the level of protein expression did not necessarily correlate with T cell recognition of tumors. This is not surprising since it has been observed that although protein over expression is a pre requisite for antibody or drug mediated targeting, it is not a critical factor for MHC class I processing and presentation of the T cell epitope which primarily correlates with the level of degradation of proteins associated with misfolding, cryptic translation and other causes of high turnover [1,2,17]. Our approach to antigen identification differs from the more common approach which is to perform a gene expression analysis. However, gene expression may not reflect the protein level or stability and modifications of the protein [18,19].

In summary, the characterization of EDDR1 as an immunotherapy target as well as a target for small molecule inhibitors may open up the possibility of combined approaches utilizing novel small molecules and cancer vaccines. Ongoing studies are evaluating the immunologic efficacy of EDDR1 in human cancer vaccines. Indeed the T cell epitope derived from EDDR1 has been evaluated for safety and CTL responses in patients with breast and ovarian cancers in a phase I clinical study

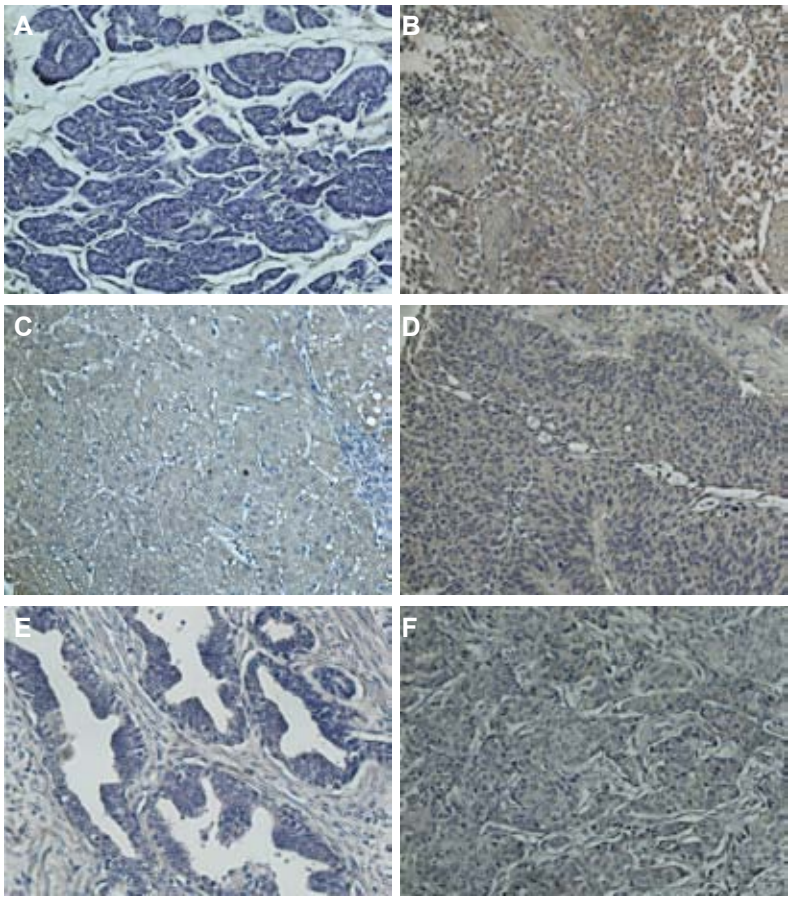

Figure 5: Immunohistochemical analysis of expression of EDDR1 in normal and cancer tissues: Tissue arrays comprising of matched normal or cancer specimens (A and B- normal and cancer breast, C and D- normal and cancer ovarian and E and F- normal and cancer prostate tissues) and a panel of normal tissues (Figure S1: duodenum mucosa, spleen, pancreas, liver, lymph node and uterus myometrium) were stained with a EDDR1 specific monoclonal antibody followed by treatment with an HRP-conjugated secondary antibody reagent. Slides were developed with DAB substrate and tissues were counterstained with Meyer's hematoxylin. Stained tissue sections were analyzed using a fluorescent microscope and micrographs were captured at 200× magnification.

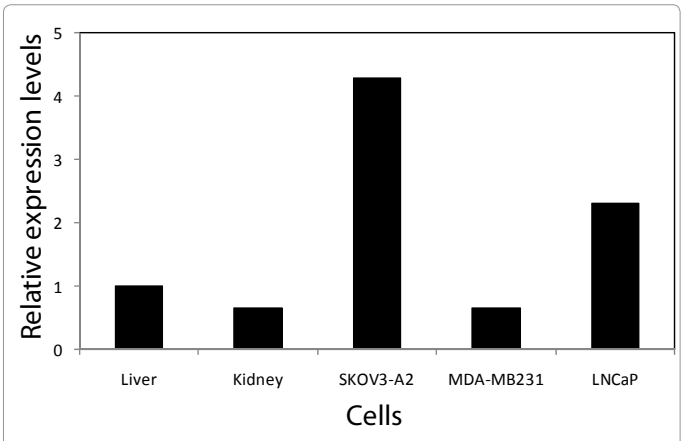

Figure 6: Expression level of EDDR1 in normal and cancer cells by Realtime-qRT-PCR analysis: Realtime-qRT-PCR analysis was carried out using RNA isolated from normal tissues (liver and kidney) or cancer cell lines (ovarian cancer cell line SKOV3-A2, breast cancer cell line MDA-MB231 and prostate cancer cell line LNCaP) to determine the transcript levels of EDDR1. GAPDH was used as internal control. Data analysis was carried out employing $\Delta\Delta C_t$ method. Results are presented as fold difference of EDDR1 expression in cancer cell lines over the control cells obtained from a normal liver tissue.

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