Differential expression and methylation of the TNFSF11 gene in hepatosplenic T-cell lymphoma.

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Hepatosplenic T-cell lymphoma (HSTCL) is a rare, aggressive, and almost uniformly lethal cancer with limited treatment options (1, 2). To understand the key features of HSTCL at the transcriptional level, we compared the transcriptomes of 35 cancer cell lines to that of the DERL-2 hepatosplenic T-cell line using a public dataset (3). We identified tumor necrosis factor (TNF) superfamily member 11, TNFSF11, also known as RANKL (receptor activator of NF- κ B ligand) as among the genes most differentially expressed in the DERL-2 HSTCL cell line. Comparing global DNA methylation profiles of tumor tissue from patients with HSTCL of the $\alpha\beta$ and $\gamma\delta$ subtypes to that of benign CD4+, CD8+ and $\gamma\delta$ T-cells using a separate published dataset (4) revealed a significant increase in methyl marks at the Tnfsf11 locus in HSTCL tumors of the $\gamma\delta$ subtype. The Tnfsf11 locus is hyper-methylated in the tumors of patients with $\gamma\delta$ HSTCL, TNFSF11 is among the genes whose expression is most different between DERL-2 HSTCL cells and a panel of 35 cancer cell lines and TNFSF11 RNA message is present in significantly higher quantities in DERL-2 HSTCL cells than all 35 cancer cell lines studied. TNFSF11 represents a target of interest in HSTCL.

Keywords: hepatosplenic T-cell lymphoma, HSTCL, TNFSF11, differential gene expression, systems oncology, tumor transcriptome analysis, targeted therapeutics in hepatosplenic T-cell lymphoma.

Hepatosplenic T-cell lymphoma (HSTCL) is a rare peripheral T-cell lymphoma with poor treatment outcomes (1, 2). The affected organs most often include the spleen and the liver, with sinusoidal infiltration of the spleen and hepatosplenomegaly (1). Bone marrow involvement and thrombocytopenia are common (1). HSTCLs, like T-lymphocytes that express T-cell receptors (TCRs), can be sub-classified into $\alpha\beta$ and $\gamma\delta$ subtypes based on their TCR expression (1). Though a relatively infrequent cancer, considered rare within peripheral T-cell lymphomas (which themselves account for 7-10% in total of all non-Hodgkin's lymphomas) (5), the advent of TNF monoclonal antibody therapy in patients with inflammatory bowel disease has generated a new profile of patients with HSTCL (6, 7). These are young adolescent males with Crohn's Disease or Ulcerative Colitis co-treated with infliximab, an anti-TNF monoclonal antibody, in conjunction with 6-mercaptopurine or azathioprine (8-11). HSTCL otherwise has arisen in patients with chronic immune suppression such as after organ transplant (1). Patients diagnosed with HSTCL possess a median overall survival of 8 months and a 5-year survival rate of 7%, the lowest of all peripheral T-cell lymphomas (5).

Only two HSTCL cancer cell lines, or *in vitro* models of HSTCL exist - DERL-2 and DERL-7 cells, isolated from the same male diagnosed with HSTCL (12). We studied the global gene expression in DERL-2 cells by comparing its transcriptome to that of 35 other cancer cell lines using a public dataset (3) to ask in an unbiased manner what were the most significant changes in gene expression in HSTCL cells compared to other cancer cell lines *in vitro*. We found that TNFSF11 was among the genes whose expression changed most significantly between DERL-2 cells and each of the 35 cancer cell lines paneled. DERL-2 cells expressed significantly higher quantities of messenger RNA for the TNF superfamily member 11 TNFSF11 than each of these 35 cancer cell lines, from the blood, the prostate, the colon, the breast, the brain, and the lung. Analysis of a separate dataset (4) revealed that Tnfsf11 gene was among the genomic loci most differentially methylated in the tumor tissue of HSTCL patients of the $\gamma\delta$ subtype. TNFSF11 is a molecule of interest in the biology of HSTCL.

Methods

Datasets GSE110081 (3) and GSE57083 (4) were utilized for this study in conjunction with GEO2R. Bergmann et al. generated dataset GSE110081 (3) using bisulfite conversion of DNA paired with Illumina Infinium 450k Human Methylation Beadchip technology. The Astra Zeneca Internal Cell Line database (GSE57083) (4), which was mined here, was generated using Affymetrix Human Genome U133 Plus 2.0 Array. In the analysis of dataset GSE110081, all tumor samples were from the spleen except for one bone marrow sample and one peripheral blood sample, both of which were from patients with $\gamma\delta$ HSTCL. A statistical test was performed to evaluate (a) the significance of difference in methylation levels at the *Tnfsf11* locus between $\alpha\beta$ HSTCL tumor tissue, $\gamma\delta$ HSTCL tumor tissue, and control T-cell groups, as well as (b) the significance of difference in mean mRNA expression levels of *TNFSF11* between the DERL-2 cell line and the 35 other cancer cell lines (4) paneled here. The results of these tests, a one-way ANOVA (analysis of variance) with Tukey's multiple comparisons, is depicted on each graph as a p-value. In Figure 1, only the statistically significant p-values ($p < 0.05$) are shown.

Results

TNFSF11 is one of the most differentially expressed genes when comparing the transcriptome of DERL-2 cells to a panel of 35 other cancer cell lines.

We compared the global gene expression profiles of a HSTCL cell line, DERL-2, that that of 35 other cancer cell lines from the blood, the prostate, the colon, the breast, the brain, and the lung to identify the most significant differences in HSTCL cells *in vitro*. We identified *TNFSF11* as one of the genes whose expression is most different in DERL-2 cells when compared to the remaining 35 cancer cell lines representing 6 tissue types. *TNFSF11* was the 40th most differentially expressed gene out of 54675 total RNA transcripts compared between this cancer cell line transcriptome comparison, and the global differential expression of *TNSF11* in HSTCL DERL-2 cells was statistically significant (Table 1; $p = 2.47E-17$).

1 HSTCL DERL-2 cells relatively over-express TNFSF11 messenger RNA.

2 We then extracted the exact mRNA expression values of TNFSF11 from each of 36 cancer cell
3 lines used in this study, including the DERL-2 HSTCL cell line. We found that DERL-2 cells expressed
4 higher amounts of TNFSF11 mRNA than all 35 cancer cell lines analyzed. The difference in TNFSF11
5 mRNA levels in DERL-2 cells was statistically significant when compared to mean mRNA level of
6 TNFSF11 in each of the 6 tissue types represented by the 35 cancer cell line panel, including the blood,
7 the prostate, the colon, the breast, the brain, and the lung (Figure 1; $p < 0.0001$ for all comparisons to
8 HSTCL DERL-2).

9 Thus, we found that TNFSF11 was among the genes most differentially expressed in the DERL-2
10 HSTCL cancer cell line when compared to 35 other cancer cell lines, and that TNFSF11 was expressed at
11 significantly higher levels in DERL-2 HSTCL cells when compared to each of the 35 cancer cell lines
12 paneled here (Figure 1).

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15 The Tnfsf11 genomic locus is among the genes whose DNA methylation level changes most significantly
16 in the tumors of patients with $\gamma\delta$ HSTCL.

17 Next, we used a separate published dataset that profiled DNA methylation globally, in tumor
18 tissue from patients with HSTCL of the $\alpha\beta$ subtype (n=4) as well as the $\gamma\delta$ subtype (n=6), comparing
19 global DNA methylation in HSTCL to that of “normal” (benign) $\alpha\beta$ and $\gamma\delta$ T-cells. This control T-cell
20 group (n=12) included CD4+ T-cells (n=4), CD8+ T-cells (n=4), and $\gamma\delta$ T-cells (n=4). The tumor tissue
21 sampled in 8 of 10 HSTCL patients was from the spleen, with 1 sample of peripheral blood and 1 sample
22 of bone marrow from patients with $\gamma\delta$ HSTCL.

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25 We identified a probe recognizing the Tnfsf11 genomic locus as one of the most differentially
26 methylated probes in the tumors of patients with $\gamma\delta$ HSTCL but not the tumors of patients with $\alpha\beta$
27 HSTCL. Differential DNA methylation of the Tnfsf11 locus in $\gamma\delta$ HSTCL compared to the
28 transcriptomes of $\alpha\beta$ HSTCL tumor tissue and benign $\alpha\beta$ and $\gamma\delta$ T-cell subsets ranked 215 most

1 differentially methylated. This was out of 65534 total sites probed in the genome, and was statistically
2 significant (Table 1; $p=8.47E-12$).

3
4 A site in the Tnfsf11 genomic locus is hyper-methylated in the tumors of patients with $\gamma\delta$ HSTCL.

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6 Finally, we obtained quantitative information for DNA methylation at the Tnfsf11 locus by
7 extracting DNA methylation values for the differentially methylated Tnfsf11 probe in each patient tumor
8 as well as from each control $\alpha\beta$ and $\gamma\delta$ T-cell subset. We found that the tumor tissues of patients with $\gamma\delta$
9 HSTCL (spleen, bone marrow and peripheral blood) possessed a significantly higher quantity of methyl
10 marks at the Tnfsf11 genomic locus when compared to benign $\alpha\beta$ and $\gamma\delta$ T-cells as well as when
11 compared to tumor tissues from patients with $\alpha\beta$ HSTCL (Figure 2; $p<0.0001$ for both comparisons).
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14 DNA methylation at Tnfsf11 was not significantly different when comparing $\alpha\beta$ HSTCL tumor tissues to
15 control, benign $\alpha\beta$ and $\gamma\delta$ T-cells (Figure 2; $p=0.9966$).
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18 Thus, by mining a published dataset, we found that in the tumors of patients with $\gamma\delta$ HSTCL, a
19 site at the Tnfsf11 genomic locus was among the most differentially methylated loci out of over 65,000
20 sites genome-wide when comparing global DNA methylation profiles to that of benign $\alpha\beta$ and $\gamma\delta$ T-cells.
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22 The Tnfsf11 locus possessed significantly higher quantities of methyl marks in the tumors of patients with
23 $\gamma\delta$ HSTCL than the same site at the Tnfsf11 locus in benign $\alpha\beta$ and $\gamma\delta$ T-cells. Differential hyper-
24 methylation at Tnfsf11 was specific to the tumors of patients with $\gamma\delta$ HSTCL, and not found in tumors
25 from patients with $\alpha\beta$ HSTCL.
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Discussion

HSTCL is a rare and aggressive peripheral T-cell lymphoma that offers a 5-year survival rate of 7%. Novel treatment strategies are needed for the patients that will develop HSTCL. Identifying targets of biologic and therapeutic interest in cancers has been facilitated by systems-level analysis of the cancer transcriptome or methylome in reference to the transcriptome or methylome of its tissue or cell of origin. HSTCL tumors, like TCR⁺ T-lymphocytes, can be classified as belonging to $\alpha\beta$ or $\gamma\delta$ subtypes based on TCR expression.

In this study, we integrated transcriptome and epigenome data from datasets (public (4) or published (3)) that profiled global mRNA expression and DNA methylation in the DERL-2 HSTCL cell line, and in tumors from patients with $\alpha\beta$ and $\gamma\delta$ HSTCL and identified the tumor necrosis factor superfamily member 11, TNFSF11, as differentially expressed and methylated in HSTCL. In the DERL-2 HSTCL cell line, TNFSF11 was one of the most differentially expressed genes when comparing its transcriptome to that of 35 cancer cell lines from the blood, the brain, the breast, the liver, the lungs and the prostate (4). TNFSF11 mRNA was expressed at significantly higher levels in DERL-2 HSTCL cells than in each of 35 cancer cell lines studied. Using a separate dataset (3), we identified a site at the Tnfsf11 genomic locus as among the most differentially methylated DNA genome-wide in the tumors of patients with $\gamma\delta$ HSTCL but not in the tumors of patients with $\alpha\beta$ HSTCL. This site at the Tnfsf11 locus possessed significantly higher levels of DNA methylation than the same site in benign $\alpha\beta$ and $\gamma\delta$ T-cells. Differential hyper-methylation of Tnfsf11 specific to the tumors of patients with $\gamma\delta$ HSTCL and was not found in the tumors of patients with $\alpha\beta$ HSTCL.

TNFSF11, also known as RANK ligand (RANKL), TRANCE, or osteoprotegerin (OPG) (13) has functions in the immune system and in the skeletal system (14). RANKL plays a key role in skeletal biology through its involvement in osteoclastogenesis, and is expressed by osteocytes, osteoclasts, osteoblasts, as well as bone marrow stromal cells (15). RANKL is also expressed in lymphocytes, both

by B-cells and T-cells. TNFSF11-null mice (16) display reduced skeletal growth, fail to erupt teeth, and possess osteopetrosis - brittle, dense and poorly formed bones (17). The immune functions of TNFSF11 include a role in the generation of lymph nodes, as TRANCE knockout mice manifest defects in lymph node development (18). In dendritic cells, TRANCE signaling induces secretion of the interleukins IL-1, IL-6, IL-12 and IL-15 to support DC survival and pro-inflammatory signaling (19). Structurally, TNFSF11/TRANCE/RANKL is a type II membrane protein whose extracellular domain is most similar to the Fas ligand (FasL) and the TNF-related apoptosis inducing ligand (TRAIL) (20). The human homolog of TRANCE/TNFSF11 shares 83% identity with the ectodomain of murine TRANCE/TNFSF11 (20).

This is the first report to document a relationship between TNFSF11 and hepatosplenic T-cell lymphoma (HSTCL) by combining systems-level transcriptome data from an *in vitro* model of HSTCL (4) with epigenome data from the tumor tissues of patients diagnosed with HSTCL. Gene expression of TNFSF11 in the HSTCL DERL-2 cell line was among the most significantly different globally when comparing the DERL-2 transcriptome to the transcriptomes of 35 cancer cell lines from 6 tissue types, the *Tnfsf11* locus was one of the genomic sites most differentially methylated in the tumors of patients with $\gamma\delta$ HSTCL, and the tumors of patients with $\gamma\delta$ HSTCL possessed significantly higher levels of methyl marks at the *Tnfsf11* locus when compared to the same site in $\alpha\beta$ T-cells, in $\gamma\delta$ T-cells, and in the tumors of patients with the $\alpha\beta$ subtype of HSTCL.

TNFSF11 over-expression in $\gamma\delta$ HSTCL should be validated in patient tumors at the mRNA and protein level, and the effect of TNFSF11 depletion on DERL-2 proliferation and viability *in vitro* should be assessed. HSTCL xenograft models are virtually non-existent other than the CBTL-81777 patient-derived xenograft (PDX) in NSG mice (21) but TNFSF11 deletion using CRISPR or TNFSF11 silencing by short hairpin RNA interference in the Id3 knock-out Rag1-deficient adoptive transfer mouse model (22) or in the CBTL-8177 PDX model (21) can begin to evaluate any roles for TNFSF11 in HSTCL biology *in vivo*.

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Rank	ID	p-value	F	Gene	Gene name
mRNA expression globally (GSE57083)					
40	210643_at	2.47E-17	76.8481	TNFSF11	tumor necrosis factor superfamily member 11
DNA methylation globally (GSE110081)					
215	NM_003701; NM_033012	8.47E-12	114	TNFSF11;TNFSF11	tumor necrosis factor superfamily member 11

Table 1: TNFSF11 is differentially expressed in the DERL-2 HSTCL cell line, and the Tnfsf11 genomic locus is differentially methylated in the tumors of patients with $\gamma\delta$ HSTCL.

The rank of global differential gene expression for the TNFSF11 mRNA message (out of 54675 total mRNA transcripts compared) and the rank of global differential DNA methylation at the TNFSF11 locus (out of 65534 genomic loci probed and compared), the p-value with respect to global differential expression or global differential methylation, the F statistic, the gene symbol and gene name are shown in this chart (obtained from GEO2R).

Figure 1: TNFSF11 is expressed at higher levels in the DERL2 hepatosplenic T-cell lymphoma (HSTCL) cell line than in 35 cancer cell lines of the blood, the prostate, the colon, the breast, the brain, and the lung.

The expression of TNFSF11 in the DERL2 HSTCL cell line, is graphically represented in conjunction with the expression of TNFSF11 in 35 other cancer cell lines, from the blood (n=11), the prostate (n=3), the colon (n=7), the breast (n=6), the brain (n=5), and the lung (n=3). A one-way ANOVA with multiple comparisons was performed to evaluate the statistical significance in TNFSF11 mRNA expression between the mean expression values of every group, and all statistically significant results are shown on the graph as a p-value.

Figure 2: The Tnfsf11 locus is hyper-methylated in the tumors of patients with $\gamma\delta$ HSTCL.

DNA methylation at the Tnfsf11 locus in benign T-cells (CD4+ (n=4), CD8+ (n=4), and $\gamma\delta$ (n=4); n=12), in the tumors of patients with $\alpha\beta$ HSTCL (n=4), and in the tumors of patients with $\gamma\delta$ HSTCL (n=6) is graphically represented with the standard error of the mean (error bars). A one-way ANOVA was performed to evaluate the statistical significance of differences in DNA methylation levels between control T-cells, $\alpha\beta$ HSTCL and $\gamma\delta$ HSTCL cell types, and the result of each of the multiple comparisons is shown as a p-value.