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Ectopic expression of cancer testis antigens in Cutaneous T-Cell Lymphoma (CTCL) patients

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Abstract

Purpose—The pathogenesis of CTCL remains only partially understood. A number of recent studies attempted to identify novel diagnostic markers and future therapeutic targets. One group of antigens, cancer-testis (CT) antigens, normally present solely in testicular germ cells, can be ectopically expressed in a variety of cancers. Currently only a few studies attempted to investigate the expression of CT antigens in CTCL.

Experimental Design—In the present work we test the expression of CT genes in a cohort of CTCL patients, normal skin samples, skin from benign inflammatory dermatoses and in patient-derived CTCL cells. We correlate such expression with the p53 status and explore molecular mechanisms behind their ectopic expression in these cells.

Results—Our findings demonstrate that SYCP1, SYCP3, REC8, SPO11 and GTSF1 genes are heterogeneously expressed in CTCL patients and patient-derived cell lines, while cTAGE1 was found to be robustly expressed in both. Mutated p53 status did not appear to be a requirement for the ectopic expression of CT antigens. While T cell stimulation resulted in a significant upregulation of STAT3 and JUNB expression, it did not significantly alter the expression of CT antigens. Treatment of CTCL cells *in-vitro* with Vorinostat or Romidepsin Histone Deacetylase inhibitors resulted in a significant dose-dependent upregulation of mRNA, but not protein. Further expression analysis demonstrated that SYCP1, cTAGE1 and GTSF1 were expressed in CTCL, but not in normal skin or benign inflammatory dermatoses.

Conclusions—A number of CT genes are ectopically expressed in CTCL patients and can be used as biomarkers or novel targets for immunotherapy.

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Keywords

Cutaneous T Cell Lymphoma (CTCL); Cancer Testis Antigens; GTSF1; SYCP1 and cTAGE1

INTRODUCTION

Cutaneous T-cell Lymphoma (CTCL) is a rare cancer with the documented incidence rate of 4–8 cases per million(1–4). A number of studies documented a ~3 fold increase in the incidence of CTCL in the last 25–30 years(2, 4). CTCL represents a heterogeneous group of non-Hodgkin lymphomas with Mycosis Fungoides (MF) and its leukemic variant Sézary Syndrome (SS) being the most common variants(5). In Caucasians MF/SS primarily affects individuals over 55 years of age, while in African-Americans, Hispanics and Arabic individuals this disease presents at a significantly younger age (i.e. 20s and 30s) (1, 4, 6). Furthermore, CTCL was reported to have a higher predilection for males and African-Americans, where disease typically presents with higher clinical stage and follows a more aggressive clinical course(4, 6).

In the early disease stages, which can last several years, MF presents as flat erythematous skin patches resembling benign inflammatory diseases, whereas in the later stages, MF cells gradually form plaques or tumors and may disseminate to the lymph nodes and internal organs (5). The early stages of CTCL are often difficult to distinguish clinically and even histologically from other benign entities including chronic eczema, psoriasis and pityriasis rubra pilaris. In advanced disease cancerous cells in some patients may appear in the peripheral blood, leading to the leukemic stage of CTCL. A subset of leukemic CTCL, known as SS, is characterized by a triad of erythroderma, lymphadenopathy and detection of malignant T cells with convoluted/cerebriform nuclei on a peripheral blood smear (5).

Recent advances in tumor immunology have led to the isolation of several genes and gene families encoding antigens capable of eliciting autologous T-cell responses in cancer patients (7). One group of antigens, Cancer-Testis (CT) antigens, are named after their typical pattern of expression since they are present in a variety of cancers, but in normal adult tissues they are only expressed in germ cells of the testis (8, 9). Several CT antigens have been successfully employed as target antigens in various vaccine-based clinical trials (10, 11). Though knowledge of their biology and function is not known, the aberrant CT antigen expression in cancer appears to reflect the reactivation of a normally silenced gametogenic program, conferring some of the central characteristics of malignancy to the tumor (12, 13). The present catalog of CT antigens comprises more than 100 distinct CT genes and/or gene families(14). The expression of several CT antigens has been analyzed in a variety of malignant neoplasms on the mRNA level and, to a lesser extent, on the protein level. Highest expression was found in melanoma and carcinomas of the bladder, lung, liver, certain types of sarcomas, and multiple myelomas (15). Currently, there are only a few studies evaluating the expression of CT antigens in CTCL.

Limited pilot studies attempted to test the expression of CT antigens serologically and by RT-PCR in CTCL patients or immortalized cell lines(16–19). This work suggested that a subset of CT genes were upregulated in a subset of patients with CTCL(16–19). However,

the biological role that these genes might be playing or how the ectopic expression of these genes is regulated remains unknown.

In the current work we test the expression of a subset of CT genes that were previously suggested to be expressed in CTCL in the historic cohort of 60 CTCL patients and compare such ectopic expression in CTCL to the expression in normal skin and benign inflammatory dermatoses. We further evaluate the expression of a number of CT genes in 11 patient-derived CTCL cell lines and confirm the ability of HDAC and HAT inhibitors to modulate or induce the expression of these genes.

PATIENTS, MATERIALS AND METHODS

Patients and Samples

All patients were enrolled in an IRB-approved study protocol with informed consent. CTCL patients were recruited from the Cutaneous Lymphoma Clinic at the Dana Farber Cancer Institute (DFCI)/Brigham and Women's Hospital (BWH). All tissue samples were obtained and processed as previously described(20). Briefly, punch biopsies from involved skin were collected from 60 CTCL patients between January 26, 2003 and June 1, 2005. The obtained 6 mm biopsies were immediately snap-frozen in liquid nitrogen. Tissue was powdered in liquid nitrogen (Cryo-Press; Microtec Co, Chiba, Japan), and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and converted to cDNA using the iScript RT-PCR kit (Bio-Rad, Hercules CA) according to the manufacturer's instructions. The biopsy samples analyzed in this report are the same samples that were analyzed in previous studies(20–24). Also, the isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Sézary patients (n = 13) and control patients with benign inflammatory dermatoses (n = 8) was performed in accordance with the IRB approved protocol and with informed consent as previously described(21). The diagnosis and clinical staging were established according to the diagnostic criteria of CTCL(25). Similarly, volunteers with normal healthy skin (N=5) and benign inflammatory dermatoses (N=19) were recruited from the outpatient dermatology clinic of the University of British Columbia (Vancouver, Canada) with informed consent. These included 8 cases of psoriasis and 11 patients with benign chronic dermatitis. With informed consent, full-thickness lesional skin punch biopsies were obtained under local anesthesia as described previously (21, 24).

Cell Culture

HH, H9, Hut78, MJ and Hut102 patient-derived CTCL cell lines were previously described (26, 27) and were purchased from the American Tissue Culture Collection (ATCC). H9 is a clonal derivative of Hut78 cell line(28). MyLa, PB2B, Mac2A, SZ4, SeAx, Sez4 were a generous gift from professors K. Kaltoft and N. Ødum (Copenhagen, Denmark) and were previously described elsewhere (29–33). MJ, Hut78 cells were serially passaged in IMDM media (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen). HH, H9, Hut102, MyLa, Mac2A and SZ4 cells were grown in RPMI media containing 10% FBS. Finally, Sez4 and SeAx cells were grown in RPMI media containing 10% FBS, 5 ng/mL of recombinant human IL-2 and IL-4 (R&D Systems, Minneapolis, MN). All cells were grown in 5% CO_2, 95% air humidified incubator at 37°C. For stimulation, malignant T cells were

treated with 10 ng/mL of PMA (phorbol 12-myristate 13-acetate) and 1 μM of ionomycin (Tocris Bioscience, Bristol, UK) or with CD3/CD28 Dynabeads® (Invitrogen) according to manufacturer's instructions. To inhibit histone deacetylase (HDAC) activity cells were treated with 2.5–10 μM of Suberoylanilide Hydroxamic Acid (SAHA also known as Vorinostat, Santa Cruz, Dallas, TX) or 250nM-1μM Romidepsin (Adooq Bioscience, Irvine CA). To block histone acetyltransferase (HAT) activity cells were treated with 25–50 μM of Anacardic Acid (Sigma-Aldrich, St. Louis, MO). mRNA from cell lines was isolated using Quiagen (Valencia, CA) and was converted into cDNA using Bio-Rad iScript cDNA synthesis kit. Lysates for western blotting were obtained and quantitated as previously described(34). TP53 sequencing was performed by the Molecular Genetics Laboratory, The Hospital For Sick Children (Toronto, ON) as previously described (35, 36).

Quantitative Real-Time Reverse Transcription-PCR Gene Expression Analysis

Based on previous reports and our recent analyses(16–20, 22) we have selected 16 CT genes to be tested in the CTCL patients lesional skin, normal skin form healthy volunteers, lesional skin from patients with benign inflammatory dermatoses, and in patient-derived CTCL cell lines. Primers for candidate human genes were designed using Primer 3 web software (37) and were purchased from Invitrogen Inc. Primer pair sequences used in this experiment are listed in supplementary table 1. RT-PCR was performed utilizing the obtained cDNA from patients and iScript RT-PCR mix (Bio-Rad) on Bio-Rad iCycler as previously described (22–24). The expression was standardized using genorm method(38) utilizing ACTB, SDHA and GAPDH housekeeping genes. The obtained data was analyzed using XLSTAT 2009 software.

Western Blotting

Western blotting was performed as described previously(34). cTAGE1 and Rec8 rabbit polyclonal antibodies were purchased from Proteintech (Chicago, IL). GTSF1 rabbit polyclonal antibody was purchased from Abnova (Walnut, CA). SYCP1 rabbit polyclonal antibody was purchased from GenTex (Irvine, CA). SPO11 rabbit polyclonal antibody was purchased from Abcam (Cambridge, MA). STAT antibodies were purchased from Cell Signaling (Danvers, MA) as part of Stat Antibody Sampler kit (Catalog #9939). Also, STAT3 (79D7) Rabbit mAb (Catalog number #4904) was used in our Western Blot experiements. Chemiluminescent detection reagents (ECL) were purchased from Amersham Biosciences (Piscataway, NJ).

RESULTS

Expression of CT genes in CTCL lesional skin

A number of critical seminal studies using serological detection of CTCL antigens suggested that a number of Cancer Testis (CT) genes might be heterogeneously expressed in CTCL patients(16–18, 39). Furthermore, additional testing by RT-PCR and Northern Blot confirmed the expression of some CT antigens in a subset of CTCL lesional skin(18, 19). In the current work, we tested by RT-PCR the expression of selected 16 CT antigens in a larger previously described cohort of 60 CTCL patients. Our findings demonstrated that, while most CT genes were not detectable in lesional CTCL skin, 5 genes (cTAGE1, REC8,

SYCP1, SYCP3 and GTSF1) demonstrated ectopic expression in a subset of CTCL patients (Figure 1A, Supplementary Figure 1). Out of these genes, cTAGE-1 demonstrated the most robust and uniform expression in all CTCL patients, while SYCP3 was strongly expressed only in <10% of patients.

Expression of CT genes in a panel of patient-derived CTCL cell lines

To confirm that the above observed ectopic expression of CT genes takes place in malignant T cells and to further interrogate their mechanisms for ectopic expression we tested the expression of cTAGE1, REC8, SYCP1, and GTSF1 in a panel of 11 patient-derived immortalized CTCL cell lines. As documented by RT-PCR (Figure 1B and Supplementary figure 2) and Western Blot (Figure 1C) a number of the CT genes including SYCP1, cTAGE-1 and GTSF1 demonstrated robust expression in the majority of CTCL cell lines. cTAGE-1 mRNA and protein were expressed in all tested cell lines.

Chronic inflammation is one of the critical hallmarks for CTCL. Hence, we wanted to test whether a 24-hour T cell activation with PMA (phorbol 12-myristate 13-acetate) and ionomycin (P+I) or with CD3/CD28 Dynabeads® would lead to upregulation of the CT genes in patient-derived cell lines. As demonstrated in Figure 2, in commonly used HH and MyLA cells by RT-PCR and Western Blot, T cell activation via PMA and ionomycin (P+I) did not lead to a significant upregulation in expression of CT genes, but, as a positive control, resulted in upregulation of STAT3 and JUNB. Both HH and MyLa cells express significant levels of STAT3 and JUNB proteins, whose expression is significantly upregulated with T cell stimulation. Expression results for all genes tested across 11 cell lines further suggest that in most samples these genes are not upregulated by P+I T cell stimulation (Supplementary Figure 3). Similar results were obtained using CD3/CD28 Dynabeads® activation (data not shown).

The normal function of SYCP1 and REC8 is to control meiosis during gametogenesis(40). Previous reports suggested that during mitotic catastrophe in p53-mutated lymphoma cells after irradiation several meiosis-prophase CT antigens were upregulated (40). These studies further indicated that mutated p53 played a permissive role to enable CT gene expression and function(40). Hence, we wanted to interrogate the status of p53 in our cell lines. Sequencing analysis was performed for p53 gene and results are summarized in Table 1. Hut78 and its clonally derived variant, H9, had a nonsense mutation of p53 in exon 6. SeAx cells had a deleterious Gly245Ser mutation in exon 7. HH cells harbor a c560-1G>A mutation, which is predicted to affect splicing in intron 5 and lead to partial loss of function. Many cell lines had a silent c75–80C>G polymorphism in intron 2, which is predicted not to affect gene expression or function.

It is of interest that all cell lines with mutated p53 (i.e., Hut78, H9 and SeAx) expressed detectable SYCP1 and REC8 by RT-PCR and Western Blot (Figures 1B and 1C). A number of cells with wild-type p53 such as PB2B and Mac2A strongly expressed SYCP1, but only weakly expressed REC8. Furthermore, two of the cell lines with wild-type p53 (HUT102 and MJ) expressed both CT genes, suggesting that mutation in p53 is not a requirement for ectopic upregulation of meiotic CT genes.

Molecular mechanisms of regulation for ectopic CT antigen expression

Recently, epigenetic changes became a significant focus of basic and clinical research in CTCL. A number of previous studies documented methylation/histone acetylation abnormalities in malignant CTCL cells(41, 42). In fact, two of the commonly used medications for advanced stages of this cancer are HDAC inhibitors (Romidepsin and Vorinostat) (43). Hence, we hypothesized that ectopic expression of CT genes was due to a loss of epigenetic transcriptional repression of these genes. To test whether histone acetylation mediates the expression of these genes we treated Hut78, H9 and Mac2A CTCL cell lines for 24 hours with HDAC inhibitors, Romidepsin and Suberoylanilide Hydroxamic Acid (SAHA also known as Vorinostat). By blocking the deacetylation of histones these agents promote an open (i.e., acetylated) chromatin structure and thereby upregulate the expression of a number of genes. As demonstrated by RT-PCR, treatment with Vorinostat and Romidepsin dramatically upregulated the expression of SYCP1, cTAGE-1, Rec8 CT genes in a dose dependent manner, but not the B2M “housekeeping gene”, in HH and Hut78 cells, while Mac2A cells demonstrated only a modest upregulation in expression of these CT genes (Figure 3A, B and Supplementary figure 4).

To potentiate the efficacy of immunotherapy it may be desired to induce the expression of novel CT genes. Hence, we questioned whether these treatments are able to induce the expression of CT genes that are commonly not expressed in CTCL patients. As a proof of principle, we tested the effect of these drugs on a meiosis regulator SPO11 gene that was detected in only 3 out of 60 CTCL patients (Supplementary Figure 1). The tested cell lines exhibited low detectable expression of SPO11, which was dramatically increased in Hut78 and H9 cells after the 24 hour treatment with the HDAC inhibitors (Figure 3A).

We further hypothesized that if HDAC inhibition leads to an upregulation of mRNA expression of CT genes, then HAT inhibition should produce an opposite effect. Hence, we treated these cells with 25 and 50 μM of anacardic acid and tested the expression of SYCP1 and SPO11 in these cells. As demonstrated in Figure 3C, anacardic acid significantly blocked the ectopic expression of SPO11 in all tested cell lines (Figure 3C) and also blocked the expression of SYCP1 in Mac2A cells (Supplementary Figure 5).

Many meiotic genes are subject to extensive post-transcriptional and post-translation regulation(44). Hence, in addition to the above analysis of mRNA expression we analyzed protein expression. While Vorinostat and Romidepsin produced an upregulation of REC8 expression, for other genes (cTAGE, GTSF1, SPO11 and SYCP1) a downregulation in expression was observed (Figure 3D). The posttranscriptional/posttranslational mechanisms for the observed downregulation remain to be elucidated. These combined results highlight the importance of epigenetic, posttranscriptional and posttranslational regulation for this family of genes.

Comparison of CT antigen expression between CTCL lesional skin, normal skin and skin from patients with benign inflammatory dermatoses

Since a number of CT antigens may potentially be used as diagnostic markers or serve as novel targets for immunotherapy, we compared the expression of SYCP1, cTAGE1, GTSF1

and REC8 between CTCL lesional skin, normal skin from healthy donors and skin affected by benign inflammatory dermatoses that often clinically mimic CTCL (i.e., psoriasis, pityriasis rubra pilaris and chronic eczema). This RT-PCR analysis revealed that 3 genes (GTSF1, SYCP1 and cTAGE1) were preferentially expressed in CTCL, but not in other skin samples from benign inflammatory dermatoses patients or healthy volunteers (Figure 4). As a positive control, other signaling genes (JunB and PLK1) were heterogeneously expressed at similar levels between malignant and non-malignant biopsy samples (Supplementary Figure 6). A similar expression analysis on isolated Peripheral Blood Mononuclear Cells (PBMCs) from Sézary patients vs. from patients with benign inflammatory dermatoses was performed and demonstrated that cTAGE1 and GTSF1 were preferentially expressed in CTCL (Suplementary Figure 7).

DISCUSSION

In the current work we demonstrated that a number of CT antigens are heterogeneously expressed in CTCL patients while cTAGE1, SYCP1 and GTSF1 are preferentially expressed in CTCL lesional skin when compared to benign inflammatory dermatoses or normal skin. GTSF1 is not expressed in HH, MJ and Hut102 patient-derived CTCL cell lines, while cTAGE1 and SYCP1 are expressed in all cell lines tested by RT-PCR. Consistently with the observed RT-PCR expression we document via Western Blot that most cells express protein products for the tested CT antigens. We further investigated the mechanism for ectopic expression of the tested CT genes and documented that treatment of cell lines with HDAC inhibitors results in upregulation in mRNA expression, while treatment with HAT inhibitors led to downregulation of mRNA expression in a subset of cells. This suggests that aberrant epigenetic control of methylation/histone acetylation may play an important role in producing ectopic expression of CT genes in CTCL.

It was reported that in other cancers upregulation of CT genes corresponded with advanced pathological stage and worse prognosis(45–48). In a variety of cancers, CT antigens are suspected to play an important role in maintaining cell survival (i.e., inhibition of apoptosis) (49–51), promote resistance to various forms of chemo- and radio-therapy(52, 53) and contribute to oncogenesis by downregulating p53 and p21 tumor suppressor genes(12, 54). Also, considering that the normal function of several CT genes (such as SYCP1, REC8, SPO11 and others) is to regulate generation of double strand DNA breaks, chromosomal recombination/crossing over in meiosis, it was suggested that these genes may promote aneuploidy and genomic instability in cancers by producing aberrant chromosomal recombination(40).

Experimental molecular reports indicate that during mitotic catastrophe in p53-mutated lymphoma cells after irradiation several meiosis-prophase CT antigens were upregulated, localized to the centromeres in the nucleus, which led to an emergence of endopolyploid cells(40). Hence, it is possible that this subset of CT antigens regulates the extent of arrested mitosis and polyploidy. In order to elucidate this further we tested the p53 mutation status in the patient-derived CTCL cell lines. Based on our findings we discovered that p53 was mutated in H9, Hut78 and SeAx cells. While the presence of p53 mutation is not a requirement for ectopic expression of CT genes, concomitant loss of p53 function may

augment the ability of CT genes to accelerate cancerogenesis. Notably as documented by Western Blot the cell lines that harbored p53 mutations expressed SYCP1, SPO11, REC8 and other CT genes that might be contributing to carcinogenesis.

Another intriguing gene, GTSF1 (Gametocyte Specific Factor 1), was reported to be a part of a molecular signature that is specific to CTCL (55). The findings in our study further underscore the importance of this gene as a potential diagnostic marker and a putative therapeutic target.

Previous studies suggested based on serology and limited PCR in 20 patients that cTAGE1 (Cutaneous T-Cell Lymphoma-Associated Antigen 1) is expressed only in 30% of CTCL patients(17, 39). However, in our study we found this gene to be robustly expressed in a majority of CTCL patients and patient-derived cell lines. One possibility that might explain this discrepancy is the presence of various splicing variants for this gene(39). Hence, use of different primers targeting different portions of the gene may produce discrepancies. Thus, additional testing in new populations of CTCL patients will be necessary to confirm high incidence of ectopic expression for this CT gene.

In summary, CT genes represent potential targets for immunotherapy, may play an important role in cancerogenesis and contribute to treatment resistance by malignant tumors. Further investigations into the molecular mechanism of action of this family of genes will lead to improved understanding of CTCL biology and may lead to a development of novel therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Translational Relevence

Ectopic expression of Cancer Testis (CT) antigens was previously found in carcinomas of the bladder, lung, liver, certain types of sarcomas and multiple myelomas. Several CT antigens have been successfully employed as target antigens in various vaccine-based clinical trials. The expression of CT genes in CTCL is not well documented. In the current work we define the expression for a number of CT genes in CTCL and explore molecular mechanisms behind their expression. These genes may be used as targets for immunotherapy or as novel diagnostic markers for CTCL.

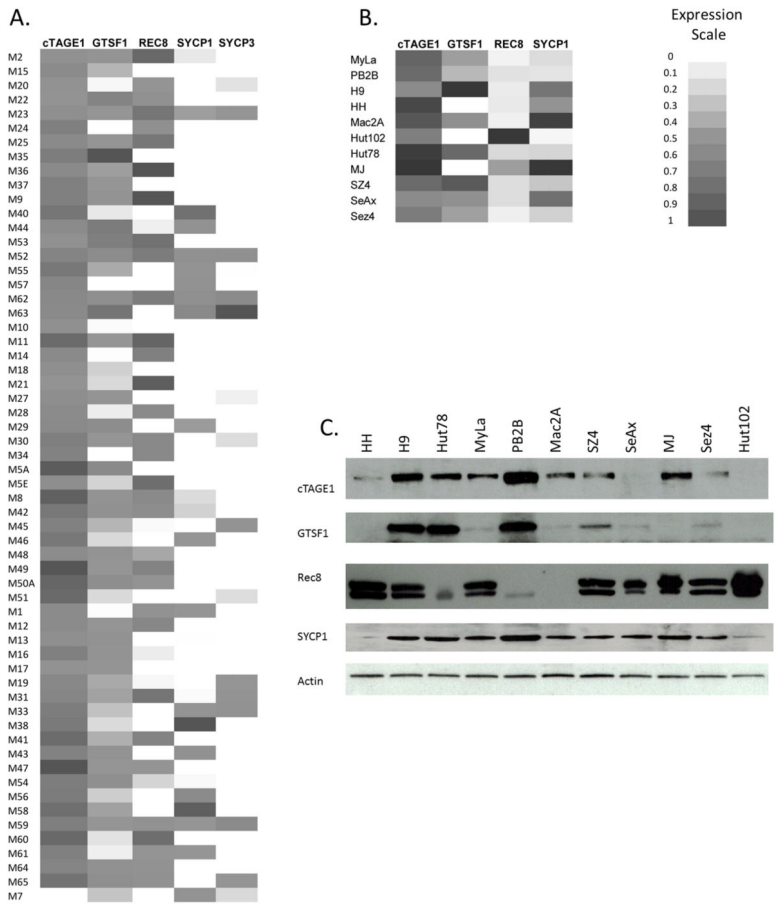

Figure 1.

Analysis of ectopic expression of CT genes in CTCL patients by RT-PCR(A) and in CTCL patient-derived cell lines by RT-PCR (B) and Western Blot (C).

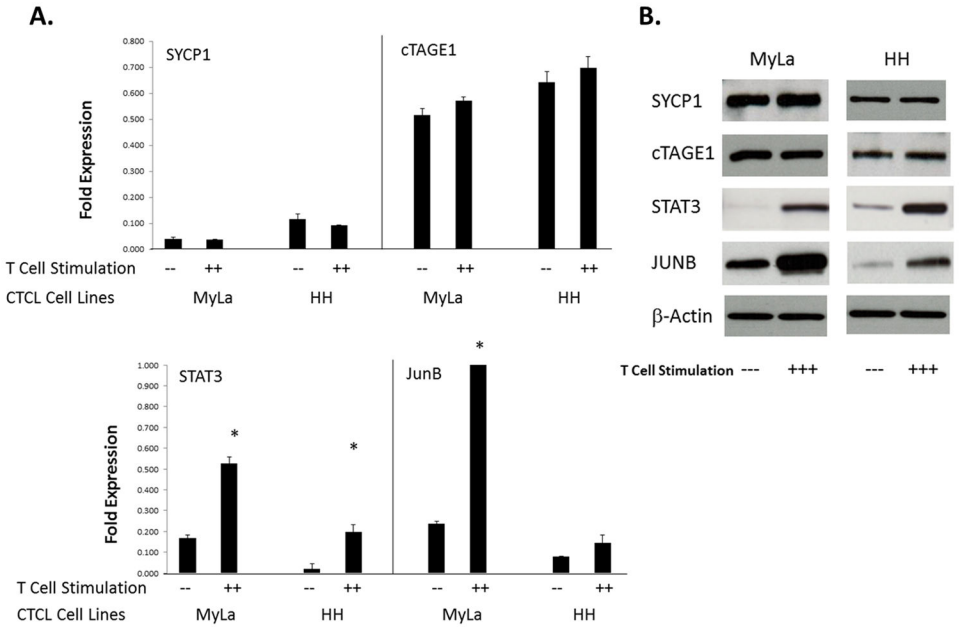

Figure 2.

Effect of T cell stimulation with (P+I) on CT gene expression and (as a positive control) on JUNB and STAT3 expression by RT-PCR (A) and Western Blot (B). Note: Both cell lines (HH and MyLa) express significant amounts of STAT3 and JunB proteins. However, low exposure images were used to highlight upregulation of these proteins with T cell stimulation.

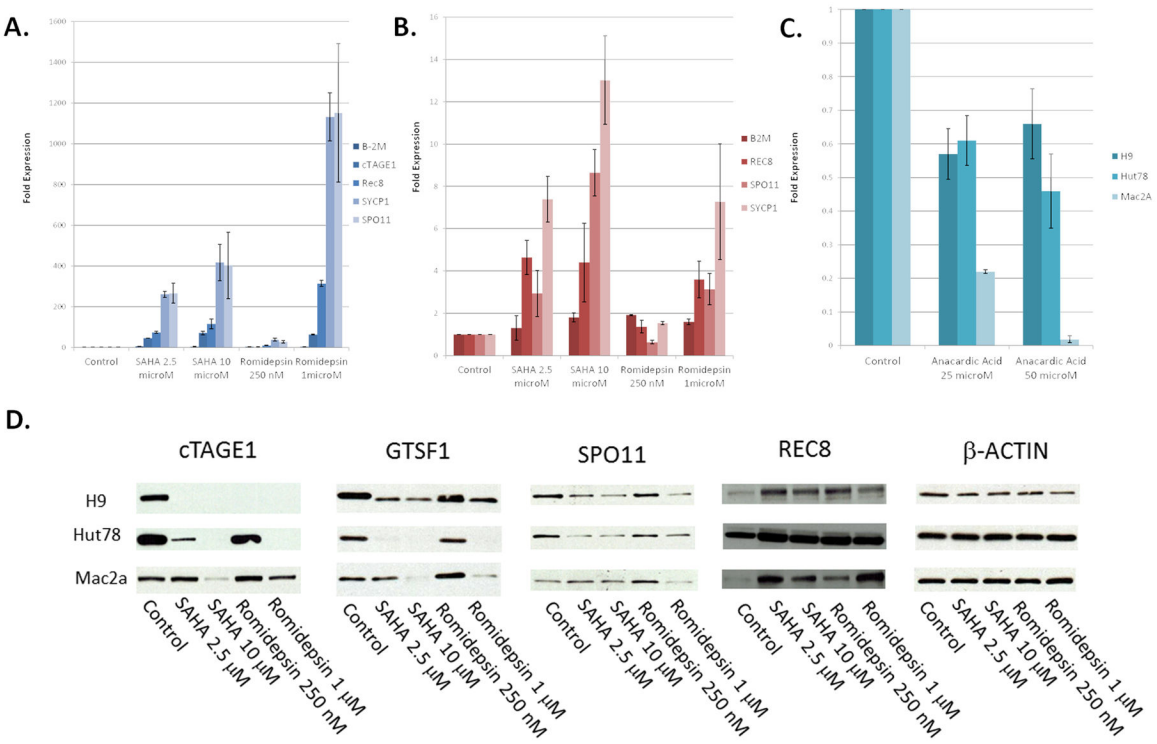

Figure 3.

Effect of HDAC inhibitors on mRNA expression of CT antigens in H9 (A) and Mac2A (B) cells. C. Effect of anacardic acid on mRNA expression of SPO11 in CTCL patient-derived cell lines. D. Effect of HDAC inhibitors on CT protein product expression in CTCL cell lines.

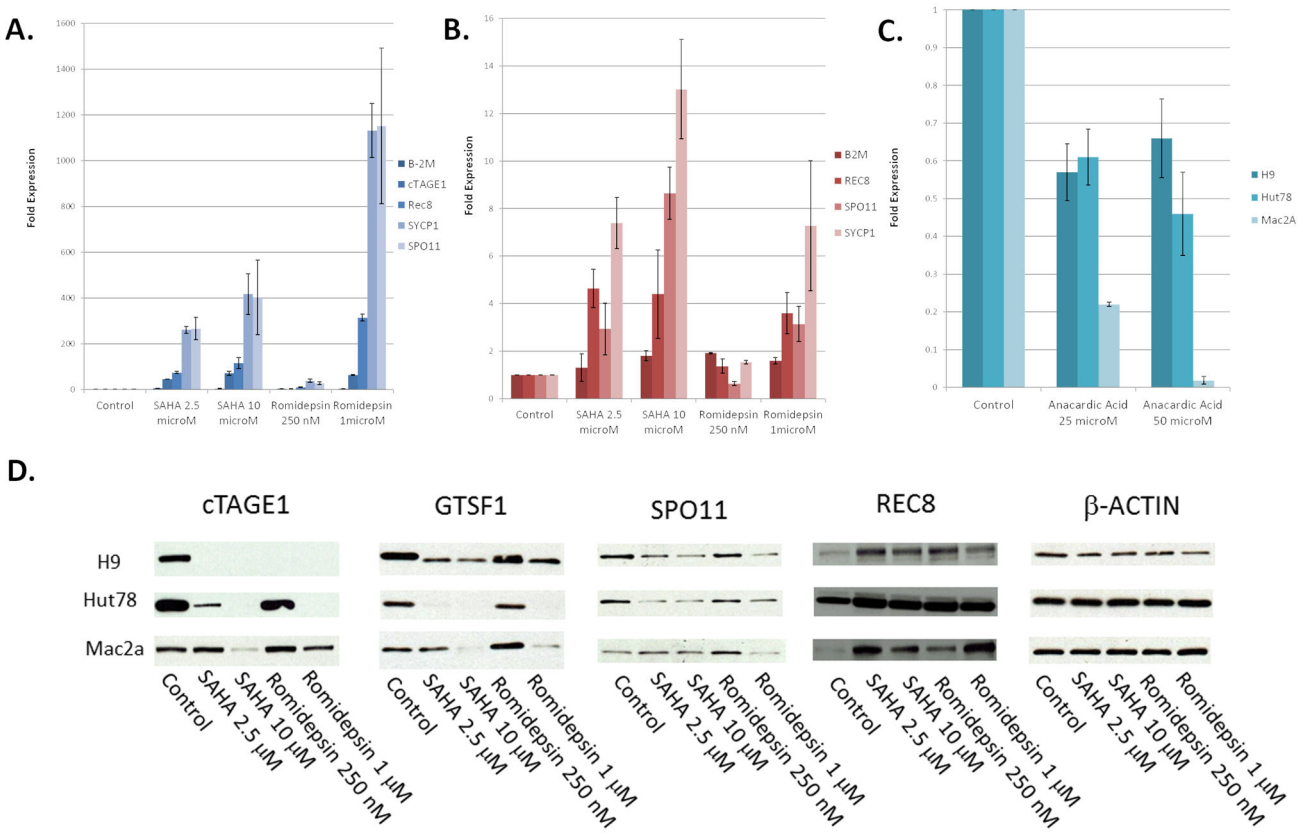

Figure 4.

cTAGE1, GTSF1 and SYCP1 CT genes are preferentially expressed in CTCL lesional skin when compared to normal skin from healthy donors or lesional skin from patients with benign inflammatory dermatoses.

Table 1

TP53 mutation status in patient-derived CTCL cell lines. Wild type is denoted by a "+" sign.

Cell line	TP53 Status			
	cDNA		Phenotype	
	Allele 1	Allele 2	Allele 1	Allele 2
HH	c.560-1G>A Intron 5	c.560-1G>A Intron 5	Splicing affected	Splicing affected
H9	c.75–80 C>G Intron 2	c.75–80 C>G Intron 2	Arg196X Nonsense mutation	p.Arg196X Nonsense mutation
	c.586 C>T Exon 6	c.586 C>T Exon 6		
Hut78	c.75–80 C>G	c.75–80 C>G	p.Arg196X Nonsense mutation	p.Arg196X Nonsense mutation
	c.586 C>T Exon 6	c.586 C>T Exon 6		
MyLa	c.75–80 C>G Intron 2	+	+	+
PB2B	c.75–80 C>G Intron 2	+	+	+
Mac2A	c.75–80 C>G Intron 2	+	+	+
SZ4	c.75–80 C>G Intron 2	c.75–80 C>G Intron 2	+	+
SeAx	c.733G>A Exon 7	c.733G>A Exon 7	p.Gly245Ser Deleterious mutation	p.Gly245Ser Deleterious mutation
MJ	c.75–80 C>G Intron 2	+	+	+
Sez4	c.75–80 C>G Intron 2	c.75–80 C>G Intron 2	+	+
Hut102	c.75–80 C>G Intron 2	+	+	+

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