Ectopic Expression of Cancer–Testis Antigens in Cutaneous T-cell Lymphoma Patients

Ivan V. Litvinov, Brendan Cordeiro, Yuanshen Huang, Hanieh Zargham, Kevin Pehr, Marc-André Doré, Martin Gilbert, Youwen Zhou, Thomas S. Kupper, and Denis Sasseville

Abstract

Purpose: The pathogenesis of cutaneous T-cell lymphoma (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 patients with CTCL, 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 patients with CTCL and patient-derived cell lines, whereas cTAGE1 (cutaneous T-cell lymphoma-associated antigen 1) 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 patients with CTCL and can be used as biomarkers or novel targets for immunotherapy.

Introduction

Cutaneous T-cell lymphoma (CTCL) is a rare cancer with the documented incidence rate of 4 to 8 cases per million (1–4). A number of studies documented about 3-fold increase in the incidence of CTCL in the past 25 to 30 years (2, 4). CTCL represents a heterogeneous group of non-Hodgkin lymphomas with mycosis fungoides and its leukemic variant Sézary syndrome being the most common

variants (5). In Caucasians, mycosis fungoides/Sézary syndrome primarily affects individuals older than 55 years, whereas in African Americans, Hispanics, and Arabic individuals, this disease presents at a significantly younger age (i.e., 20s and 30s; refs. 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, mycosis fungoides presents as flat erythematous skin patches resembling benign inflammatory diseases, whereas in the later stages, mycosis fungoides cells gradually form plaques or tumors and may disseminate to the lymph nodes and internal organs (3). 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 CICL, known as Sézary syndrome, is characterized by a triad of erythroderma, lymphadenopathy, and detection of malignant T cells with convoluted/cerebriform nuclei on a peripheral blood smear (3).
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 used as target antigens in various vaccine-based clinical trials. The expression of CT genes in cutaneous T-cell lymphoma (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.

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 patients with cancer (7). One group of antigens, cancer–testis (CT) antigens, are named after their typical pattern of expression as 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 used as target antigens in various vaccine-based clinical trials (10, 11). Although 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 patients with CTCL 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 biologic 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 patients with CTCL 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 histone deacetylase (HDAC) and histone acetyltransferase (HAT) inhibitors to modulate or induce the expression of these genes.

Patients, Materials, and Methods

Patients and samples

All patients were enrolled in an Institutional Review Board (IRB)-approved study protocol with informed consent. Patients with CTCL were recruited from the Cutaneous Lymphoma Clinic at the Dana Farber Cancer Institute (DFCI)/Brigham and Women’s Hospital (BWH; Boston, MA). All tissue samples were obtained and processed as previously described (20). Briefly, punch biopsies from involved skin were collected from 60 patients with CTCL 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), and total RNA was extracted using TRizol (Invitrogen) and converted to cDNA using the iScript RT-PCR Kit (Bio-Rad) 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 (PBMC) from patients with Sézary (\( n = 13 \)) 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 ATCC. H9 is a clonal derivative of Hut78 cell line (28). MyLa, PB2B, Mac2A, SZ4, SeAx, and Sez4 were a generous gift from professors K. Kaltoft and N. Ødum (Copenhagen, Denmark) and were previously described elsewhere (29–33). MJ and Hut78 cells were serially passaged in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) containing 10% 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 and 5 ng/mL of recombinant human IL2 and IL4 (R&D Systems). All cells were grown in 5% CO2, 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 μmol/L of ionomycin (Tocris Bioscience) or with CD3/CD28 Dynabeads (Invitrogen) according to manufacturer’s instructions. To inhibit HDAC activity, cells were treated with 2.5 to 10 μmol/L of suberoylanilide hydroxamic acid (SAHA also known as vorinostat; Santa Cruz) or 250 nmol/L to 1 μmol/L romidepsin (Adooq Bioscience). To block HAT activity, cells were treated with...
25 to 50 μmol/L of anacardic acid (Sigma-Aldrich). mRNA from cell lines was isolated using Qiagen 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 RT-PCR gene expression analysis

On the basis of 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 dermatomases, 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 S1. RT-PCR was performed using 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) using ACTB, SDHA, and GAPDH housekeeping genes. The obtained data were analyzed using XLSTAT 2009 software.

Western blotting

Western blotting was performed as described previously (34). cTAGE1 (cutaneous T-cell lymphoma-associated antigen 1) and Rec8 rabbit polyclonal antibodies were purchased from Proteintech. GTSF1 rabbit polyclonal antibody was purchased from Abnova. SYCP1 rabbit polyclonal antibody was purchased from GenTex. SPO11 rabbit polyclonal antibody was purchased from Abcam. STAT antibodies were purchased from Cell Signaling as part of Stat Antibody Sampler kit (Catalog #9939). Also, STAT3 (79D7) rabbit mAb (Catalog number #4904) was used in our Western blot experiments. Chemiluminescent detection reagents (ECL) were purchased from Amersham Biosciences.

Results

Expression of CT genes in CTCL lesional skin

A number of critical seminal studies using serologic detection of CTCL antigens suggested that a number of CT genes might be heterogeneous expressed in patients with CTCL (16–18, 39). Furthermore, additional testing by RT-PCR and Northern blotting 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 patients with CTCL. 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 patients with CTCL (Fig. 1A and Supplementary Fig. S1). Of these genes, cTAGE1 demonstrated the most robust and uniform expression in all patients with CTCL, whereas SYCP3 was strongly expressed only in less than 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 (Fig. 1B and Supplementary Fig. S2) and Western blotting (Fig. 1C), a number of the CT genes, including SYCP1, cTAGE1, and GTSF1 demonstrated robust expression in the majority of CTCL cell lines. cTAGE1 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 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 Fig. 2, in commonly used HH and MyLa cells by RT-PCR and Western blotting, 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 Fig. S3). 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. HHt cell had a silent c75-80C→G polymorphism in exon 7, which was 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 blotting (Fig. 1B and C). A number of cells with wild-type p53 such as PB2B and Mac2A strongly expressed SYCP1 but only weakly expressed Rec8. Furthermore, 2 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, 2 of the commonly used medications for advanced stages of this cancer are HDAC inhibitors (romidepsin...}

Figure 1. Analysis of ectopic expression of CT genes in patients with CTCL by RT-PCR (A) and in CTCL patient-derived cell lines by RT-PCR (B) and Western blotting (C).
and vorinostat; ref. 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 SAHA (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, and Rec8 CT genes in a dose-dependent manner, but not the B2M "housekeeping gene", in HH and Hut78 cells, whereas Mac2A cells demonstrated only a modest upregulation in expression of these CT genes (Fig. 3A and B and Supplementary Fig. S4).

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 patients with CTCL. As a proof of principle, we tested the effect of these drugs on a meiosis regulator SPO11 gene that was detected in only 3 of 60 patients with CTCL (Supplementary Fig. S1). 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 (Fig. 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 μmol/L of anacardic acid and tested the expression of SYCP1 and SPO11 in these cells. As demonstrated in Fig. 3C, anacardic acid significantly blocked the ectopic expression of SPO11 in all tested cell lines (Fig. 3C) and also blocked the expression of SYCP1 in Mac2A cells (Supplementary Fig. S5).

Many meiotic genes are subject to extensive posttranscriptional and posttranslation 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 (Fig. 3D). The posttranscriptional/posttranslational mechanisms for the observed downregulation remain to be elucidated. These combined results highlight the importance of epigenetic,

![Figure 2](image-url)
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

Because 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 (Fig. 4). As a positive control, other signaling genes (JunB and PLK1) were heterogeneously expressed at similar levels between malignant and nonmalignant biopsy samples (Supplementary Fig. S6). A similar expression analysis on isolated PBMCs from patients with Sézary versus from patients with benign inflammatory dermatoses was performed and demonstrated that cTAGE1 and GTSF1 were preferentially expressed in CTCL (Supplementary Fig. S7).

Discussion

In the current work, we demonstrated that a number of CT antigens are heterogeneously expressed in patients with CTCL, whereas cTAGE1, SYCP1, and GTSF1 are preferentially expressed in CTCL lesional skin when compared with benign inflammatory dermatoses or normal skin. GTSF1 is not expressed in HH, MJ, and Hut102 patient-derived CTCL cell lines, whereas cTAGE1 and SYCP1 are expressed in all cell lines tested by RT-PCR. Consistent with the observed RT-PCR expression, we document via Western blotting 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, whereas 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

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<th>Allele 2</th>
<th>TP53 status</th>
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NOTE: Wild-type is denoted by a "+" sign.
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 pathologic 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; refs. 49–51), promote resistance to various forms of chemo- and radiotherapy (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. To elucidate this further, we tested the p53 mutation status in the patient-derived CTCL cell lines. On the basis of 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 blotting, 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 on the basis of serology and limited PCR in 20 patients that cTAGE1 is expressed only in 30% of patients with CTCL (17, 39). However, in our study, we found this gene to be robustly expressed in a majority of patients with CTCL 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 patients with CTCL 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 the development of novel therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I.V. Litvinov, B. Cordeiro, Y. Huang, Y. Zhou, D. Sasseville
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I.V. Litvinov, B. Cordeiro, H. Zargham, Y. Zhou, D. Sasseville

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Acknowledgments
The authors thank Mr. Gregory Cormack for his technical assistance in performing molecular experiments.

Grant Support
This work was supported by the Canadian Dermatology Foundation research grants to I.V. Litvinov, D. Sasseville, and Y. Zhou; the Fonds de la recherche en santé du Québec (FRSQ) research grant to D. Sasseville; Canadian Institutes of Health Research to Y. Zhou; and the NIH SPORE program (P50 CA093683) to T.S. Kupper.

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Received February 4, 2014; revised April 29, 2014; accepted May 16, 2014; published OnlineFirst May 21, 2014.

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