BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

***HACE1*, a Potential Tumor Suppressor Gene on 6q21, Is Not Involved in Extranodal Natural Killer/T-Cell Lymphoma Pathophysiology**

Nouhoum Sako,^{*†} Valérie Dessirier,^{*†} Martine Bagot,^{*†‡} Armand Bensussan,^{*†} and Christian Schmitt^{*†}

From INSERM, UMR-S 976,* Paris; the University of Paris Diderot—Paris 7,[†] Sorbonne Paris Cité, Paris; and the Department of Dermatology,[‡] Hospital Saint-Louis, Paris, France

Accepted for publication
July 25, 2014.

Address correspondence to
Christian Schmitt, Ph.D.,
INSERM UMR-S 976, Hôpital
Saint-Louis, Pavillon Bazin,
F-75475, Paris, France. E-mail:
christian.schmitt@inserm.fr.

Extranodal natural killer—T-cell lymphoma (NKTCL) of nasal type is a malignant disorder of cytotoxic lymphocytes of natural killer or more rarely T cells, associated with clonal Epstein—Barr virus infection. NKTCL is an aggressive neoplasm with very poor prognosis. Although the pathogenesis of NKTCL is little understood, some insight has been gained in the recent years, especially from genome-wide studies, which revealed a deletion on chromosome 6q21 in more than 50% of patients. Of interest, this deleted region contains four candidate tumor suppressor genes whose decreased expression has been confirmed at the mRNA level: *PRDM1*, *ATG5*, *AIM1*, and *HACE1*. Mutations and methylation in *PRDM1*, *ATG5*, and *AIM1* have been reported in NKTCL cell lines. We investigated the involvement of *HACE1* in NKTCL pathophysiology. Even though the hypermethylation of CpG-177 island located directly upstream of *HACE1* locus led to down-regulation of *HACE1* mRNA, the protein product was expressed at nearly normal levels and was functional in the NKTCL cell lines regardless of 6q21 deletion (and indeed no double deletion of 6q21 and no nonfunctional mutations have been reported). Furthermore, contrary to previous report, overexpression of *HACE1* by transduction of recombinant protein did not affect proliferation or survival of NKTCL cell lines. We therefore conclude that *HACE1* is not directly involved in NKTCL pathophysiology. (*Am J Pathol* 2014, 184: 2899–2907; <http://dx.doi.org/10.1016/j.ajpath.2014.07.011>)

The term extranodal natural killer/T-cell lymphoma (NKTCL) refers to a group of clonal proliferations of cytotoxic lymphocytes of natural killer (NK) or, more rarely, T-cell types, with similar clinicopathologic features, arising as destructive tumors in the upper airway or other extranodal sites.^{1,2} One important feature of NKTCL is its close association with Epstein—Barr virus (EBV), and its incidence parallels the geographic distribution of EBV infection: prevalence is greatest in Asia and in Central and South America, where it accounts for up to 10% of non-Hodgkin lymphomas. Importantly, tumor lymphocytes are clonally infected by EBV, which suggests a possible oncogenic role of the virus.³ The characteristic expression of cytotoxic molecules such as granzyme B, perforin, and T-cell-restricted intracellular antigen-1 (TIA-1) reflects proliferation of activated cytotoxic cells in this lymphoma.⁴

In nasal areas, its main location, NKTCL results in destructive damage by affecting the nasal septum and hard

palate in the typical form or through the development of an apparently isolated nasal tumor. Ischemic necrosis is often present, associated with angioinvasion and vascular obstruction. It has been proposed that angiocentricity is linked to the release of chemokines CXCL9 (alias MIG) and CXCL10 (alias IP-10).⁵ Despite progress in therapeutics, this lymphoma is an aggressive disease with poor prognosis. In this context, the search for new therapeutic targets is essential.^{6–9}

Genome-wide studies have led to the identification of several chromosomal abnormalities in NKTCL; the most common of these is loss of heterozygosity in 6q21~q25, observed in approximately half of patients.^{10–13} This chromosomal region contains several potential tumor suppressor genes: *POPDC3*,

Supported by grants from INSERM (C.S.), University of Paris Diderot—Paris 7 (A.B.), and French Society of Dermatology grant SDF2010 (Société Française de Dermatologie; C.S.).

Disclosures: None declared.

PREP, *PRDM1*, *ATG5*, *AIM1*, *LACE1*, *FOXO3*, and *HACE1*. In particular, *HACE1* was identified by characterization of the 6q21 breakpoint in a translocation frequently involved in sporadic Wilms' tumor.^{14,15} A down-regulation of this gene has since been observed in other tumors.^{15–18} *HACE1* encodes a HECT domain and ankyrin repeat containing E3 ubiquitin ligase that tags specific target proteins for degradation by the proteasome or to control their subcellular localization.¹⁴

HACE1 is expressed in multiple tissues, including brain, lung, kidney, heart, liver, skeletal muscle, and spleen.¹⁹ The antitumor mechanism of *HACE1* is not yet established, but overexpression of *HACE1* protein inhibits proliferation of the 293T human epithelial kidney cell line through degradation of phosphorylated cyclin D1, suggesting a possible role of *HACE1* in the cell cycle.¹⁵

Recently, it was found that *HACE1* regulates Golgi membrane association during cell division²⁰ and promotes RAC1 degradation by its ubiquitination.²¹ Also, *HACE1* overexpression leads to cell-cycle arrest and apoptosis in NK cell lines.²² It has therefore been proposed that *HACE1* may be an important tumor suppressor gene in NKTCL and other NK cell neoplasms. Here, however, we show that, despite a significant expression at the transcriptional level due to loss of heterozygosity and CpG island methylation, *HACE1* expression is at nearly normal levels and the protein is functional. Moreover, we observed that increased expression of *HACE1* protein in the cytoplasm of NKTCL cell lines neither altered the cell cycle nor induced apoptosis. Our previous study of NKTCL using gene-expression profiling and array comparative genomic hybridization pointed to a 6q21 deletion and a possible involvement of *HACE1*, but lacked a functional analysis. Here, we show that *HACE1* can be ruled out as a gene critical to NKTCL pathophysiology.¹⁰

Materials and Methods

Cell Culture

Six NKTCL cell lines were used: SNK-6, SNT-8, SNT-15, and SNT-16 (obtained from Dr. Norio Shimizu, Tokyo Medical and Dental University),^{23,24} MEC04 (obtained from Prof. Paul Coppo, University of Paris—6, Inserm U832, Paris, France),²⁵ and the natural killer leukemia cell line YT (DSMZ cell collection, ACC#: ACC-434; Leibniz Institute DSMZ, Braunschweig, Germany). Cell lines used for control included including the *HACE1* down-regulation model SK-NEP1 (Wilms' tumor; ATCC, Manassas, VA), normal *HACE1*-expressing NK3.3 cells (long-term cultured non-transformed NK cells; Saint Louis University, St. Louis, MO), and HeLa cells (ATCC). PBL-IL2 cells [ie, peripheral blood lymphocytes (PBLs) from a healthy donor, activated by phytohemagglutinin and then cultured with IL-2] contained a mixture of T and NK cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine; the medium was changed twice weekly.

Exogenous IL-2 was added to maintain proliferation and survival of all NKTCL cell lines except YT.

RT-qPCR

For quantitative real-time RT-PCR (RT-qPCR), total RNA was extracted using an RNeasy micro kit (Qiagen, Hilden, Germany; Valencia, CA) according to the manufacturer's instructions. The total RNA concentration and quantity were assessed by absorbance at 260 nm using a NanoDrop spectrophotometer (2000c; Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using 2 µg of total RNA treated with GoScript reverse transcriptase (Promega, Charbonnière-les-bains, France; Madison, WI) and anchored oligo-dT primer (Sigma-Aldrich, Saint-Quentin-Pallavier, France; St. Louis, MO) to synthesize cDNA according to the manufacturer's recommendations. The cDNA was used to perform a real-time quantitative PCR (qPCR) for evaluation of the relative expression levels of *HACE1*, with GAPDH as internal control. The primers, synthesized by Europrim-Invitrogen (Cambridge, UK; Life Technologies, Carlsbad, CA), were as follows: 5'-AA-TGAAGACCTCCGAGAAAACA-3' (sense) and 5'-TGGCT-TTCACTTTGCTGAGA-3' (antisense) for *HACE1*; 5'-GCCC-AATACGACCAAATCC-3' (sense) and 5'-AGCCACATC-GCTCAGACAC-3' (antisense) for GAPDH. SYBR Green master mix (Roche, Mannheim, Germany) was used according to the manufacturer's recommendations. A LightCycler 480 real-time PCR system (Roche) was used for relative quantification of genes in 35 cycles. Each sample was tested in duplicate, and each experiment was repeated at least twice using cDNA samples from separate reverse transcription reactions. The data were analyzed using LightCycler 480 software version 1.5.0 (Roche) for relative quantification of genes, as described previously.²⁶

Methylation Analysis

We performed methylation analysis of genomic DNA in patients and NKTCL cell lines. Genomic DNA was extracted from samples using a DNeasy blood and tissue DNA extraction kit (Qiagen). Bisulfite conversion was performed using an Applied Biosystems MethylSEQR bisulfite conversion kit (Life Technologies, Villebon-sur-Yvette, France; Carlsbad, CA) according to the manufacturer's recommendations. Finally, the CpG177 island sequence was PCR-amplified from all samples using GoTaq DNA polymerase (Promega). Primers used to perform endpoint PCR were a methyl-sensitive primer pair CpG177 5'-GAAAAGAAGATTTGGGTTTAGATATG-3' (sense) and 5'-TAAACCTTCCATTCAATTCCAAAA-3' (antisense) and a non-methyl-sensitive control primer pair CpG177C 5'-GAATGGAAGGTTTAATTTTCGC-3' (sense) and 5'-CTAAAACCCTACGTCAACCG-3' (antisense). Two pairs of *PDGFRA* promoter primers were also used, to control bisulfite conversion efficiency: 5'-AGCCTAAAAACCCG-GTTTTC-3' (sense) and 5'-TCCCTTGAAGTTACTTATC-TG-3' (antisense) and 5'-TCAGAGAGCGATGAAGGTGA-3'

(sense) and 5'-TGTTTTTCGGGGTTATTTGG-3' (antisense). For demethylation assay, we cultured the various cell lines with 5-aza-2'-deoxycytidine (decitabine) (Sigma-Aldrich) for 3 days, as described previously.²⁷ Control cells were treated with an equivalent amount of dimethyl sulfoxide. After 72 hours of culture, transcriptional and proteomic studies of cells from the different conditions described above were performed.

Western Blot Analysis

The cells were washed with phosphate-buffered saline, and lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with proteases inhibitor cocktail (Sigma-Aldrich), according to the manufacturer's recommendations. The resulting lysate was centrifuged at $20,000 \times g$ for 10 minutes to obtain a clear lysate. Total protein was estimated using the Bradford method. Equal amounts of protein were loaded onto 10% SDS-PAGE gels and then transferred to Immunoblot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Marne-la-coquette, France; Hercules, CA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline and 0.1% Tween 20. After probing with primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated monoclonal anti-rabbit and anti-mouse antibodies (Bio-Rad Laboratories) and revealed by enhanced chemiluminescence (GE Healthcare, Velizy Villacoublay, France; Chalfont St. Giles, UK). The primary antibodies used were anti-HACE1 rabbit polyclonal antibody ABIN631377 (Antibodies-online, Aachen, Germany), anti-Rac1 mouse monoclonal antibody (Millipore Merck, Germany; Billerica, MA), and anti-ubiquitin monoclonal antibody (P4D1; Enzo Life Sciences, Villeurbanne, France; Farmingdale, NY). Anti- β -actin (Sigma-Aldrich) was used as an internal control, to confirm equal protein loading. Images of blots were analyzed and quantified with a luminescence image analyzer (ImageQuant LAS 4000; GE Healthcare). HACE1 protein was quantified from the Western blot results using ImageQuant software version 1.1 and was normalized to β -actin levels.

TAT-HA-HACE1 Expression

The pEB-TAT-2 vector was kindly provided from the laboratory of Steven F. Dowdy (Howard Hughes Medical Institute, Chevy Chase, MD). This vector contains a 6 $\times$ HIS sequence and allows the expression of recombinant proteins in a prokaryotic system under the control of a T7 promoter. HACE1 cDNA was amplified by PCR from normal activated PBLs and cloned into the vector EcoRI and KpnI sites. The TAT-HACE1 fusion protein was produced by BL21 bacteria, as described previously.²⁸ TAT-HACE1 was purified on a nickel-agarose column (Qiagen). For immunofluorescence studies, TAT-HACE1 was conjugated using an Alexa Fluor 488 labeling kit (Life Technologies) according to the manufacturer's recommendations. For transduction, cells were incubated for 4 hours with 25 μ g/mL of recombinant protein in a 96-well plate. After a

Figure 1 Transcriptional regulation of HACE1. **A:** Assessment of relative endogenous HACE1 mRNA expression by quantitative real-time RT-PCR (RT-qPCR) compared to PBL-IL2 (solid line). **B:** Methyl-sensitive PCR detects methylation status of CpG177 island for the HACE1 promoter. The CpG177 primer pair amplifies methylated DNA; CpG177C, a control primer pair, amplifies a non-cytosine-rich zone of the CpG177 island. **C:** Two primer pairs for the PDGFRA promoter were used to determine conversion control efficiency. **D:** Relative mRNA HACE1 expression assessed by RT-qPCR after genomic DNA demethylation by decitabine for 72 hours in four NKTCL cell lines and in the HACE1-down-regulated cell line SK-NEP1. GAPDH was used as a housekeeping gene. For each cell line, fold change was calculated relative to the dimethyl sulfoxide control (solid line). **E:** Western blotting detects HACE1 protein after demethylation with decitabine (+); dimethyl sulfoxide served as control (−). Data are expressed as means $\pm$ SEM of three independent experiments. ** $P < 0.01$; *** $P < 0.001$. AZA, 5-aza-2'-deoxycytidine (decitabine).

washing, cells were cultured for fluorescence microscopy and for 48-hour survival kinetics.

Microscopy

Transmission electron microscopy was performed as described previously.²⁹ Golgi stacks and clusters were identified using morphological criteria. Golgi stack images were captured at $\times 60,000$ magnification, to obtain a better view of the stacks. The longest cisterna was measured as the cisternal length of a Golgi stack, using the ruler and line tools in ImageJ software version 1.46r (NIH, Bethesda, MD), both of which gave essentially identical results. At least 20 cells were quantified in each experiment, and the results represent at least three independent experiments. Electron microscopy was performed at the microscopy platform of the Cochin Institute (Paris, France).

Figure 2 HACE1 protein expression in NKTL cells (MEC04, SNK-6, SNT-15, SNT-16, and YT), in cells with normal (NK3.3) and down-regulated (SK-NEP1) HACE1 expression, and in peripheral blood lymphocytes cultured with or without IL-2 (PBL-IL-2, PBL). **A:** Western blot analysis of HACE1 expression, using total cell lysates. **B:** Quantification of endogenous HACE1 protein levels. Data are expressed as means $\pm$ SEM of seven experiments. * $P < 0.05$, ** $P < 0.01$.

Fluorescence microscopy was performed with an Axiovert 200 M inverted microscope (Carl Zeiss Microscopy, Jena, Germany). MEC04 cells were transduced with Alexa Fluor 488-conjugated TAT-HACE1 (TAT-HACE1-A488) for 4 hours, and cultured overnight. The cells were spun down with a Shandon Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Runcorn, UK; Waltham, MA) and fixed with 4% paraformaldehyde and permeabilized with Triton X100. Cells were also incubated with mouse anti-GM130 antibody conjugated with BD Pharmingen Alexa Fluor 647 (BD Biosciences, Le pont de Claix, France; San Jose, CA), which recognizes the Golgi membrane protein GM130. Cell nuclei were stained with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame CA).

Apoptosis and Cell-Cycle Analysis

Cells were incubated for 4 hours with TAT-HACE1, washed twice with RPMI 1640 medium, and cultured overnight (for cell-cycle analysis) or for 48 hours (for apoptosis assay). After culture, cells were washed twice with phosphate-buffered saline. The same sequence of TAT recombinant protein (Sigma-Aldrich) was used as negative control for different experiments. Apoptosis was detected using 7-aminoactinomycin D staining (BD Pharmingen 7-AAD; BD Biosciences) staining. Etoposide (VP-16-213; Sigma-Aldrich) at 2 μ M final concentration was used as a positive control for apoptosis. Cell-cycle analysis was performed by incubation with 25 μ g/mL propidium iodide (Sigma-Aldrich) at 4°C in citrate buffer (3.5 mmol/L

Tris-sodium citrate, 9 mmol/L NaCl, 3.3% NP-40, 1 mg/mL RNase) and were analyzed on a cytometer (FC500; Beckman Coulter, Villepinte, France; Brea, CA).

Statistical Analysis

For quantitative analysis of cell proliferation and mortality, data are expressed as means $\pm$ SEM of at least three separate experiments. The statistical significance of differences between experimental variables was determined using

Figure 3 Functional test of endogenous HACE1 protein. Immunoprecipitation of total cell lysates was performed with anti-RAC1 antibodies. **A:** Western blot analysis of immunoprecipitated material with anti-HACE1, anti-ubiquitin, or anti-RAC1 antibodies. The membrane was incubated with Restore Plus (Thermo Fisher Scientific) Western blot stripping buffer for stripping before each redeveloping. **B:** Representative electron micrographs of the Golgi apparatus in MEC04 cells (NKTL) and SK-NEP1 cells (HACE1 down-regulated; Wilms' tumor). **C:** Quantification of the length of Golgi cisternae, using ImageJ software with electron microscopy images. Data are expressed as means $\pm$ SEM of three experiments. *** $P < 0.001$. Ub, ubiquitin. Scale bar = 200 nm. HELA, cervical cancer cells (HeLa).

the unpaired Student's *t*-test, and $P < 0.05$ was considered as significant.

Results

HACE1 mRNA Expression and Promoter Methylation Studies

Genome-wide studies with NKTCL patients have led to identification of recurrent chromosomal abnormalities, including 6q21 deletion, in 40% to 70% of patients. In the present study, *HACE1*, one of the potential tumor suppressor genes located in this region, had a diminished mRNA expression by RT-qPCR in NKTCL cell lines and in the Wilms' tumor cell line SK-NEP1, compared with IL-2-activated normal PBL (Figure 1A). This decreased expression of *HACE1* mRNA level was observed in cell lines in which monoallelic deletion in 6q21 was detected (SNK-6) or remained undetected (MEC04) in array comparative genomic hybridization analysis. Such decreased expression has also been observed in fresh cells from NKTCL patients.¹⁰

One of the proposed mechanisms for this reduced expression of *HACE1* transcripts is inactivation of gene transcription by promoter methylation (CpG island 177). We therefore examined the methylation status of this region in fresh tumor samples from six NKTCL patients and four cell lines by the bisulfite conversion method (Figure 1B). After conversion of the genomic DNA, four primer pairs were used to perform endpoint PCR. The primer pair CpG177, which surrounded the CG-rich island area, was used for methylation-specific amplification, whereas the CpG177C primer pair was used to hybridize with a cytosine-free sequence to verify the integrity of the converted genomic DNA used (Figure 1B). Two pairs of control primers for

PDGFRA promoter native DNA were also used, to verify efficacy of the bisulfite conversion (Figure 1C). With all DNA samples, a strong amplification could be obtained with the CpG177 primer pair, indicating that the CpG177 island is indeed hypermethylated in these *HACE1* promoters.

To evaluate the significance of the above results, we tested the action of the demethylation agent decitabine on cells in culture, looking for increased expression at the mRNA level (Figure 1D) and the protein level (Figure 1E). *HACE1* transcription was significantly increased after decitabine treatment in all of the cell lines tested, compared with sham treatment with dimethyl sulfoxide treatment, although not to the extent observed with the Wilms' tumor cell line SK-NEP1 (*HACE1* down-regulated).

At the protein level, except for SK-NEP1 (for which methylation is reported as a *HACE1*-controlling factor), in repeated attempts using NKTCL cell lines we observed only marginal increases of *HACE1* protein levels in the presence of decitabine; furthermore, base levels of *HACE1* expression in Western blots were already high, compared with SK-NEP1 cells (Figure 1E). This may suggest that control of *HACE1* transcription regulation may vary between lymphoid and nonlymphoid cells and that mRNA levels may not directly correlate with *HACE1* protein levels.

Analysis of Endogenous HACE1 Protein Expression

In view of the above results, we decided to investigate in greater depth the level of *HACE1* protein expression in NKTCL cell lines, in comparison with IL-2-activated PBL (PBL-IL-2) from healthy donors. Careful quantification of total proteins from lysed cells from repeated experiments was analyzed by immunoblotting with a specific anti-*HACE1* antibody (Figure 2). Again, although SK-NEP1 cells exhibited a clear diminution of *HACE1* expression, compared with

Figure 4 Characterization and functional testing of recombinant TAT-*HACE1* protein. TAT-*HACE1*, produced in *Escherichia coli*, was conjugated with Alexa Fluor 488 (A488). Cells were incubated for 4 hours with conjugated recombinant protein (TAT-*HACE1*-A488), washed twice with RPMI 1640 medium, and cultured overnight. **A:** Western blot analysis indicates *HACE1* expression in TAT-*HACE1*-expressing SK-NEP1 cells. **B:** Localization of recombinant protein TAT-*HACE1*-A488 in the Golgi apparatus of MEC04 cells by fluorescence microscopy. Anti-Golgi membrane antibody (anti-GM130) conjugated to Alexa Fluor 647 was used to label the Golgi apparatus in the cell. Nuclei were stained with DAPI. **C:** After 24 hours of culture, the lysate of TAT or TAT-*HACE1* transduced MEC04 cells was immunoprecipitated with anti-HA (IP HA) or anti-RAC1 antibodies (IP RAC1); the membrane was then probed with anti-*HACE1*, anti-RAC1, or anti-ubiquitin antibodies (IP HA) or with anti-HA, anti-*HACE1*, or anti-Rac1 antibodies (IP RAC1).

Figure 5 Kinetic analysis of cell survival after overexpression of TAT-HACE1-A488. The TAT-HACE1-A488 transduced cells were cultured for 48-hour kinetics and then were analyzed by flow cytometry. Apoptotic cell populations were identified using 7-AAD staining. Etoposide treatment was used as positive control for apoptosis. Control cells were transduced with the TAT peptide under the same conditions as TAT-HACE1-A488. **A:** Flow cytometry profile of 7-AAD-positive cells after TAT-HACE1 transduction in the MEC04 cell line. **B:** Kinetics of apoptotic cells (7-AAD positive) for SNK-6, SNT-15, or SK-NEP1 cell lines and PBL-IL-2 cells. Data are expressed as means $\pm$ SEM of three independent experiments. 7AAD, 7-aminoactinomycin D; VP16, etoposide (VP-16-213).

PBL-IL-2, none of the NKTCL cell lines exhibited a statistically significant HACE1 relative expression, compared with PBL-IL-2, in seven independent repeated experiments ($P > 0.05$). SNK-6,¹⁰ but not MEC04,²⁵ has a monoallelic deletion in 6q21, and both cell lines originate from patients with NK cell lymphomas, nasal type. These results suggest that, unlike the case with mRNA transcripts, HACE1 protein levels are not down-regulated in NKTCL cell lines (as opposed to the Wilms' tumor cell line SK-NEP1).

Endogenous HACE1 Protein Is Functional

Although expression of HACE1 protein was within the normal range in the NKTCL cell lines tested, and despite the absence of loss-of-function mutation reported in this disease, it remains possible that endogenous HACE1 was not biologically active. HACE1 ubiquitinates a number of substrates to induce their degradation via the proteasome. Among these, RAC1 (a member of the Rho superfamily of small GTPase signaling proteins) appears to be preferentially ubiquitinated by HACE1.²¹ HACE1 preferentially binds GTP-bound RAC1 (active form) to catalyze its polyubiquitination and degradation. We therefore tested for this activity in the NKTCL cell lines by Western blot analysis. Anti-RAC1 immunoprecipitated material from total cell lysates contained HACE1, in addition to RAC1 and polyubiquitinated RAC1 proteins (Figure 3A).

Although the levels of endogenous RAC1 did not differ among the cell lines, comparison of tumor cells (MEC04, SNK-6, or YT) versus nontransformed NK cells (NK3.3) yielded similar results in terms of RAC1-immunoprecipitated HACE1 protein. This confirmed that endogenous HACE1 in NKTCL cell lines can bind to and ubiquitinate RAC1.

To confirm this result, we studied the activity of HACE1 in the Golgi apparatus, where HACE1 has been described as an essential component in disassembly and reassembly during the mitotic process via interaction with Rab proteins.²⁰ Depletion of HACE1 or loss of its ubiquitin ligase activity reduces the length of cisternae in the stacks and affects the overall Golgi structure, as exemplified in SK-NEP1 and unlike NKTCL cell lines (Figure 3B). Quantification in NKTCL cell lines (SNT-16, MEC04, and SNK-6) and in controls (PBL-IL-2, NK-IL-2) indicated significant differences, compared with SK-NEP1 (Figure 3C). In conclusion, the data we obtained militate for normal expression and normal function of endogenous HACE1 in NKTCL.

TAT-HACE1 Is Functional in NKTCL Cell Lines

Ectopic gene expression has been used in attempts to compensate for low expression of tumor suppressor genes. Although the mechanisms of action of HACE1 tumor suppression are still elusive, it has been suggested that apoptosis

Figure 6 Cell-cycle analysis after overexpression of TAT–HACE1-A488. Cells were transduced for 4 hours with TAT–HACE1 recombinant protein and then cultured overnight. Cells were washed twice with phosphate-buffered saline and then incubated for 2 hours at 4°C in 300 μ L of a solution of propidium iodide in citrate buffer. Histograms include the different phases of the cell cycle (G₀/G₁, S, and G₂/M). FSC, forward scatter.

inhibition and decrease of cell cycling could be responsible for neoplastic transformation.^{15,21,22,30} We explored the effects of HACE1 up-regulation in NKTCL cell lines. Because of the observed dissociation between HACE1 mRNA and protein expression, we used a system of TAT-induced protein transduction to allow HACE1 entry into the cells, with rapid and efficient protein transfer in a majority of cells.

We were able to transduce the cells efficiently in 4 hours with 2.5 μ mol/L TAT–HACE1 (Figure 4A); these results were also confirmed by immunofluorescence using Alexa Fluor 488–conjugated TAT–HACE1 (data not shown). To assess the functionality of the transduced TAT–HACE1 protein, we first tested its ability to localize in the Golgi apparatus (Figure 4B). The fluorescent TAT–HACE1 protein colocalized to the Golgi region in the perinuclear area identified by the Golgi membrane protein GM130. Immunoprecipitation and Western blot analysis confirmed that the transduced TAT–HACE1 [identified by its hemagglutinin (HA) tag] can immunoprecipitate Rac1 and ubiquitinate Rac1, and that Rac1 can immunoprecipitate TAT–HACE1 (Figure 4C). These experiments allowed us to conclude that NKTCL cell lines can be efficiently transduced with functional HACE1 protein.

HACE1 Protein Overexpression Does Not Induce Apoptosis in NKTCL Cells

We then studied the effect of TAT–HACE1 overexpression on NKTCL apoptosis. We used flow cytometry analysis to look for labeling with 7-AAD in TAT–HACE1-A488 transduced cells. For controls, we used cells transduced with the TAT peptide alone or nontransduced cells treated with etoposide. After 4 hours of transduction, almost all MEC04 cells incorporated TAT–HACE1-A488 and maintained the recombinant protein during the 48 hours of the experiment (Figure 5A). Although more than 40% of apoptotic cells in the etoposide condition could already be detected at 18 hours, at no time point did the level of 7-AAD–positive cells exceed 3.5% in the TAT–HACE1-transduced cells or 6% in the TAT-transduced control. Similar results were obtained for other cell lines (Figure 5B), confirming the above

observations and leading to the conclusion that increased HACE1 protein expression in NKTCL cell lines does not significantly increase their susceptibility to apoptosis.

To complete these studies, we analyzed the cell cycle of the NKTCL cell lines transduced by TAT–HACE1 or TAT alone. The cell-cycle profile of TAT–HACE1 cells overlaps almost perfectly with that of the TAT-transduced control (Figure 6). We did not observe G₂/M cell-cycle arrest under HACE1 overexpression. None of the cell lines tested exhibited statistically significant differences between TAT–HACE1 and control at any stage of the cell cycle (Table 1).

Discussion

NKTCL is an extranodal EBV⁺ lymphoma affecting NK or (more rarely) T cells. In approximately half of cases, a del(6q21~q25) is observed, a localization site for candidate tumor-suppressor genes, including *PRDM1*, *ATG5*, *AIM1*, and *HACE1*.^{9–11,22,31} Down-regulation of *HACE1* mRNA expression, initially described in Wilms' tumor, has since been reported in other types of cancer.^{14,15,17,18} Besides monoallelic expression, *HACE1* promoter methylation (CpG177) has been thought to be involved in tumoral transformation.^{11,16,22} Consistent with previous findings,^{10,22} we observed reduced expression of HACE1 transcripts by qPCR, associated with methylation of the CpG177 island in various NKTCL cell lines and patient tumor samples.

Nonetheless, analysis at the protein level showed that, although HACE1 expression in SK-NEP1 cells was low and was significantly increased by treatment with a demethylating agent (decitabine), this was not the case for the NKTCL cell lines. The levels of endogenous HACE1 protein in NKTCL cells were similar to those observed in non-transformed cells, such as PBL–IL-2 (containing both activated T cells and NK cells) or NK3.3 (a nontumoral human NK cell line). Therefore, although HACE1 was down-regulated at the mRNA level, its protein expression was normal. To our knowledge, this is the first time that HACE1 expression has been evaluated at the protein level. Such

Table 1 Percentage of Cells at Different Stages of the Cell Cycle Did Not Differ after Transduction with TAT or TAT–HACE1 Proteins in NKTCL and Control Cell Lines

Cell line	Cells at G ₀ /G ₁ (%)		Cells at S (%)		Cells at G ₂ /M (%)	
	TAT	TAT–HACE1	TAT	TAT–HACE1	TAT	TAT–HACE1
NKTCL						
SNK-6	19.7	21.7	5.3	5.9	6.6	7.3
SNT-15	62.6	62.0	13.5	13.6	14.0	13.5
MEC04	38.8	37.0	19.1	18.0	14.8	14.2
Control*						
SK-NEP1	60.9	54.8	22.1	28.0	15.0	14.9
NK3.3	21.9	18.9	9.2	8.4	8.0	6.2

*HACE1 is down-regulated in SK-NEP1 cells and is expressed normally in NK3.3 cells.

discrepancies between mRNA and protein levels are not exceptional and have been reported for other genes.^{32–37} The discrepancy can be explained by post-transcriptional HACE1 stabilization to maintain protein activity sufficient for critical cell functions. In this regard, the fact that no complete loss-of-function mutation or deletion has ever been reported in NKTCL is an indication that HACE1 activity is certainly important for maintaining cell metabolism, even in tumoral cells.^{14,22} Indeed, when we investigated the biological activities of endogenous HACE1 protein, we found that the protein was fully active.

HACE1 binds the active (GTP) form of RAC1 and poly-ubiquitinates it to drive its degradation by the proteasome.^{21,30} Our Western blot analysis revealed that RAC1 immunoprecipitation material contained both HACE1 and ubiquitinated RAC1 in NKTCL cell lines. HACE1 is also important in the cell cycle, for assembly of the Golgi apparatus. Part of HACE1 is targeted to the Golgi membrane through interaction with Rab proteins. During mitosis, Golgi disassembly allows its apportioning in the daughter cells. During the postmitotic phase, monoubiquitination of Rab proteins allows membrane fusion and Golgi reassembly.^{20,38} In HACE1-deprived cells (eg, SK-NEP1 cells), the Golgi apparatus is fragmented, with reduced length of cisternae. Electronic microscopy analysis of Golgi fragmentation did not reveal such signs of HACE1 deprivation. Thus, not only is HACE1 protein expressed normally in NKTCL, but it also has normal activity.

The various targets identified for HACE1 activity point to a potential involvement in cell cycling and survival, two functions important in lymphomagenesis. Recently, it has been reported that HACE1 up-regulation can lead to apoptosis and G₂/M cell-cycle arrest.²² To avoid ectopic expression of HACE1 transgene, we used TAT–HACE1 recombinant protein to efficiently deliver functional protein into NKTCL cells. Fluorescent recombinant protein was used to monitor protein transduction and its ability to localize in the Golgi apparatus. Recombinant protein also associates with RAC1 and is capable of ubiquitinating it. Under these conditions, we were unable to detect apoptosis or cell-cycle modifications in TAT–HACE1–transduced NKTCL cell lines.

In conclusion, *HACE1*, which is a potential tumor suppressor gene involved in Wilms' tumor and located on 6q21, has been implicated in NKTCL lymphomagenesis because of the high frequency of del(6q21) and down-regulation of HACE1 transcripts through promoter methylation observed in NKTCL. We found that, despite low mRNA expression, HACE1 protein is normally expressed and is functional in NKTCL cell lines. In addition, overexpression of HACE1 protein did not (despite previous report) implicate apoptosis or cell-cycle arrest. However, HACE1 has been described as a tumor suppressor gene in other neoplasms, and we cannot rule out the possibility that it may mediate some effect through cooperation with other tumor suppressor genes in 6q21, such as *PRDM1*; however, any such mechanisms remain to be elucidated.

Acknowledgments

We thank Prof. Philippe Gaulard for helpful discussion and for access to DNA of patients, Alain Schmitt for performing the electronic microscopy at the Cochin Institute electron microscopy platform, and Prof. Steven F. Dowdy for providing the pTAT-2.1 plasmid.

References

1. Jaffe ES, Nicolae A, Pittaluga S: Peripheral T-cell and NK-cell lymphomas in the WHO classification: pearls and pitfalls. *Mod Pathol* 2013, 26(Suppl 1):S71–S87
2. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (Eds): *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues*. ed 4. Lyon, IARC Press, 2008
3. Cohen JJ, Kimura H, Nakamura S, Ko YH, Jaffe ES: Epstein-Barr virus-associated lymphoproliferative disease in non-immunocompromised hosts: a status report and summary of an international meeting, 8–9 September 2008. *Ann Oncol* 2009, 20:1472–1482
4. Kanavaros P, Boulland ML, Petit B, Arnulf B, Gaulard P: Expression of cytotoxic proteins in peripheral T-cell and natural killer-cell (NK) lymphomas: association with extranodal site, NK or Tgammadelta phenotype, anaplastic morphology and CD30 expression. *Leuk Lymphoma* 2000, 38:317–326
5. Teruya-Feldstein J, Jaffe ES, Burd PR, Kanegane H, Kingma DW, Wilson WH, Longo DL, Tosato G: The role of Mig, the monokine induced by interferon-gamma, and IP-10, the interferon-gamma-inducible protein-10, in tissue necrosis and vascular damage associated with Epstein-Barr virus-positive lymphoproliferative disease. *Blood* 1997, 90:4099–4105
6. Bajor-Dattilo EB, Pittaluga S, Jaffe ES: Pathobiology of T-cell and NK-cell lymphomas. *Best Pract Res Clin Haematol* 2013, 26:75–87
7. Huang Y, de Leval L, Gaulard P: Molecular underpinning of extranodal NK/T-cell lymphoma. *Best Pract Res Clin Haematol* 2013, 26:57–74
8. Rouget-Quermalet V, Giustiniani J, Marie-Cardine A, Beaud G, Besnard F, Loyaux D, Ferrara P, Leroy K, Shimizu N, Gaulard P, Bensussan A, Schmitt C: Protocadherin 15 (PCDH15): a new secreted isoform and a potential marker for NK/T cell lymphomas. *Oncogene* 2006, 25:2807–2811
9. Schmitt C, Sako N, Bagot M, Huang Y, Gaulard P, Bensussan A: Extranodal NK/T-cell lymphoma: toward the identification of clinical molecular targets. *J Biomed Biotechnol* 2011, 2011:790871
10. Huang Y, de Reyniès A, de Leval L, Ghazi B, Martin-Garcia N, Travert M, Bosq J, Brière J, Petit B, Thomas E, Coppo P, Marafioti T, Emile JF, Delfau-Larue MH, Schmitt C, Gaulard P: Gene expression profiling identifies emerging oncogenic pathways operating in extranodal NK/T-cell lymphoma, nasal type. *Blood* 2010, 115:1226–1237
11. Iqbal J, Kucuk C, Deleeuw RJ, Srivastava G, Tam W, Geng H, Klinkebiel D, Christman JK, Patel K, Cao K, Shen L, Dybkaer K, Tsui IF, Ali H, Shimizu N, Au WY, Lam WL, Chan WC: Genomic analyses reveal global functional alterations that promote tumor growth and novel tumor suppressor genes in natural killer-cell malignancies. *Leukemia* 2009, 23:1139–1151
12. Nakashima Y, Tagawa H, Suzuki R, Karnan S, Karube K, Ohshima K, Muta K, Nawata H, Morishima Y, Nakamura S, Seto M: Genome-wide array-based comparative genomic hybridization of natural killer cell lymphoma/leukemia: different genomic alteration patterns of aggressive NK-cell leukemia and extranodal NK/T-cell lymphoma, nasal type. *Genes Chromosomes Cancer* 2005, 44:247–255
13. Yoon J, Ko YH: Deletion mapping of the long arm of chromosome 6 in peripheral T and NK cell lymphomas. *Leuk Lymphoma* 2003, 44:2077–2082

14. Anglesio MS, Evdokimova V, Melnyk N, Zhang L, Fernandez CV, Grundy PE, Leach S, Marra MA, Brooks-Wilson AR, Penninger J, Sorensen PH: Differential expression of a novel ankyrin containing E3 ubiquitin-protein ligase, HACE1, in sporadic Wilms' tumor versus normal kidney. *Hum Mol Genet* 2004, 13:2061–2074
15. Zhang L, Anglesio MS, O'Sullivan M, Zhang F, Yang G, Sarao R, Mai PN, Cronin S, Hara H, Melnyk N, Li L, Wada T, Liu PP, Farrar J, Arceci RJ, Sorensen PH, Penninger JM: The E3 ligase HACE1 is a critical chromosome 6q21 tumor suppressor involved in multiple cancers. *Nat Med* 2007, 13:1060–1069
16. Hibi K, Sakata M, Sakuraba K, Shirahata A, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y: Aberrant methylation of the HACE1 gene is frequently detected in advanced colorectal cancer. *Anticancer Res* 2008, 28:1581–1584
17. Sakata M, Kitamura YH, Sakuraba K, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K: Methylation of HACE1 in gastric carcinoma. *Anticancer Res* 2009, 29:2231–2233
18. Sakata M, Yokomizo K, Kitamura Y, Sakuraba K, Shirahata A, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Hibi K: Methylation of the HACE1 gene is frequently detected in hepatocellular carcinoma. *Hepatogastroenterology* 2013, 60:781–783
19. Nagase T, Kikuno R, Ishikawa KI, Hirose M, Ohara O: Prediction of the coding sequences of unidentified human genes. XVI. The complete sequences of 150 new cDNA clones from brain which code for large proteins in vitro. *DNA Res* 2000, 7:65–73
20. Tang D, Xiang Y, De Renzis S, Rink J, Zheng G, Zerial M, Wang Y: The ubiquitin ligase HACE1 regulates Golgi membrane dynamics during the cell cycle. *Nat Commun* 2011, 2:501
21. Torrino S, Visvikis O, Doye A, Boyer L, Stefani C, Munro P, Bertoglio J, Gacon G, Mettouchi A, Lemichez E: The E3 ubiquitin-ligase HACE1 catalyzes the ubiquitylation of active Rac1. *Dev Cell* 2011, 21:959–965
22. Küçük C, Hu X, Iqbal J, Gaulard P, Klinkebiel D, Cornish A, Dave BJ, Chan WC: HACE1 is a tumor suppressor gene candidate in natural killer cell neoplasms. *Am J Pathol* 2013, 182:49–55
23. Nagata H, Numata T, Konno A, Mikata I, Kurasawa K, Hara S, Nishimura M, Yamamoto K, Shimizu N: Presence of natural killer-cell clones with variable proliferative capacity in chronic active Epstein-Barr virus infection. *Pathol Int* 2001, 51:778–785
24. Zhang Y, Nagata H, Ikeuchi T, Mukai H, Oyoshi MK, Demachi A, Morio T, Wakiguchi H, Kimura N, Shimizu N, Yamamoto K: Common cytological and cytogenetic features of Epstein-Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. *Br J Haematol* 2003, 121:805–814
25. Coppo P, Gouilleux-Gruart V, Huang Y, Bouhlal H, Bouamar H, Bouchet S, Perrot C, Vieillard V, Dartigues P, Gaulard P, Agbalika F, Douay L, Lassoued K, Gorin NC: STAT3 transcription factor is constitutively activated and is oncogenic in nasal-type NK/T-cell lymphoma. *Leukemia* 2009, 23:1667–1678
26. Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002, 30:e36
27. Vallot C, Stransky N, Bernard-Pierrot I, Herault A, Zucman-Rossi J, Chapeaublanc E, Vordos D, Laplanche A, Benhamou S, Lebret T, Southgate J, Allory Y, Radvanyi F: A novel epigenetic phenotype associated with the most aggressive pathway of bladder tumor progression. *J Natl Cancer Inst* 2011, 103:47–60
28. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF: Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat Med* 1998, 4:1449–1452
29. Franciszkiewicz K, Le Floch A, Boutet M, Vergnon I, Schmitt A, Mami-Chouaib F: CD103 or LFA-1 engagement at the immune synapse between cytotoxic T cells and tumor cells promotes maturation and regulates T-cell effector functions. *Cancer Res* 2013, 73:617–628
30. Castillo-Lliva S, Tan CT, Daugaard M, Sorensen PH, Malliri A: The tumour suppressor HACE1 controls cell migration by regulating Rac1 degradation. *Oncogene* 2013, 32:1735–1742
31. Karube K, Nakagawa M, Tsuzuki S, Takeuchi I, Honma K, Nakashima Y, Shimizu N, Ko YH, Morishima Y, Ohshima K, Nakamura S, Seto M: Identification of FOXO3 and PRDM1 as tumor-suppressor gene candidates in NK-cell neoplasms by genomic and functional analyses. *Blood* 2011, 118:3195–3204
32. Bamford RN, Battista AP, Waldmann TA: IL-15: the role of translational regulation in their expression. *J Leukoc Biol* 1996, 59:476–480
33. Clayton LK, D'Adamio L, Howard FD, Sieh M, Hussey RE, Koyasu S, Reinherz EL: CD3 eta and CD3 zeta are alternatively spliced products of a common genetic locus and are transcriptionally and/or post-transcriptionally regulated during T-cell development. *Proc Natl Acad Sci USA* 1991, 88:5202–5206
34. Kordek R, Potemski P, Kusinska R, Pluciennik E, Bednarek A: Basal keratin expression in breast cancer by quantification of mRNA and by immunohistochemistry. *J Exp Clin Cancer Res* 2010, 29:39
35. Niemir ZI, Stein H, Noronha IL, Kruger C, Andrassy K, Ritz E, Waldherr R: PDGF and TGF-beta contribute to the natural course of human IgA glomerulonephritis. *Kidney Int* 1995, 48:1530–1541
36. Ranganathan S, Maffei M, Kern PA: Adipose tissue ob mRNA expression in humans: discordance with plasma leptin and relationship with adipose TNFalpha expression. *J Lipid Res* 1998, 39:724–730
37. Zhang GJ, Xiao HX, Tian HP, Liu ZL, Xia SS, Zhou T: Upregulation of microRNA-155 promotes the migration and invasion of colorectal cancer cells through the regulation of claudin-1 expression. *Int J Mol Med* 2013, 31:1375–1380
38. Cabrera-Poch N, Pepperkok R, Shima DT: Inheritance of the mammalian Golgi apparatus during the cell cycle. *Biochim Biophys Acta* 1998, 1404:139–151