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Tumorigenesis and Neoplastic Progression

Epigenetic Silencing of a Proapoptotic Cell Adhesion Molecule, the Immunoglobulin Superfamily Member IGSF4, by Promoter CpG Methylation Protects Hodgkin Lymphoma Cells from Apoptosis

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The malignant Hodgkin/Reed-Sternberg (HRS) cells of Hodgkin lymphoma (HL) are believed to derive from germinal center (GC) B cells, but lack expression of a functional B cell receptor. As apoptosis is the normal fate of B-cell receptor–negative GC B cells, mechanisms that abrogate apoptosis are thus critical in HL development, such as epigenetic disruption of certain pro-apoptotic cancer genes including tumor suppressor genes. Identifying methylated genes elucidates oncogenic mechanisms and provides valuable biomarkers; therefore, we performed a chemical epigenetic screening for methylated genes in HL through pharmacological demethylation and expression profiling. *IGSF4/CADM1/TSLC1*, a pro-apoptotic cell adhesion molecule of the immunoglobulin superfamily, was identified together with other methylated targets. In contrast to its expression in normal GC B cells, *IGSF4* was down-regulated and methylated in HL cell lines, most primary HL, and microdissected HRS cells of 3/5 cases, but not in normal peripheral blood mononuclear cells and seldom in normal lymph nodes. We also detected *IGSF4* methylation in sera of 14/18 (78%) HL patients but seldom in normal sera. Ectopic *IGSF4* expression decreased HL cells survival and increased their sensitivity to apoptosis. *IGSF4* induction that normally follows heat shock stress treatment was also abrogated in methylated lymphoma cells. Thus, our data demonstrate that *IGSF4* silencing by CpG methylation provides an anti-apoptotic signal to HRS cells important in HL pathogenesis. ***(Am J Pathol 2010, 177:1480–1490; DOI: 10.2353/ajpath.2010.100052)***

Hodgkin lymphoma (HL) is a tumor derived from the germinal center (GC) or the post-GC stages of B cell differentiation, and is unusual among the B cell lymphomas in that the malignant Hodgkin/Reed-Sternberg (HRS) cells lack a functional B-cell receptor (BCR). Because apoptosis is the normal fate of BCR-negative GC B cells, mechanisms that abrogate apoptosis are likely to be critical in the development of HL.[1] Such mechanisms might be expected to include the disruption of cancer genes with pro-apoptotic activity like tumor suppressor genes (TSGs).[2]

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Epigenetic silencing of TSGs has been shown to be a frequent and critical cause involved in tumor pathogenesis,[3,4] including hematological malignancies though they are commonly driven by genetic mutations like chromosomal translocations.[5] To identify critical TSGs aberrantly methylated/silenced in HL cells, we used a chemical epigenetic approach[6–9] through pharmacological demethylation of HL cells using 5-aza-2′-deoxycytidine (Aza) followed by genome-wide microarray expression profiling. This analysis identified a group of candidate TSGs including *IGSF4* (also known as *TSLC1, CADM1, SynCAM1, necl-2, RA175, SgIGSF*). *IGSF4* encodes a transmembrane protein whose extracellular domain shows close homology to the immunoglobulin superfamily cell adhesion molecules (Ig-CAM), particularly with the neural cell adhesion molecules, N-CAM1 and N-CAM2,[10] and the prostate tumor-suppressor TSLL2/IGSF4C.[11] Silencing of *IGSF4*,[12] is frequently observed in solid tumors including lung,[13] prostate,[10] pancreatic, gastric, breast, nasopharyngeal,[14] and cervical cancers,[15,16] as well as nasal NK/T-cell lymphoma.[17] Restoration of *IGSF4* expression decreases epithelial cell scattering and tubulogenesis and suppresses lung cancer metastasis in nude mice,[13,18–20] IGSF4 also reduces the anchorage-independent growth and tumorigenicity of cervical cancer cells.[16] As cell–cell contact and cross-linking of surface immunoglobulins is important in signaling B cells to death,[21] changes of cell adhesion and motility mediated by loss of receptor expression like *IGSF4* would result in aberrant regulation of the cell fate of GC B cells and HRS cells.[10,20] Moreover, we and others have already reported that *IGSF4* is indeed a stress-responsive gene capable of inducing apoptosis.[17,22] Thus, we have selected *IGSF4* for further study of its abnormal loss in protecting Hodgkin lymphoma cells from apoptosis.

Materials and Methods

Cell Lines and Tumor Samples

Lymphoma cell lines studied included BL cell lines (BJAB, CA46, Rael, Namalwa, Raji, AG876); diffuse large B-cell lymphoma (DLBCL) cell lines (OCI-Ly1, Ly3, Ly7, Ly8, Ly18); T-cell lines (Ly13.2, Ly17); and HL cell lines: KM-H2, L428, L540, L591, HD-MY-Z, HD-LM2, L1236 (DSMZ cell collection, Braunschweig, Germany). Some nasopharyngeal and breast carcinoma cell lines and a normal immortalized but non-transformed nasopharyngeal epithelial cell line (NP69), a transformed human embryonic kidney cell line (HEK293), and a normal lymphoblastoid cell line (CCL-256.1) were used as controls. Cells were maintained in RPMI 1640 or Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum (Invitrogen, Paisley, Scotland) and 1% streptomycin/penicillin at 37°C in 5% CO_2.[8] For demethylation experiments, cell lines were treated with 5 μmol/L of demethylating agent Aza (Sigma, St. Louis, MO), which is a cytosine analog that demethylates DNA by inhibiting DNA methyltransferase, for 3 days.[8] The collection and processing of lymphoma biopsy samples used in the present study have been described previously.[2,8,23]

Normal peripheral blood mononuclear cells (PBMCs), sera from healthy individuals and HL patients, cells microdissected from normal germinal centers, and lymph node samples taken from individuals without any malignancy, and in which histological examination revealed either normal histology or reactive hyperplasia, were collected as previously described.[2] DNA and RNA were extracted from cell lines and primary tumors using TriReagent (Molecular Research Center, Cincinnati, Ohio) as previously described.[8,23]

Microarray Expression Profiling

KM-H2 cells were suspended at 1×10^5 cells/ml in cRPMI-1640 and allowed to grow overnight. Aza was added to the desired concentrations (5 μmol/L, dissolved in H_2O), with distilled H_2O instead of drug as the control. Cells were treated for 3 days, with fresh medium containing Aza replaced every 24 hours. After the treatment, cells were pelleted and washed with PBS, and DNA and RNA were extracted. Affymetrix Human Genome Focus Arrays (Affymetrix, Santa Clara, CA) were used for all experiments. Total RNA was used to prepare biotinylated RNA as indicated by manufacturer protocol (Affymetrix, Santa Clara, CA). The 3′/5′ ratios for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were within acceptable limits (GAPDH 0.74 to 0.87, β-actin 0.80 to 1.08), and BioB spike controls were present on 3/6 chips, with BioC, BioD, and CreX also present in increasing intensity. When scaled to a target intensity of 100 (Affymetrix MAS 5.0), scaling factors for all arrays were within acceptable limits, as were backgrounds, Q values and mean intensities. Images of GeneChips were analyzed by Affymetrix Microarray Suite 5.0. Probe level quantile normalization and robust multi-array analysis[24] on the raw.CEL files were performed using the Affymetrix package of the Bioconductor (*htttp://www.bioconductor.org*, last accessed July 29, 2010) project. Differentially expressed probe sets were identified using SAM[25,26]; only those with changes $\geq$1.8-fold and false discovery rate $\leq$1% were included. Hierarchical clustering was performed using dChip (*http://www.dchip.org*, last accessed July 29, 2010).

Semiquantitative Reverse Transcription-PCR

For reverse transcription (RT)-PCR, total RNA and AmpliTaq Gold (PE Biosystem, Foster City, CA) were used.[27] The PCR program used initial denaturation at 95°C for 10 minutes, followed by 35 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 30 s), with a final extension at 72°C for 10 minutes.[9] The primers for *IGSF4* were: A: 5′-TCCGGCTTCTGCTGTTGC-3′; B: 5′-TTTCCTGTGGGGGATCGG-3′. The signals were quantified by densitometry using the ImageJ software (*http://rsbweb.nih.gov/ij/index.html*) developed by Wayne Rasband (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland).

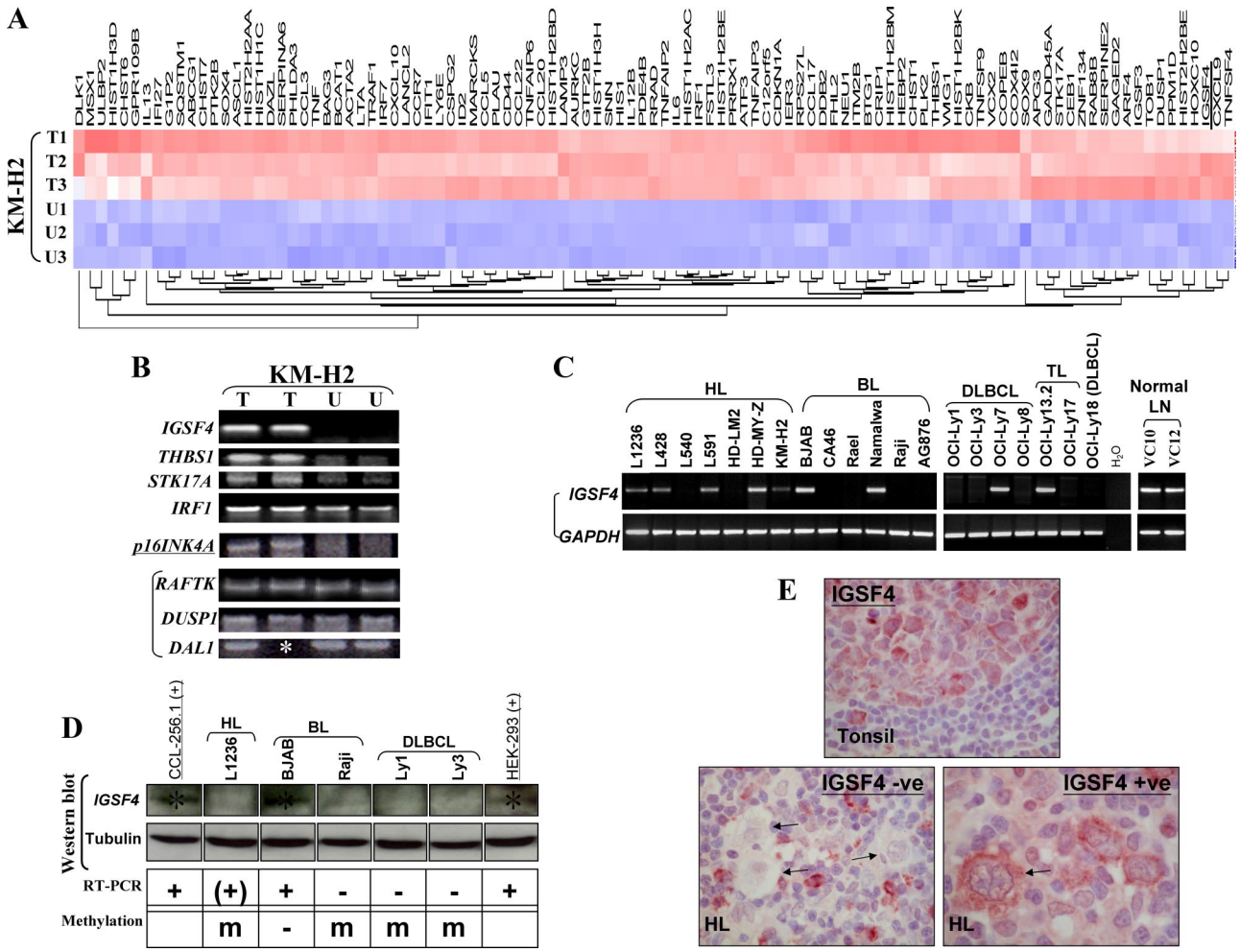

Figure 1. **A:** Heat map of genes up-regulated in KM-H2 cells following demethylating treatment with Aza. T1–3: treated samples; U1–3: controls. *IGSF4* is underlined. **B:** Validation of microarray results by RT-PCR of selected genes up-regulated in KM-H2 cells after Aza treatment (T) compared with untreated cells (U). Upregulation of mRNA for *IGSF4, THBS1, STK17A* and *IRF1*, and unchanged expression of *RAFTK, DUSP1,* and *DAL1* are shown. *p16INK4A* was not picked up by the microarray screening, however its expression was up-regulated after Aza treatment. **Asterisk** indicates that no RNA left in one sample. **C:** *IGSF4* down-regulation in HL and NHL cell lines by RT-PCR. RT-PCR analysis showed that when compared with two normal lymph nodes, *IGSF4* expression was completely lost or reduced in 6/7 HL, 4/5 DLBCL, and 4/6 BL cell lines. **D:** IGSF4 protein expression correlated with its mRNA expression, absent in methylated cell lines: one HL cell line (L1236), one BL (Raji), and two DLBCL cell lines (Ly1 and Ly3). HEK293 and CCL-256.1 were used as positive controls. **Asterisk** indicates the +v band for IGSF4 protein. **E:** Immunohistochemistry with an IGSF4-specific monoclonal antibody in normal lymphoid tissues and HL. **Upper panel** shows normal expression of IGSF4 protein in GC B cells. In contrast, HRS cells in most HL cases lacked expression or showed lower levels of expression. A representative example of the absence of IGSF4 expression is shown in the **lower left panel**. HRS cells are indicated with **arrows**. A minority of cases showed normal expression of IGSF4 in HRS cells (**lower right panel**). These results were confirmed using two separate chicken polyclonal antibodies directed to IGSF4 (data not shown). See Table 4 for correlation with MSP analysis.

Methylation-Specific PCR and Bisulfite Genomic Sequencing

Genomic DNA was treated with sodium metabisulfite (Sigma) as previously described,[27] but without prior EcoRI digestion. Methylation-specific PCR (MSP) was performed according to our previous method and can detect 1% methylated alleles in an unmethylated DNA background.[2] Primers used were as follows: for the methylated promoter (142-bp product), TSLC1m12 (5′-AGCGAGGTTTTTCGAGAGTC-3′) and TSLC1m11 (5′-CCTACCTCAAACTAACGACG); for the unmethylated promoter (144-bp product), TSLC1u12 (5′-GAGTGAGGTTTTTTGAGAGTT-3′) and TSLC1u11 (5′-ACCTACCTCAAACTAACAACA-3′). One ul of bisulfite-treated DNA (~50 ng measured before bisulfite treatment) was PCR amplified using 0.3125 U of AmpliTaq Gold (Perkin Elmer-Cetus, Norwalk, CT) with 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP and 0.6 umol/L each primer in a 12.5 μl reaction volume. MSP was conducted with hot-start at 95°C for 10 minutes, then 38 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 30 s), followed by 72°C for 5 minutes. MSP products were analyzed on a 1.8% agarose gel. GeneRuler 100-bp Plus DNA ladder was used as a marker to indicate the size of the MSP products on the gel. MSP primers were shown not to amplify DNA not treated with bisulfite thus specific and selected MSP products were sequenced.

Bisulfite genomic sequencing (BGS) was performed as described previously.[27] The strand-specific primers for the bisulfite-converted single stranded DNA of the *IGSF4* promoter were: BGS3, 5′-GGATTTTTTTAAGGGAGATTTTTT-3′, BGS2A, 5′-CRAACACCTACCTCAAACTAA-3′. These two primers amplify a 422-bp region with 35 CpG sites in the *IGSF4* promoter. For BGS, at least 6 to 8 bacterial colonies were analyzed for each DNA sample.

Table 1. Thirty Known and Candidate TSGs Up-Regulated Following Aza Demethylation of KM-H2 HL Cells

Gene name	Activity/tumor suppressor function
IGSF4/CADM1/TSLC1	Known TSG, inactivated by methylation in lung and other tumors
CDKN1A (p21)	Known TSG, sometimes inactivated by methylation in cancers, also induced by 5-aza-^{d}C treatment
CCL3	Expression decreases tumorigenesis in the colorectal tumor model CMT93[34]
CCL20	Expression decreases tumorigenesis in the colorectal tumor model CMT93[34]
CD44	Metastasis suppressor, inactivated by methylation in several tumors, including prostate and gastric cancer, neuroblastoma[30]
CRIP1	Methylated and growth suppressive in esophageal squamous cell carcinoma[7]
DLK1	Methylated and growth suppressive in neuroblastoma and renal cell carcinoma[35]
IRF1	Known TSG[32]; promoter methylation not described
IRF7	Known TSG, inactivated by methylation in astrocytoma and hepatocellular carcinoma[31]
IER3/IEX-1	Induces apoptosis in HeLa cells[36]
ZMAT3/WIG1	Growth inhibitory zinc finger protein[33]
BTG-1	Anti-proliferative function[37]
THBS1	Known TSG, methylated in gastric cancer, nasopharyngeal carcinoma and others[29]
FHL2/DRAL	Pro-apoptosis function[38]
DDB2	Feline homolog implicated as TSG in B cell lymphoma[39]
LY6E/RIG-E	Down-regulation associated with development of hepatocellular carcinoma[40]
MSX1	Interacts with p53, overexpression induces apoptosis of cancer cells harboring nonfunctional wild-type p53 and suppressed growth of human tumor xenografts in nude mice[41]
GADD45A	Induced by 5-azacytidine, growth arrest
HOXC10	Degradation associated with cell cycle progression[42]
STK17A/DRAK1	Pro-apoptosis function[43]
KLF6/COPEB/BCD1	Candidate TSG, mutated in some cancers (prostate and colon)[34]
RRAD	Methylated in malignant mesothelioma[44]
TOB1	Putative TSG, mice lacking *Tob1* are predisposed to cancer development[45], loss of expression associated with lung cancer development
SOX9	Overexpression suppresses growth and tumorigenicity of prostate cancer cells[46]
LAMP3/CD63	Decreased expression of CD63 elevates invasive potential of human melanoma cells
ITM2B	Candidate TSG in prostate cancer
MARCKS	Candidate TSG in melanoma
FSTL3	Potential TSG. Down-regulated in endometrial cancer
PRRX1	Potential TSG. Inhibits transforming properties of Maf[47]
PLK2	Cell cycle checkpoint, inhibits oncogenic transformation

Methylation Analysis of Microdissected HRS Cells and Serum DNA by Nested-MSP

Fifty HRS cells were microdissected from each case and DNA was extracted using the QIAamp MiniElute virus kit. The DNA was bisulfite-treated and amplified using primers TSLC1BGS7 (5′-ATGTTATTAGTATTTTATTAGTTGTT-3′) and TSLC1BGS8 (5′-CACACCTACCTATAAAAAT-CAATA-3′) (60 cycles), using 0.625 U of AmpliTaq Gold in a 12.5 μl reaction volume. Five μl of the 10× diluted PCR products were used for a nested-MSP with methylation-specific primers TSLC1m5 and TSLC1m8 (109-bp product), TSLC1m5: 5′-TAGTATTTTATTAGTTGTTCGT-TC-3′, TSLC1m8: 5′-GCACACTAAAATCCGCTCG-3′; or unmethylated-specific primers TSLC1u5 and TSLC1u8 (112-bp product), TSLC1u5: 5′-TTAGTATTTTATTAGTT-GTTTGTTT-3′, TSLC1u8: 5′-CCACACACTAAAATCCAC-TCA-3′. Nested-MSP was conducted at 95°C for 10 minutes, then 45 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 30 s), followed by 72°C for 5 minutes, using 0.625 U of AmpliTaq Gold in a 12.5 μl reaction volume.

A similar approach was used to detect IGSF4 methylation in serum samples from HL patients,[2] except that primers TSLC1m12 and m11 (40 cycles, 0.625 U of AmpliTaq Gold in a 12.5 μl reaction) were used for the first round of PCR. One μl of the 10× diluted PCR products was then used in a nested-MSP with the methylation-specific primers TSLC1m10 (5′-GTGATTGGTTTGTTCG-GATTTC-3′) and TSLC1 m11 (103-bp product, 40 cycles, 0.625 U of AmpliTaq Gold in a 12.5 μl reaction). For detection of unmethylated alleles in serum samples, primers TSLC1 BGS9R and BGS2A (40 cycles, 0.625 U of AmpliTaq Gold in a 12.5 μl reaction) were used for the first round of PCR and one μl of the 10× diluted PCR products was used in the nested-MSP with methylation-specific primers TSLC1u10 (5′-TGTGATTGGTTTGTTTG-GATTTT-3′) and TSLC1u11 (105-bp product, 40 cycles, 0.625 U of AmpliTaq Gold in a 12.5 μl reaction). Nested MSP products were confirmed by direct sequencing.

Phenotypic Effects of Ectopic Expression of IGSF4 in HL Cells

Transient expression of IGSF4 in cell lines was achieved using the nucleofection system (Amaxa GmbH). Briefly, 2×10^6 cells and 5 μg of each plasmid were suspended in 100 μl of prepared Nucleofector solution kit T and pulsed using program T-01. Transfection efficiency was monitored by parallel transfection of cell lines with a GFP expression vector followed by fluorescence-activated cell sorting analysis. Transfection efficiency was >70% for HL cell lines (data not shown). Viability assays (48 hours post-transfection) using Trypan blue and proliferation assays (72 hours post-transfection) using WST1 reagent (Roche Diagnostics, Lewes, UK) were performed on transiently transfected cells. The IGSF4 expression vector was kindly provided by Professor RD Steenbergen.[16] The RT-PCR primers used for detection of *IGSF4* transcripts were as described.[16] *GAPDH* primers were as follows: GAPDH forward 5′-CCACCCATGGCAATTCCAT-

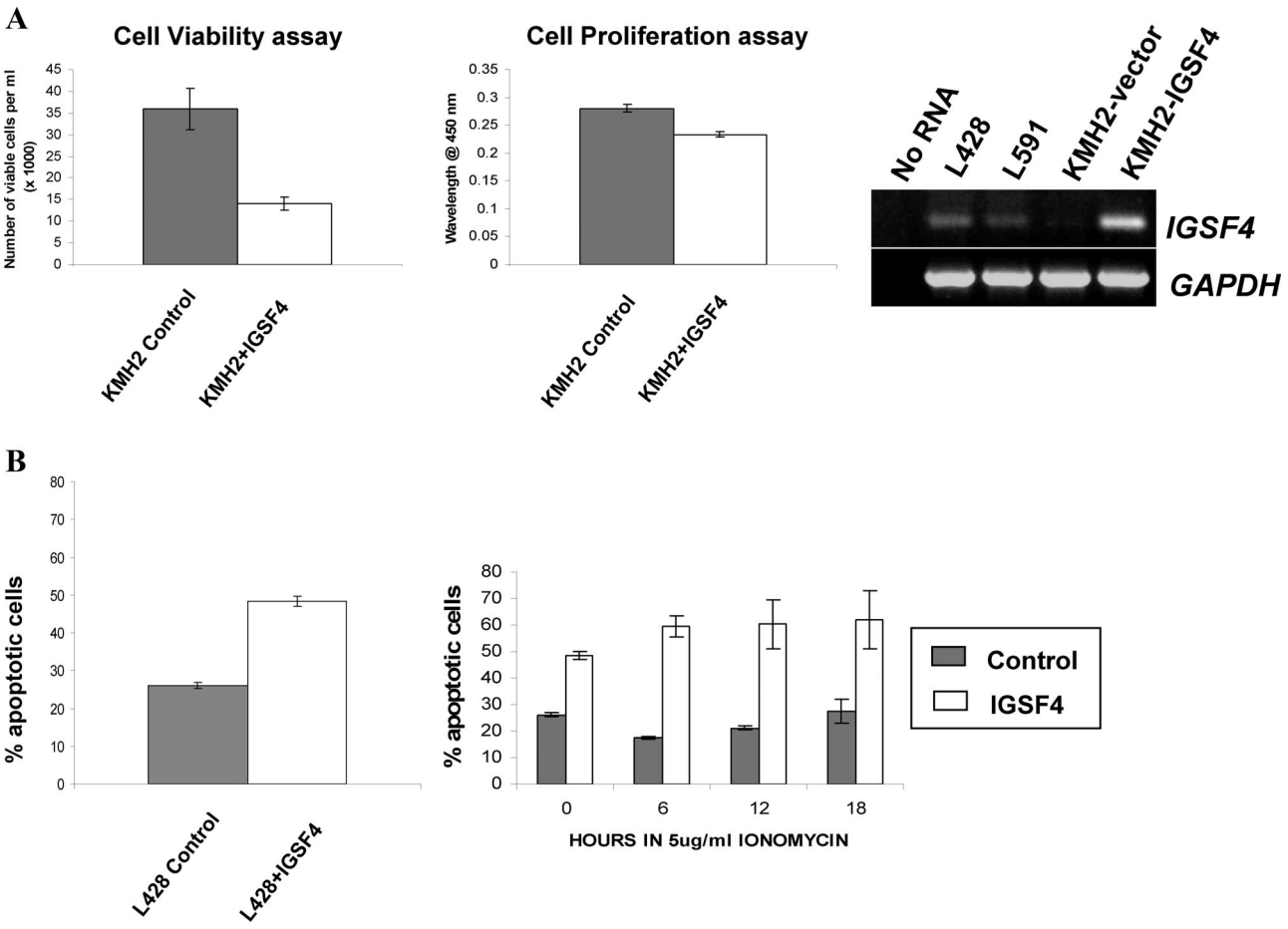

Figure 2. A: Representative phenotypic changes in HL cells following ectopic IGSF4 expression. KM-H2 cells with low levels of IGSF4 were transfected either with IGSF4 expression vector or empty vector. Cell proliferation was monitored by WST1 assay and cell viability was monitored by Trypan blue assay. **Left panel** shows reduced cell viability (48 hours) and proliferation (72 hours) following ectopic IGSF4 expression (white bars) compared with cells transfected with vector (gray bars). **Right panel** shows RT-PCR analysis of the levels of *IGSF4* mRNA following transfection. **B:** Representative data of apoptosis for L428 cells. **Left panel** shows increased apoptosis 48 hours after transfection with IGSF4 (white bars) compared with control (gray bars). **Right panel** shows percentage of apoptotic cells in untreated cells (time 0 hour) and in cells treated with the apoptosis inducer, ionomycin (5 μg/ml; after 6, 12 and 18 hours). Ionomycin treatment increased apoptosis in IGSF4-transfected cells but not in control cells.

GGCA-3′; GAPDH reverse 5′-TCTAGACGGCAGGTCA-GGTCCACC-3′. Apoptosis was determined by the standard Annexin V-PE apoptosis detection kit according to the manufacturer's instructions (BD Pharmingen Ltd) and analyzed by flow cytometry.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4 μm sections as previously described.[2] We used an IGSF4-specific monoclonal antibody, and two separate chicken polyclonal antibodies.[10,20] IGSF4 protein expression was categorized as absent/low (defined as undetectable or staining intensity in all tumor cells lower than that in GC B cells), moderate (>80% of tumor cells staining less intensely than GC B cells), or normal (>80% of tumor cells staining similarly as GC B cells).

Western Blot

Western blot analysis was performed as previously described,[28] using antibody against IGSF4 (Santa Cruz Biotechnology, CA), and antibody against tubulin from Neomarkers (Fremont, CA).

Results

Identification of IGSF4 as Methylated Target Gene in HL Cells

We used a chemical epigenetic approach by comparing gene expression profiles of KM-H2 HL cells before and after treatment with the demethylating agent Aza, using Affymetrix microarray expression analysis. This identified a total of 106 genes that were up-regulated (>1.8 fold with a false discovery rate less than 1%) following this treatment (Figure 1A), including 30 genes with known or putative tumor suppressor functions, several of which have previously been shown to be inactivated by methylation in other tumors (Table 1),[7,29–47] such as *IGSF4*, *GADD45α*, *THBS1*,[29] *CD44*,[30] and *IRF7*,[31] although these genes have not been reported to be epigenetically silenced in HL. Several up-regulated genes were known

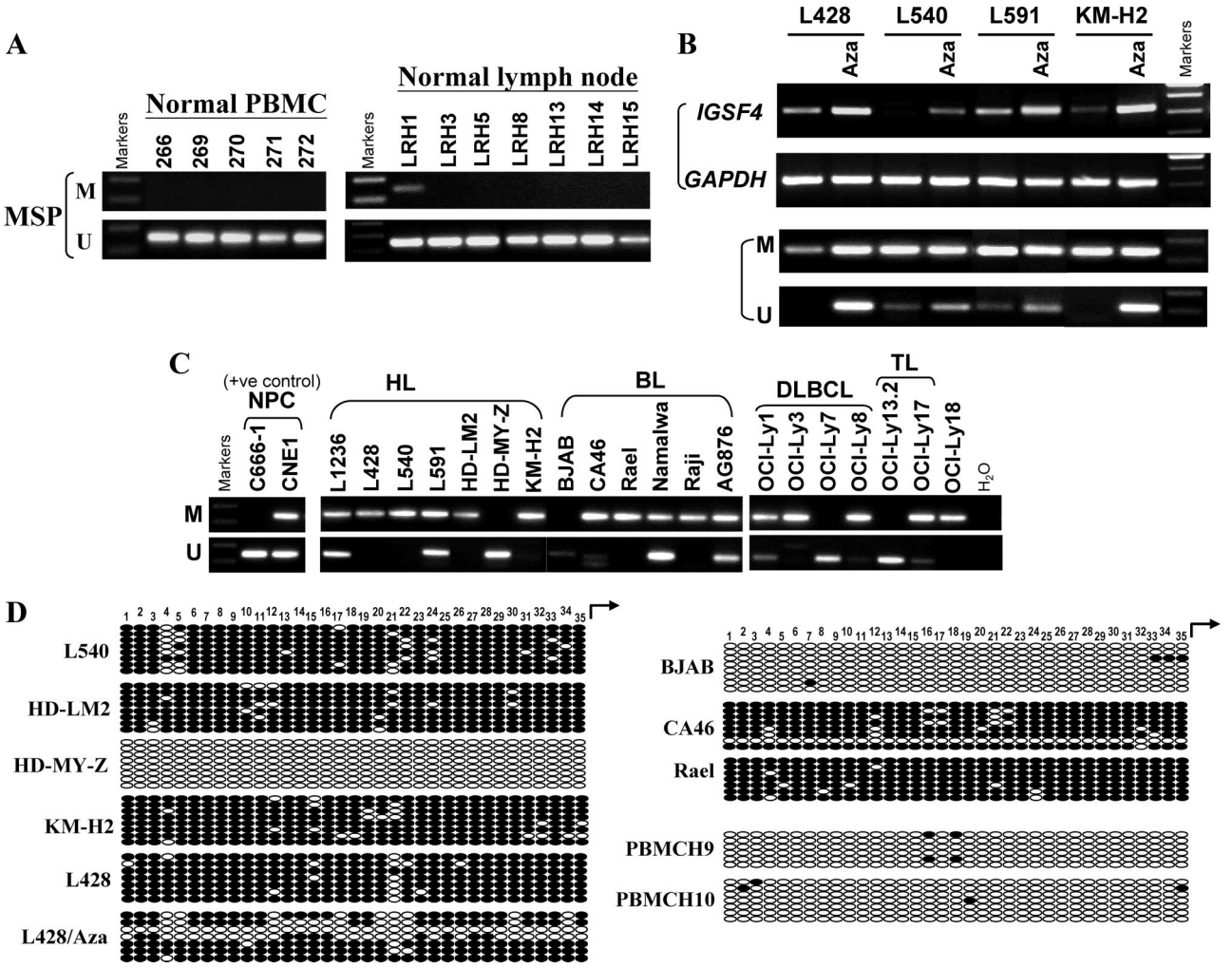

Figure 3. Analysis of *IGSF4* methylation in normal PBMC, normal lymph nodes, and lymphoma cell lines. **A:** By MSP, all PBMC and 6/7 nonmalignant lymph node samples showed only un-methylated *IGSF4* promoter; one lymph node with weak *IGSF4* methylation was detected. (M = MSP for methylated DNA; U = USP for unmethylated DNA). **B:** Up-regulation of IGSF4 mRNA with promoter demethylation by Aza treatment in HL cell lines (L428, L591 and KM-H2) with low levels of *IGSF4* and L540 lacking IGSF4 expression. **Upper panel** shows RT-PCR analysis and **lower panel** shows MSP for methylation. **C:** MSP analysis of tumor cell lines. Loss or reduced IGSF4 expression was always accompanied by promoter methylation. NPC cell lines used as positive controls. **D:** Representative BGS methylation analysis in normal PBMC and lymphoma cell lines before or after Aza treatment. CpG sites are shown on the top row as numbers. Each row of circles represents a single allele of the *IGSF4* promoter analyzed. Open circles: unmethylated CpG sites, filled circles: methylated CpG sites. *IGSF4* was unmethylated in normal PBMCs (H9, H10) and cell lines expressing high levels of IGSF4 (examples shown are BJAB and HD-MY-Z). In contrast, *IGSF4* was almost completely methylated in silenced cell lines (L540, HD-LM2, Rael, CA46, L428 and KM-H2). Promoter of L428 was partly demethylated following Aza treatment.

tumor suppressors, including *IRF1*,[32] p53 target gene *ZMAT3/WIG1,*[33] and *KLF6/BCD-1*.[48] But their inactivation by aberrant methylation has not been reported. We validated the upregulation of several of these genes including *IGSF4*, following treatment with Aza by RT-PCR analysis, together with several genes without apparent changes in expression like *RAFTK*, *DUSP1* and *DAL1* (Figure 1B). *IGSF4* was selected for further investigation on the basis of its described anti-apoptotic activities.[22]

Loss of IGSF4 Expression in Hodgkin Lymphoma Cells

We then studied the expression of *IGSF4* mRNA in HL-derived cell lines by RT-PCR (Figure 1C). When compared with two normal lymph nodes, *IGSF4* mRNA levels were found to be lower in four HL cell lines (KM-H2, L428, L591, L1236), and undetectable in an additional two (HD-LM2, L540) (Figure 1C). Down-regulation was not unique to HL cell lines because 4/6 BL cell lines and 4/5 DLBCL cell lines also lacked *IGSF4* expression. We next studied IGSF4 expression in primary HL tissues using an IGSF4-specific monoclonal antibody.[49] We observed strong expression of IGSF4 in GC B cells of reactive lymphoid tissues (Figure 1E, upper panel), but absent or low level expression in HRS cells of 7/10 HL cases (Figure 1E, lower left panel). These results were confirmed using two separate chicken polyclonal antibodies directed to IGSF4[50] (data not shown). We conclude that IGSF4 expression is down-regulated in HRS cells in the majority of cases. We also studied the protein expression level of IGSF4 in one HL cell line (L1236), two BL cell lines (Raji and BJAB), and two DLBCL cell lines (OCI-Ly1 and OCI-

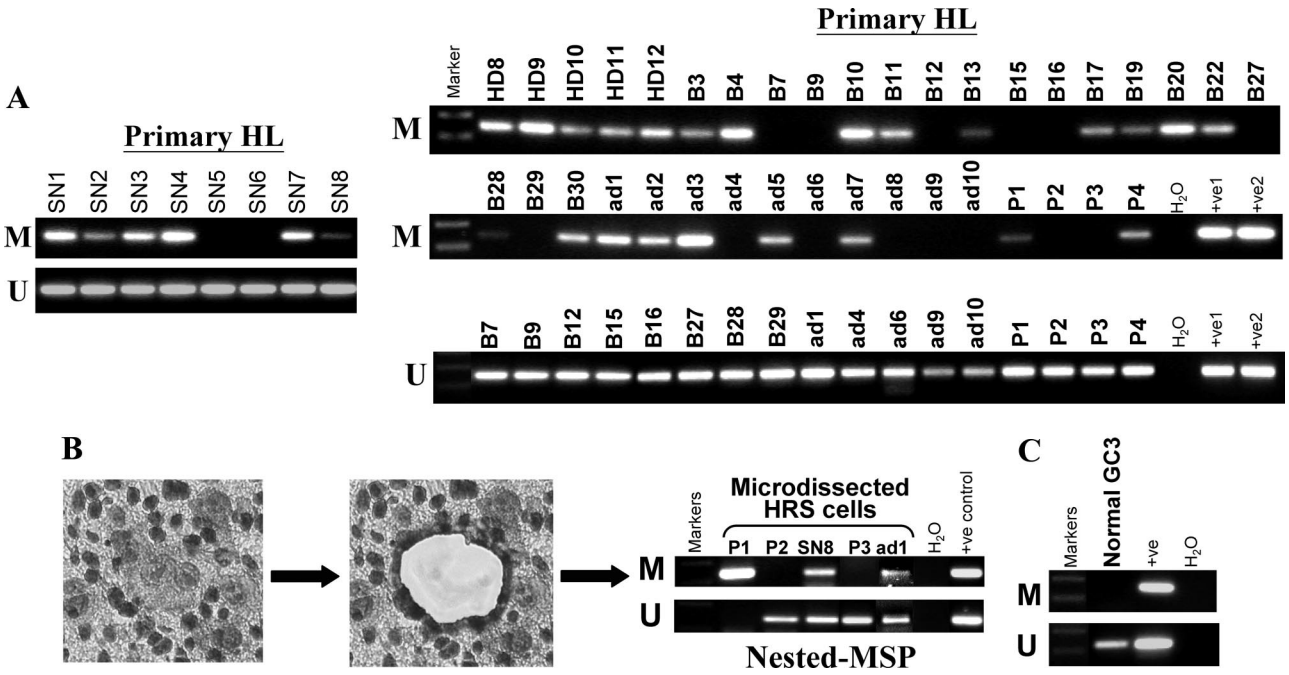

Figure 4. Analysis of *IGSF4* methylation in primary HL. **A:** By analysis of whole tumor tissue, *IGSF4* methylation was detectable in 29/45 cases of HL. L591 and L1236 were used as +ve1 and +ve2 control for MSP and USP. **B:** Microdissection of HRS cells was performed for five HL biopsies (Table 4) following CD30 staining of HRS cells. HRS cells from three tumors (P1, SN8 and ad1) showed methylation by nested MSP analysis; all were also methylated by MSP of whole tumors (Table 2). HRS cells from two further tumors, which are unmethylated by analysis of whole tumors (P2, P3), lacked detectable *IGSF4* methylation in HRS cells. L591 was used as a positive control for MSP and USP. **C:** *IGSF4* methylation could not be detected in GC B cells microdissected from reactive lymph nodes. L591 was used as a positive control for MSP and USP.

Ly3), and detected its absence in cell lines, correlated with its RNA expression (Figure 1D).

Re-Expression of IGSF4 in HL Cells Enhances Their Sensitivity to Apoptosis

We next studied the phenotypic consequences of re-expressing *IGSF4* in HL cells. KM-H2 cells were transfected with either an *IGSF4*-expressing vector or an empty vector as a control. When compared with control cells, ectopic *IGSF4* expression led to marked reduction in cell viability and a small reduction in proliferation (Figure 2A). We next examined if *IGSF4* re-expression in HL cells influenced their sensitivity to apoptosis. Figure 2B (left panel) shows that L428 HL cells expressing exogenous *IGSF4* had higher baseline levels of apoptosis, demonstrating that the reduction in survival was at least partly due to increased apoptosis. We next treated L428 cells with the apoptosis-inducing agent, ionomycin. Figure 2B (right panel) shows that compared with control cells, ionomycin treatment significantly increased apoptosis in the *IGSF4*-expressing HL cells. We conclude that *IGSF4* expression increases the sensitivity of HL cells to apoptosis.

Down-Regulation of IGSF4 in Hodgkin Lymphoma Is Associated with Promoter Methylation

We next studied if methylation of the *IGSF4* promoter was associated with reduced expression. MSP analysis showed no *IGSF4* methylation in five PBMC samples from normal donors nor in 6/7 normal lymph node samples. One normal lymph node sample showed weak methylation (Figure 3A). In contrast, *IGSF4* methylation was observed in all of the lymphoma cell lines with low or absent *IGSF4* mRNA (Figure 3C). Reduced expression and promoter methylation were also accompanied by loss of IGSF4 protein in all cases (Figure 1D). To further test whether *IGSF4* methylation inhibits its gene expression, HL cell lines were treated with demethylating agent, Aza. Following this treatment, *IGSF4* transcription was induced and accompanied by an increase in unmethylated alleles of the *IGSF4* promoter (Figure 3B).

We then used BGS to analyze the methylation of CpG islands of the *IGSF4* promoter. We found that virtually all CpG sites in the promoter were unmethylated in normal PBMCs and in cell lines expressing high levels of *IGSF4* (eg, BJAB and HD-MY-Z), but almost completely methylated in cell lines with silenced *IGSF4*. Following treatment with Aza, the *IGSF4* promoter in the HL cell line L428 was partly demethylated, which was consistent with the MSP result (Figure 3D).

We next examined *IGSF4* methylation in primary HL. Analysis of whole tumor extracts from HL biopsies showed methylation in 29/45 (64%) cases (Figure 4A). Only two cases of lymphocyte-predominant HL showed methylation (2/5), in contrast to classic HL cases (27/40; Tables 2 and 3). There was no association between *IGSF4* methylation and the status of Epstein-Barr virus infection of primary tumors (Table 2). *IGSF4* methylation was also detected in the majority of cases of primary NHL (Table 3; Supplemental Figure 1 at *http://ajp.amjpathol.org*).

Table 2. Histology, EBV Positivity, and *IGSF4* Methylation Status of Primary HL Tumors Analyzed by MSP of Whole Tumor Sections

HL case number	Histology	EBV positivity (subtype)	*IGSF4* methylation status
HD8	UC	A	M
HD9	UC	–	M
HD10	UC	–	M
HD11	UC	–	M
HD12	UC	–	M
B3	NS	A	M
B4	NS	A	M
B7	LP	–	U
B9	NS	A	U
B10	LD	A	M
B11	NS	A	M
B12	LP	–	U
B13	Interfollicular	A	M
B15	NS	–	U
B16	NS	A	U
B17	NS	–	M
B19	NS	–	M
B20	NS	–	M
B22	NS	–	M
B27	LP	–	U
B28	LP	–	M
B29	NS	–	U
B30	NS	–	M
ad1*	NS	–	M
ad2	MC	A	M
ad3	NS	–	M
ad4	NS	–	U
ad5	MC	A	M
ad6	MC	A	U
ad7	LP	–	M
ad8	MC	A	U
ad9	NS	–	U
ad10	NS	–	U
P1*	MC	A	M
P2*	NS	–	U
P3*	MC	A	U
P4	UC	Not Known	M
SN1	UC	A+B	M
SN2	UC	A	M
SN3	UC	A	M
SN4	UC	A	M
SN5	UC	B	U
SN6	UC	A	U
SN7	UC	A	M
SN8*	UC	B	M
Total: 29/45 (64.4%)			

–, No EBV detected; A, EBV Type A; B, EBV Type B; NS, nodular sclerosis HL; MC, mixed cellularity HL; LP, lymphocyte predominant HL; LD, lymphocyte depletion HL; UC, classic HL, subtype not classified; M, methylated; U, unmethylated. *indicates tumors also analyzed for microdissected HRS cells (see Table 4).

We also assessed whether the *IGSF4* methylation found in the analysis of whole tumors was present in HRS cells. CD30+ HRS cells were microdissected from frozen sections of five HL cases that had been analyzed by MSP of whole tumor extracts and also by IHC. Nested-MSP detected *IGSF4* methylation in HRS cells in three cases in which *IGSF4* methylation had been detected by MSP of whole tumor samples, but not in two MSP-negative cases (Figure 4B). No methylation was detected in GC B cells microdissected from reactive lymph nodes (Figure 4C). Sequencing of MSP products from these reactions confirmed their specificity in all cases (Supplemental Figure 2 at *http://ajp.amjpathol.org*). The presence of unmethylated alleles in microdissected cells in most cases suggests either methylation of only one allele or the existence of subpopulations of tumor cells with differing methylation status. For all of the cases studied by either whole tumor or microdissected HRS cells, 6/7 tumors with *IGSF4* methylation also showed loss of protein expression by IHC; in contrast, of three tumors which lacked methylation only one showed reduced expression (Table 4). Thus, we conclude that methylation of the *IGSF4* promoter is associated with its transcriptional repression and loss of protein expression in primary HRS cells.

Table 3. Summary of *IGSF4* Promoter Methylation (by MSP Analysis of Whole Tumors) in Primary HL and NHL

Samples		*IGSF4* methylated (no. of cases)
HL	Classic	27/40 (67.5%)
	LP	2/5 (40%)
PTLD		4/11 (36%)
BL		6/7 (86%)
DLBCL		10/12 (83%)
FL		7/7 (100%)
ALCL		1/3 (33%)
Mantle cell lymphoma		4/4 (100%)

LP, lymphocyte predominant HL; PTLD, post-transplant lymphoproliferative disease; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; ALCL, anaplastic large cell (Ki-1^+) lymphoma.

Promoter Methylation of IGSF4 Prevents its Induction by Stress

Previously we showed that *IGSF4* is a stress-responsive gene that can be up-regulated by heat shock and other environmental stresses in carcinoma and NK lymphoma cells. We further studied in HL cell lines, the induction of

Table 4. Correlation of *IGSF4* Methylation with its Protein Expression in Primary HL

HL case no.	IGSF4 expression in HRS cells by IHC	Methylation status (whole tumor/HRS cells)
SN6	Normal	U
SN8*	Normal	Weak M/M
P3*	Moderate	U/U
SN1	Absent/low	M
SN2	Absent/low	M
SN3	Absent/low	M
SN4	Absent/low	M
SN5	Absent/low	U
SN7	Absent/low	M
P1*	Absent/low	Weak M/M
ad1*	N/A	M/M
P2*	N/A	U/U

HRS cells were microdissected from some cases and subject to nested MSP. M, methylated *IGSF4* detected; U, only unmethylated alleles detected; weak M, predominantly unmethylated alleles, but some low level methylation detected. MSP results of either whole tumor (taken from Table 2), or microdissected HRS cells from some cases (marked by an asterisk) (see Table2). N/A-paraffin sections not available for IHC.

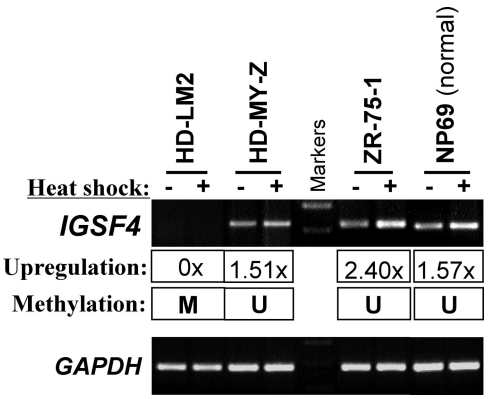

Figure 5. RT-PCR analysis of tumor cell lines shows that the response of *IGSF4* to heat shock stress is impaired in methylated lymphoma cell lines. Endogenous *IGSF4* mRNA expression could be up-regulated by heat shock in unmethylated cell lines, but this response was impaired when *IGSF4* was silenced by promoter methylation. Shown here is the induction of IGSF4 expression following 43°C heat-shock treatment in tumor cell lines, HD-MY-Z and ZR-75–1, but not in the HL cell line HD-LM2. NP69, a normal nasopharyngeal epithelial cell line was used as a positive control.

IGSF4 expression following heat shock treatment. We observed that in tumor cell lines with *IGSF4* expression (eg, HD-MY-Z, control line ZR-751) and normal cell line (NP69), *IGSF4* mRNA expression could always be up-regulated following heat shock treatment (Figure 5). We also showed that this effect was not restricted to heat shock but was also observed following serum withdrawal (data not shown). In contrast, *IGSF4* expression could not be induced in lymphoma cell lines with a methylated promoter (eg, HD-LM2). We conclude that in methylated cells, *IGSF4* cannot be induced following their exposure to environmental stress.

Frequent Detection of IGSF4 Methylation in Sera from HL Patients

Finally, we examined *IGSF4* promoter methylation in serum samples taken from 17 healthy individuals and 18 HL patients at the time of diagnosis. Using nested-MSP, we detected *IGSF4* methylation in sera of 14/18 (77.8%) cases of HL, but only weakly in 1/17 normal sera (Figure 6, A and B). *ANKRD30A,* a gene constitutively methylated in normal tissues,[51] was used as a positive control for methylated (M) specific bands and a negative control for unmethylated (U) specific bands. MSP showed that *ANKRD30A* was methylated in every normal serum (albeit weakly in three samples), while only two samples showed unmethylated specific bands, indicating that our methylation analysis system is sensitive and specific. The HL serum results were confirmed by direct sequencing of the nested-MSP products (Supplemental Figure 3 at *http://ajp.amjpathol.org*).

Discussion

The pathogenesis of HL is thought to involve protection from apoptosis that would otherwise eliminate B cells lacking functional B cell receptors. Thus, genes whose inactivation might lead to protection from cell death are likely critical candidates for a pathogenic role in HL. In this study we investigated whether IGSF4, a cell adhesion molecule of the immunoglobulin superfamily methylated in HL, could protect HRS cells from apoptosis. Although IGSF4 was originally thought to exert its tumor suppressor functions solely through changes in cell–cell interactions that facilitate metastasis, it has recently been shown to inhibit growth and proliferation of NSCLC cells through inducing their apoptosis.[22]

In contrast to normal GC B cells, we observed that IGSF4 expression was either lost or reduced in HRS cells in the majority of HL cases. Furthermore, we found that ectopic expression of IGSF4 in HL cell lines with low or absent IGSF4 expression led to their decreased survival and this was primarily a consequence of increased apoptosis. Because *IGSF4* is a stress-responsive gene that can be induced by heat shock, we also investigated the effects of this treatment on the induction of IGSF4 in lymphoma cells. We observed that IGSF4 expression could only be induced by heat shock in those cell lines in which the *IGSF4* gene was unmethylated and expressed, but not in cell lines where *IGSF4* expression was silenced by methylation. Taken together, our results suggest that loss of IGSF4 expression may contribute to the anti-apoptotic phenotype of the HRS cell and as such may represent an important pathogenic step in the development of HL.

We showed that the reduced expression of *IGSF4* in HL cell lines was at least partly due to promoter methylation, and that the *IGSF4* promoter was also frequently methyl-

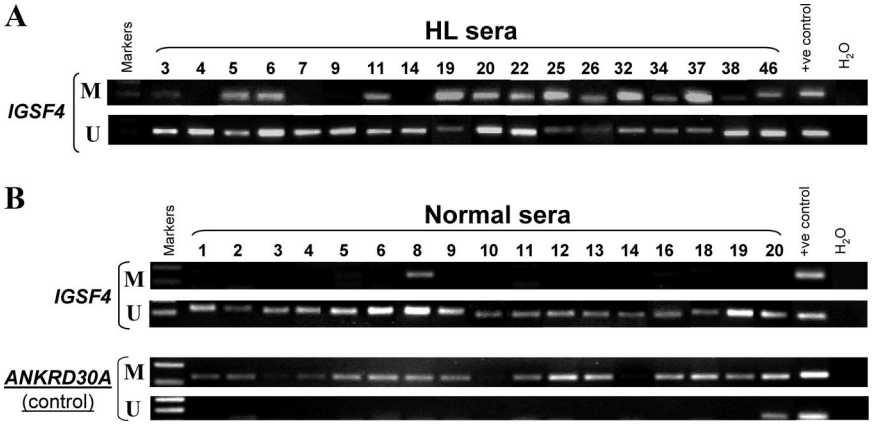

Figure 6. Analysis of *IGSF4* promoter methylation in serum samples from HL patients and normal controls using nested MSP. **A:** *IGSF4* methylation was detectable in the sera of 14/18 cases of HL. L591 was used as a positive control for MSP and USP. **B:** *IGSF4* methylation was only detectable weakly in 1/17 normal sera. MSP products were confirmed by direct sequencing (seeSupplementalFigure3at*http://ajp.amjpathol.org*). L591 was used as a positive control for MSP and USP. MSP results of *ANKRD30A* are shown here as a methylated positive and unmethylated negative control for normal sera. Human placenta was used as positive control for MSP and USP of ANKRD30A.

ated in primary HL. Furthermore, in primary tissues, *IGSF4* methylation was localized to HRS cells where it correlated with reduced protein expression, which was in contrast to the strong expression of IGSF4 in GC B cells. Loss or reduction of IGSF4 expression has been previously demonstrated only in epithelial cancers.[13] We extended our study to investigate the frequency of *IGSF4* methylation in NHL as well as HL. Unlike another TSG we previously studied for HL—*RASSF1A*—whose methylation among hematopoietic malignancies is largely restricted to HL,[2] we observed a high frequency of *IGSF4* methylation in most forms of NHL examined, but particularly in the GC derived NHLs. Therefore, IGSF4 appears to be a key target for inactivation across a broad range of lymphomas.

A series of other putative/validated TSGs were also identified in our study, including *GADD45A, THBS1, CD44, KLF6, ZMAT3/WIG1, IRF1,* and *7* (Table 1).[7,29–47] Although no further functional study was included in this report for these candidate TSGs, their reported functions in the pathogenesis of other types of cancers indicate that these candidates are likely also involved in the pathogenesis of HL. It should be noted that the demethylation approach also has limitations. For example, it is not possible to ascertain from the array whether the transcriptional upregulation of genes observed following Aza treatment is due to direct promoter demethylation and activation, to demethylation of regulators of the genes, or to mechanisms unrelated to methylation (eg, stress responses)[52,53] and thus the method is prone to false-positives. Furthermore, four TSGs previously reported to be methylated in HL: RASSF1A,[2] p16INK4a,[54] p15INK4b,[55] and p18INK4c[56] were not identified in the current analysis. These "false-negatives" could be because the analysis was performed on only one cell line since p18INK4c has been reported to be unmethylated in KM-H2 cells.[56] The microarray expression analysis may also have limited sensitivity, this might explain why *p16INK4a* was only found to be up-regulated by Aza treatment following RT-PCR analysis. The failure to identify two genes previously reported to be methylated in HL, *RASSF1A*[2] and *p14ARF* (manuscript in preparation) may be because compared to other cell lines they are only minimally induced following Aza treatment.

The presence of *IGSF4* methylation specifically in the serum of most HL patients suggests that it might serve as a useful biomarker for this disease. Furthermore, our observations might also have therapeutic relevance; we were able to reactivate IGSF4 expression following demethylation, suggesting that the treatment of HL patients with demethylating drugs might sensitize their tumor cells to apoptosis through the restoration of functions of IGSF4 and other pro-apoptotic genes.

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