MicroRNA miR-29 Modulates Expression of Immunoinhibitory Molecule B7-H3: Potential Implications for Immune Based Therapy of Human Solid Tumors

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Abstract

B7-H3, a surface immunomodulatory glycoprotein, inhibits natural killer cells and T cells. The monoclonal antibody (mAb) 8H9 is specific for 4Ig-B7-H3, the long and principal form of B7-H3. Early results from radioimmunotherapy using 8H9 have shown promise in patients with metastatic solid tumors to the central nervous system. Whereas B7-H3 transcript was ubiquitously expressed in a wide spectrum of human solid tumors as well as human normal tissues, B7-H3 protein was preferentially expressed only in tumor tissues. By quantitative reverse transcription-PCR, all three isoforms of microRNA miR-29 (a, b, and c) were highly expressed in normal tissues. However, they were down-regulated in a broad spectrum of solid tumors, including neuroblastoma, sarcomas, brain tumors, and tumor cell lines. B7-H3 protein expression was inversely correlated with miR-29 levels in both cell lines and tumor tissues tested. Using luciferase reporter assay, miR-29a was shown to directly target B7-H3 3′ untranslated region, and knock-in and knockdown of miR-29a led to down-regulation and up-regulation, respectively, of B7-H3 protein expression. The ability of miR-29 to control B7-H3 protein expression has implications in immune escape by solid tumors. Differential modulation of this key immunoinhibitory molecule in tumor versus normal tissues may advance both cell-mediated immunotherapy and antibody-based targeted strategies using the B7-H3–specific mAb 8H9. [Cancer Res 2009;69(15):6275–81]

Introduction

Human B7-H3 (also named as CD276) is a member of the B7/CD28 immunoglobulin superfamily, which provides crucial costimulatory signals that regulate T-cell functions in tumor surveillance, infections, and autoimmune diseases (1). B7-H3 was initially identified as a type I transmembrane protein with its extracellular region containing only one V-like and one C-like immunoglobulin domain (2Ig-B7-H3; ref. 2), similar to all other B7 family members. Subsequently, a second dominantly expressed form of human B7-H3 that contains tandemly duplicated V-like and C-like immunoglobulin domain (4Ig-B7-H3) was found (3, 4). The inhibitory role of B7-H3 was supported by the reports that both 2Ig and 4Ig forms of human B7-H3 inhibited T-cell proliferation and cytokine production (3). B7-H3 preferentially down-regulated T_H1-mediated immune response in B7-H3–deficient mice (5), and 4Ig-B7-H3 inhibited natural killer (NK) cell–mediated lysis of neuroblastoma cells by interacting with a putative inhibitory receptor on the surface of NK cells (6). In more recent studies of patients with prostate cancer, tumor B7-H3 expression was strongly correlated with disease spread at time of surgery, increased risk of clinical cancer recurrence, and cancer-specific death (7, 8). Moreover, tumor B7-H3 expression was correlated with patient poor survival in both clear cell renal cell carcinoma and urothelial cell carcinoma (9, 10). Earlier reports have also implicated a positive immunologic function of B7-H3; for example, human B7-H3 (2Ig form) promoted T-cell activation and IFN-γ production by binding to a putative receptor on activated T cells (2). Furthermore, antitumor response was enhanced by B7-H3 expression in murine tumor models (11). B7-H3 positivity in gastric carcinoma was also correlated with increased survival (12). It is very possible that B7-H3 has both coinhibitory and costimulatory properties depending on the receptors (13).

MicroRNAs (miRNA) represent a class of naturally occurring small noncoding RNAs, which function as gene regulators. Mature miRNAs are ∼22 nucleotide (nt) molecules cleaved from ∼70- to 100-nt hairpin pre-miRNA precursors (14). Single-stranded miRNAs bind to the 3′ untranslated region (UTR) of target mRNAs primarily through base pairing of the "seed region" of the miRNA (nt 2–7) to the cognate target (15), thereby regulating gene expression posttranscriptionally by decreasing protein translation, increasing degradation of the target message, or both (16). There is increasing evidence that miRNAs play critical roles in tumorigenesis by functioning as oncogenes or tumor suppressors (17).

Our laboratory has developed monoclonal antibody (mAb) 8H9, which targets a 58-kDa glycoprotein broadly expressed in human solid tumors, including embryonal tumors and carcinomas (18). It has shown favorable tumor uptake in xenograft models. In early-phase human clinical trials, it seems to prolong survival among high-risk patients with solid tumors suffering from central nervous system (CNS) metastasis (19, 20). In this report, we described the identification of 4Ig-B7-H3 as the target for mAb 8H9. We further investigated B7-H3 expression at the mRNA and protein levels for both human tumors and normal tissues. miRNA miR-29 was found to regulate B7-H3 protein expression with potential implications for immune-based therapy of human solid tumors.

Materials and Methods

Cell culture and human tissues. Human neuroblastoma cell line LAN-1 was provided by Dr. Robert Seeger (Children's Hospital of Los Angeles, Los Angeles, CA), and NB1691 by Dr. Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN). Human Burkitt's lymphoma cell line Daudi and adenocarcinoma cell line HeLa were purchased from American Type Culture Collection. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. Normal tissues as well as solid tumor samples of different
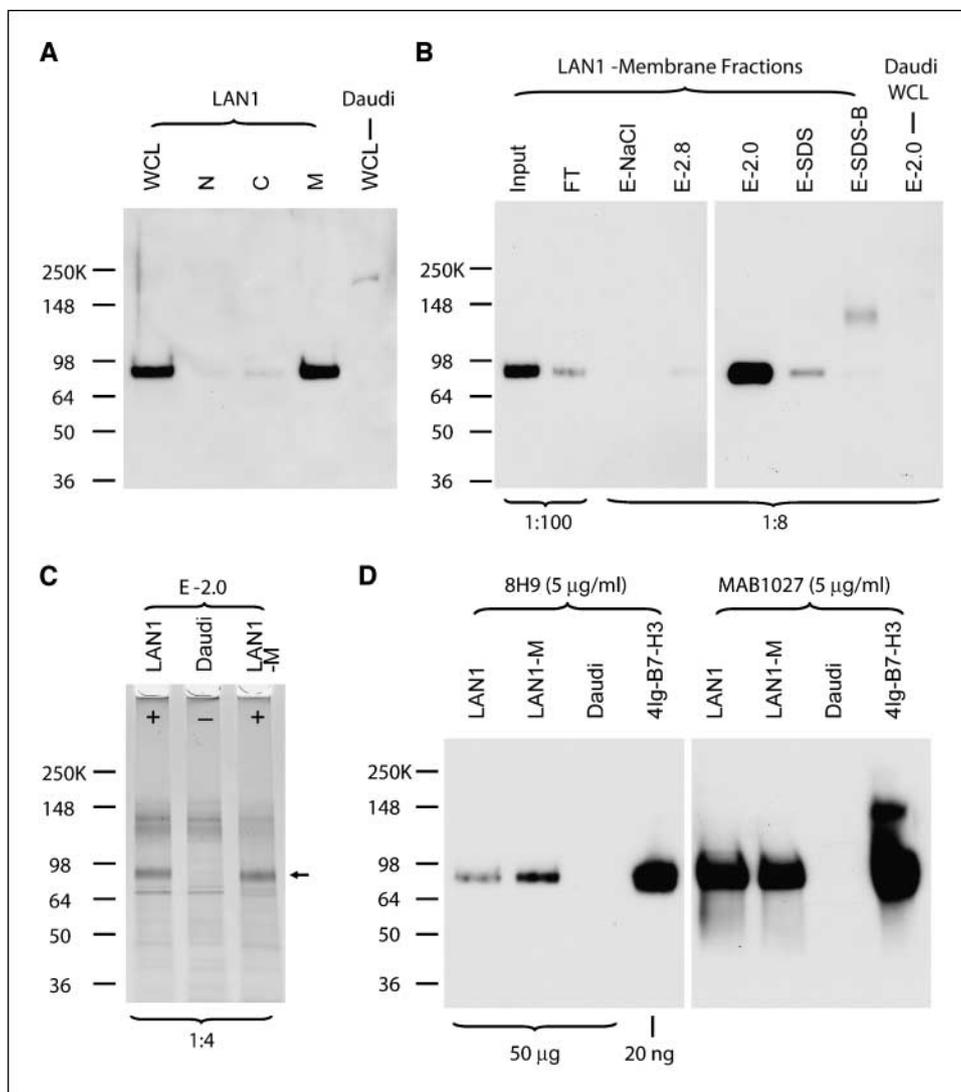
Figure 1. Identification of 8H9 antigen as 4Ig-B7-H3. *A,* Western blot detection of 8H9 antigen using 8H9. The highly glycosylated 8H9 antigen migrated at ~90 kDa on 4–15% Tris-glycine SDS PAGE under nonreducing conditions. *LAN1,* 8H9-positive cell line; *Daudi,* 8H9-negative cell line. *WCL,* whole-cell lysates; *N,* nuclei fraction; *C,* cytosolic fraction; *M,* membrane fraction. The purification procedure was monitored by 8H9 Western blot using Invitrogen SeeBlue Plus2 Pre-Stained Standard as the protein molecular weight marker (*B*) and by silver staining (*C*). Fraction designation is detailed in Materials and Methods. The amount of aliquots loaded (ratio to total amount) was indicated at the bottom. *D,* confirmation of 8H9 antigen as 4Ig-B7-H3 by Western blot using both 8H9 and MAB1027.

histologic types, obtained at Memorial Sloan-Kettering Cancer Center (MSKCC), were snap frozen in liquid nitrogen. Written informed consent was obtained from the patients and/or their guardians in accordance to the guidelines of the institutional review board of MSKCC.

Monoclonal antibodies. mAbs 8H9 (murine IgG1) and 3E7 (murine IgG2b specific for L1-CAM) were produced against human neuroblastoma in our laboratory. They were purified by protein A (GE Healthcare) affinity chromatography before use. MAB1027 (anti–B7-H3 mAb) was purchased from R&D Systems.

Whole-cell lysates and Western blot. 8H9-positive cell line LAN-1 and 8H9-negative cell line Daudi were grown to ~80% confluence. Cells were harvested using 2 mmol/L EDTA and washed with ice-cold PBS. Cells were lysed on ice (20 min) in Triton lysis buffer [50 mmol/L Tris-HCl (pH 7.2), 50 mmol/L NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail tablets]. The lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4°C. Whole-cell lysates (~25–50 μg) were analyzed by SDS-PAGE under nonreducing condition using Tris-glycine Ready Gel System (Bio-Rad). After electrophoresis, samples were transferred onto Immun-Blot polyvinylidene difluoride membrane (Bio-Rad), blocked for 1 h at room temperature with 10% dry milk in TBS/Tween 20, and incubated with primary antibodies (8H9 at 5–20 μg/mL and MAB1027 at 5 μg/mL) for 3 h at room temperature. The membrane was then washed with TBS/Tween 20 and incubated with secondary peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch). Bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Subcellular fractionation. For crude membrane preparation, LAN-1 cells were pipetted off the tissue culture dish, washed with ice-cold PBS, and lysed on ice in sucrose buffer [0.25 mol/L sucrose, 5 mmol/L Tris-HCl (pH 7.2), and protease inhibitor cocktail tablets] with a Dounce homogenizer (Kontes). On centrifugation for 10 min at 1,000 × *g* to pellet all nuclei, the supernatant was then ultracentrifuged at 100,000 × *g* for 30 min in a Beckman L-70K (25,000 rpm, SW41Ti rotor) to separate the membrane particulate from the cytosolic fraction. The cytosolic fraction was adjusted to 1% Triton, whereas crude nuclear and membrane fractions were resuspended in Triton lysis buffer and clarified before use.

8H9 antigen affinity purification. 8H9 antigen was purified from LAN-1 cell extracts by immunoaffinity chromatography using mAb 8H9. The 8H9 affinity column was prepared by covalently conjugating the Fc portion of 8H9 to protein G on the gel matrix using Protein G IgG Plus Orientation Kit (Pierce) according to the manufacturer's instructions. Four milligrams of LAN-1 whole-cell lysates or equivalent membrane fraction, prepared as described above, were incubated overnight at 4°C with 20 μL 8H9-protein G-Sepharose (3 mg bound 8H9/mL beads). After extensive washing with Triton lysis buffer, the column was eluted sequentially with 50 mmol/L Tris-HCl (pH 7.2) containing 1 mol/L NaCl (*E-NaCl*), 0.1 mol/L glycine-HCl, pH 2.8 (*E-2.8*) and pH 2.0 (*E-2.0*), SDS sample buffer (*E-SDS:* 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.005% bromophenol blue), and SDS sample buffer plus boiling in water for 5 min (*E-SDS-B*). A small aliquot of eluates was monitored for the presence of 8H9 antigen by Western blot using 8H9 antibody. Twenty-five percent of the eluate was also analyzed by

silver staining (SilverQuest Silver Staining Kit, Invitrogen). Finally, 50% of the 8H9 antigen-positive eluate (E-2.0 fraction) was analyzed by colloidal Coomassie blue staining (GelCode Blue Stain Reagent, Pierce), and the 8H9 antigen-positive band was collected and used for mass spectrometric identification by the Microchemistry and Proteomics Core Facility at MSKCC.

Detection of B7-H3 mRNA by quantitative reverse transcription-PCR. Total RNA from normal tissues, as well as solid tumors detailed in Fig. 3, was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microliter of cDNA synthesized from 1 μg of total RNA was used for real-time quantitative PCR using Applied Biosystems (ABI) Sequence Detection System 7300. B7-H3 (CD276) gene expression assay reagent (Hs00228846_m1) as well as two endogenous controls, hypoxanthine phosphoribosyltransferase 1 (HPRT1, 4326321E) and succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA, Hs00188166_ml), were purchased from ABI. Each sample was quantified using the comparative C_T method (ABI) as a relative fold difference compared with peripheral blood mononuclear cells (PBMC).

Quantitation of miR-29. miR-29a, miR-29b, miR-29c, as well as the endogenous control RNU48 were purchased from ABI. A two-step reverse transcription-PCR (RT-PCR) with reverse transcription using a miRNA-specific primer, followed by quantitative PCR with TaqMan probes, was carried out according to the manufacturer's instructions.

Protein expression by immunofluorescence and flow cytometry. These procedures were previously described (18). The blocking experiment was carried out by incubating 0 to 10 μg of recombinant human 4Ig-B7-H3 (R&D Systems) with 1 μg 8H9 or 0.1 μg 3E7 (as negative control) for 30 min at room temperature, before mixing with 10^6 M14 melanoma cells for immunofluorescence staining and flow cytometry analyses.

Luciferase reporter assay. Oligonucleotides corresponding to the miR-29a binding site in the B7-H3 3′UTR or a single-base mutant (illustrated in Fig. 6A) were synthesized (Integrated DNA Technologies) and inserted into the XbaI site immediately downstream from the stop codon of firefly luciferase of the pGL3-control vector (Promega). HeLa cells were cotransfected in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol, with 50 ng of the firefly luciferase reporter, 1 ng of the renilla luciferase reporter (pRL-CMV vector, Promega) as transfection control, and 100 nmol/L (final) miRNA mimics (ThermoFisher Scientific). Firefly and renilla luciferase activities were measured sequentially using dual-luciferase assays (Promega) 24 h after the transfection. The experiments were done in triplicate.

NB1691 transient transfection. NB1691 cells were transfected in six-well plates using DharmaFECT reagent (ThermoFisher Scientific) according to the manufacturer's instructions, with 100 nmol/L (final) of miRNA mimics, inhibitors, or mimics plus inhibitors (ThermoFisher Scientific). Twenty-four hours after transfection, cells were treated with 1 mg/mL Pronase E (E. Merck) for 30 min at 37°C to strip off B7-H3 protein already on the cell surface, and another 48 h later, newly expressed B7-H3 protein levels were measured by 8H9 immunofluorescence staining followed by flow cytometry analyses as previously described (18).

Results

Affinity purification of the antigen targeted by mAb 8H9. To monitor its affinity purification, the antigen was detected by Western blotting under nonreducing conditions using Tris-glycine SDS-PAGE. Figure 1A showed a single band (~90 kDa) detected in 8H9-positive cell line LAN-1, but not in 8H9-negative cell line Daudi. Data were not shown for two other 8H9-positive cell lines, HTB82 and U2OS, as well as control mIgG1 mAb 5F9, which did not detect a band at the same size. Binding to 8H9 was lost under reducing conditions (data not shown), suggesting that 8H9 recognizes a conformation-sensitive epitope. After subcellular fractionation, this antigen was found to be predominantly in the membrane fraction (Fig. 1A).

To identify this antigen, protein affinity purification in combination of mass spectrometric analysis was used. After

incubating either LAN-1 (and Daudi as negative control) whole-cell lysates or LAN-1 membrane fraction with 8H9-protein G-Sepharose overnight, a significant portion (>50%) of 8H9 antigen was bound to the Sepharose [Fig. 1B, input versus flowthrough (FT)]. 8H9 antigen was eluted specifically and predominantly in 0.1 mol/L glycine-HCl, pH 2.0 (E-2.0) as monitored by Western blot analysis (Fig. 1B), suggesting a very strong interaction between 8H9 antibody and its antigen. After silver staining the same eluate, a clear band (arrow position) was detected accordingly only in LAN-1 cell extracts but not in Daudi cell extracts (Fig. 1C). Approximately 10 ng of the band (visible with colloidal Coomassie staining; data not shown) were collected and used for mass spectrometric identification.

8H9 antigen identification. In-gel tryptic digested eluate was analyzed, and mass spectrometric sequencing (matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry) confirmed two peptides (P1: NPVLQQDAHSSVTITPQR and P2: SPTGAVEVQVPEDPVVALVGTDATLR). These peptides unequivocally matched the 8H9-reactive molecule to 4Ig-B7-H3 (CD276, National Center for Biotechnology Information no. 74757248), the long and principal form of B7-H3 in human tissues.

As further confirmation, both 8H9 and MAB1027 recognized a single band at ~90 kDa on Tris-glycine SDS-PAGE under
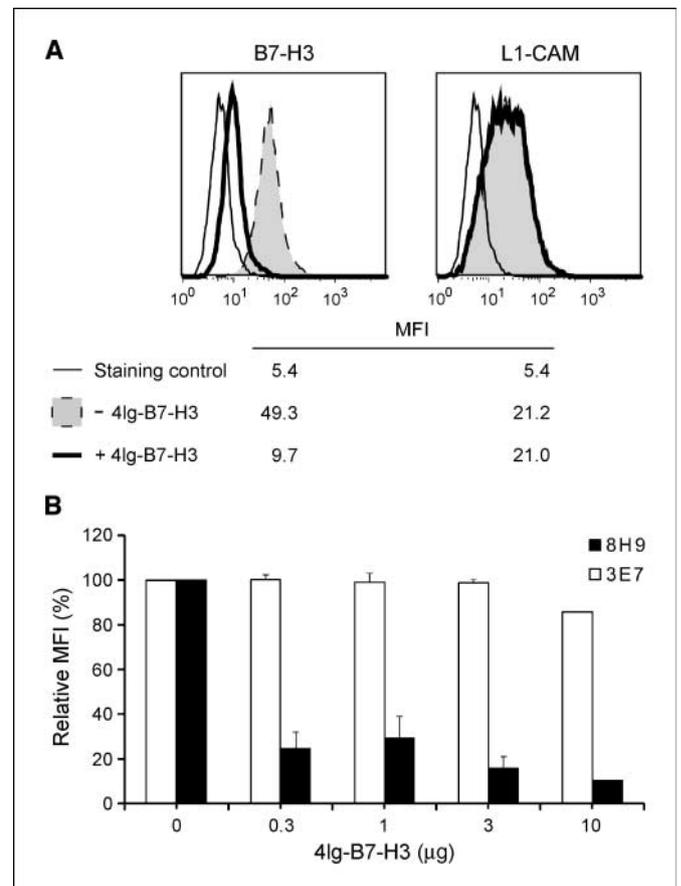

Figure 2. Blocking of 8H9 binding to natively expressed B7-H3 on M14 cells by recombinant human 4Ig-B7-H3. *A,* either 0 μg (−4Ig-B7-H3) or 3 μg (+4Ig-B7-H3) recombinant human 4Ig-B7-H3 were used for blocking. Natively expressed B7-H3 was detected by 8H9, and L1-CAM by 3E7. Representative FACS histograms with mean fluorescence intensity (*MFI*) from three independent experiments. A nonspecific mAb TIB114 served as the staining control. *B,* various amounts of recombinant human 4Ig-B7-H3 (as indicated) were used for blocking. *Columns,* mean MFI relative to 0 μg 4Ig-B7-H3; *bars,* SD.
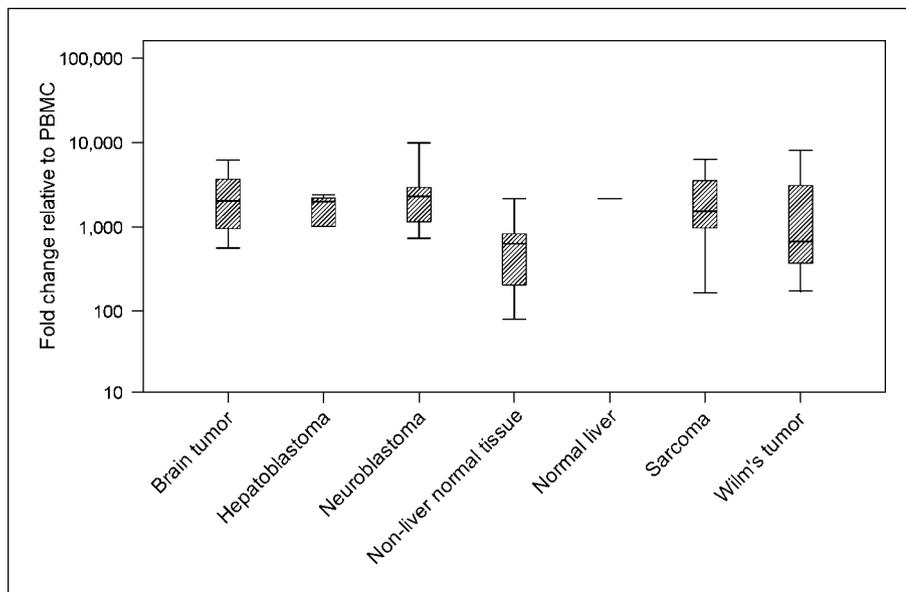
Figure 3. B7-H3 transcript was ubiquitously expressed in solid tumors and normal human tissues. mRNA levels were measured by quantitative RT-PCR. Geometric mean of HPRT1 and SDHA transcript levels served as the endogenous control, and expression levels in log scale were fold change relative to PBMCs; the number of samples for each histologic type is detailed in Results, and normal liver tissue (8H9 positive) was singled out for direct comparison.

nonreducing conditions, when either 8H9-positive cell extracts or recombinant human 4Ig-B7-H3 protein was used (Fig. 1D). This band migrated to ∼58 kDa, the calculated molecular mass for 4Ig-B7-H3, after N-glycanase treatment (data not shown). Our affinity purification strategy only identified the 4Ig form from LAN-1 cell extracts, although 8H9 was also able to recognize the 2Ig form of recombinant B7-H3 protein by Western blot analysis (data not shown). Moreover, 8H9 binding, measured by fluorescence-activated cell sorting (FACS) analyses, to the natively expressed B7-H3 on M14 melanoma cells was blocked by recombinant human 4Ig-B7-H3 (Fig. 2). In contrast, control mAb 3E7 was not affected by the presence of recombinant human 4Ig-B7-H3 (Fig. 2).

B7-H3 mRNA and protein expression. To systematically study the expression pattern of B7-H3, 18 normal tissues, including adrenal, cerebellum, frontal lobe, heart, ileum, kidney, liver, lung, lymph node, pancreas, pons, sigmoid colon, skeletal muscle, spinal cord, spleen, stomach, testes, and thyroid, were tested by quantitative RT-PCR for their level of B7-H3 mRNA expression. Solid tumors, including 15 brain tumors, 5 hepatoblastomas, 41 neuroblastomas, 11 sarcomas, and 5 Wilms' tumors, were also analyzed. Expression level as fold change relative to PBMCs was tabulated in Fig. 3. B7-H3 transcript was found to be ubiquitously

expressed in solid tumors of different histologic types, as well as normal human tissues.

In contrast to the ubiquitous abundance of mRNA expression, there was a marked differential protein expression in solid tumors versus normal tissues. Whereas solid tumors were strongly 8H9 positive by immunohistochemical staining, reflecting abundant B7-H3 protein expression, B7-H3 was undetectable in most normal tissues, including normal CNS tissues (18). Western blot analysis using 8H9 also confirmed this finding (Fig. 4), even after longer film exposure (data not shown). By contrast, the commercial antibody MAB1027 showed much higher sensitivity to B7-H3 by both immunohistochemistry (data not shown) and Western blot analysis (Figs. 1 and 4).

Relationship between B7-H3 protein and miR-29 expression among solid tumors and normal tissues. To test if miRNA can modulate differential B7-H3 overexpression in solid tumors, we used TargetScan (21) to identify miR-29 as the only broadly conserved miRNA family among vertebrates with a conserved target site in the B7-H3 3′UTR. All three isoforms of miR-29 (a, b, and c) share the same seed complementarity (nt 2–7) between miR-29 and B7-H3 3′UTR (position 1339–1346), suggesting that all isoforms potentially target B7-H3. If miR-29 is a potent regulator of
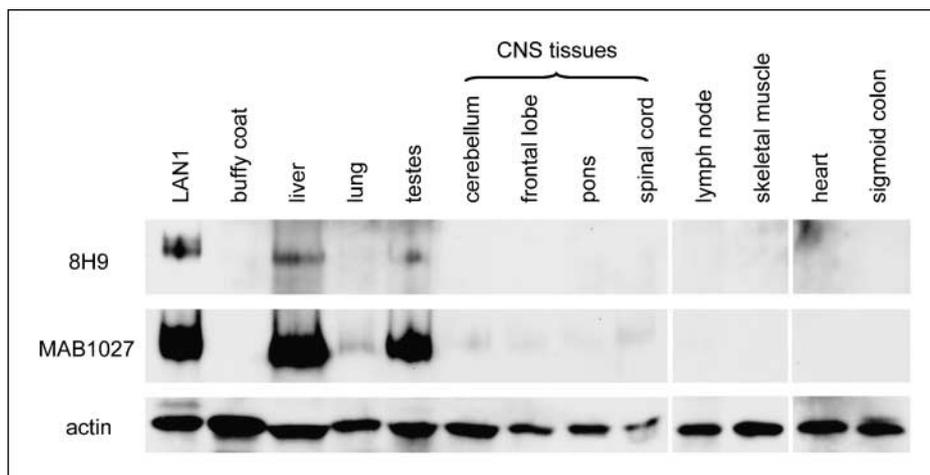

Figure 4. B7-H3 protein was not detected by 8H9 in most normal tissues, including normal CNS tissues. Protein levels were detected by Western blot using 8H9 or MAB1027. Actin levels served as loading control.

B7-H3 protein expression, then miR-29 level should be down-regulated in B7-H3–overexpressing tumor tissues. Indeed, when compared with the average of 18 normal tissues (normalized as 100%) by quantitative RT-PCR, a broad spectrum of solid tumors, as well as 8H9-positive tumor cell lines, had much lower miR-29 expression in all three isoforms (Fig. 5A). Among normal tissues, miR-29 levels in 8H9-positive liver were 2- to 9-fold (the sum of all three isoforms) lower than the levels in 8H9-negative CNS tissues (cerebellum, frontal lobe, pons, and spinal cord; Fig. 5B). All these data suggest an inverse correlation between B7-H3 protein and miR-29 expression level.

miR-29a directly targets B7-H3 3′UTR. To test if miR-29 acts directly on B7-H3 expression, we performed a luciferase reporter assay. We chose miR-29a for these studies because all three isoforms of miR-29 share the same seed complementarity to B7-H3 3′UTR and have the similar inverse relationship between B7-H3 protein and miR-29 levels. The alignment of miR-29a with the B7-H3 3′UTR target site is illustrated in Fig. 6A. The target site was cloned into the 3′UTR of the firefly luciferase gene and cotransfected with miR-29a into HeLa cells. As shown in Fig. 6B, cotransfection of wild-type luciferase construct (B7-H3-WT) with negative control (NC) miRNA only moderately reduced luciferase activity with respect to parental luciferase construct (Control), presumably due to the existence of low levels of endogenous miR-29 in HeLa cells. However, cotransfection of B7-H3-WT with miR-29a significantly reduced luciferase activity, reducing by >60% when compared with control level. This repression was reversed by a single base mutation in the binding site (B7-H3-mt). These results suggest that complementary site in the B7-H3 3′UTR is a direct target of miR-29a mediated posttranscriptional gene silencing.

Neuroblastoma cell line NB1691 showed strong B7-H3 protein expression by 8H9 staining with low endogenous miR-29a. Over-expression of miR-29a in NB1691 substantially reduced B7-H3 protein expression with a reduction of ∼60% when compared with negative control (Fig. 6C). B7-H3 mRNA level was not affected (data not shown), suggesting that modulation of B7-H3 protein levels by miR-29a was primarily due to repressed translation, and not mRNA degradation. And this repression was also reversed when miR-29a inhibitor was cotransfected (miR-29a mimic + inhibitor). Similar findings were obtained when another neuroblastoma cell line, LAN-1, was tested (data not shown). As an additional control for this experiment, the level of another cell-surface antigen L1-CAM was found to remain unchanged (data not shown), suggesting that results shown in Fig. 6C were not due to cellular nonspecific effects. These data provide direct evidence that miR-29a targeted B7-H3 mRNA and was able to modulate B7-H3 protein expression.

Discussion

In this study, we described the identification of 4Ig-B7-H3 as the target antigen for 8H9, a mAb in clinical trial for solid tumors metastatic to the CNS (NCT00089245).[1] Furthermore, we provided evidence that B7-H3 protein overexpression in tumor tissue was highly correlated with decreased expression of miR-29 as compared with normal tissues, and B7-H3 protein level could be modulated by manipulating miR-29 level in cultured cell lines. The ability of miR-29 to control 4Ig-B7-H3 protein expression has implications in

immune escape by solid tumors and may allow differential modulation of this key immunoinhibitory molecule in tumor versus normal tissues, for both cell-mediated immunotherapy and antibody-based targeted strategies using 8H9.

Both negative and positive immunologic functions for B7-H3 have been reported. These contradictory findings could be explained by the existence of antagonistic B7-H3 receptors. Indeed, a costimu-latory receptor for mouse B7-H3 that preferentially enhances mouse CD8+ T-cell activation (TREM-like transcript 2) has been identified recently (22). The association of B7-H3 expression and worse clinical outcome in prostate cancer, clear cell renal cell carcinoma, and urothelial cell carcinoma strongly implicates its potential role in inhibiting immune surveillance (7–10). Yet, coinhibitory receptor(s) for B7-H3 still remains to be found.

By Western blot and immunohistochemistry both in this report and in previous publication (18), there was a clear differential expression of B7-H3 in solid tumors versus normal tissues. Because (a) 4Ig-B7-H3 inhibits NK cell–mediated lysis of neuroblastoma cells (6), and (b) most neuroblastomas do not express surface HLA-class I molecules, thereby escaping the attack by the CTLs, it is reasonable to hypothesize that overexpression of B7-H3 on the surface of neuroblastoma and possibly other solid tumors will protect tumor cells from NK/T-cell–mediated lysis, thus escaping
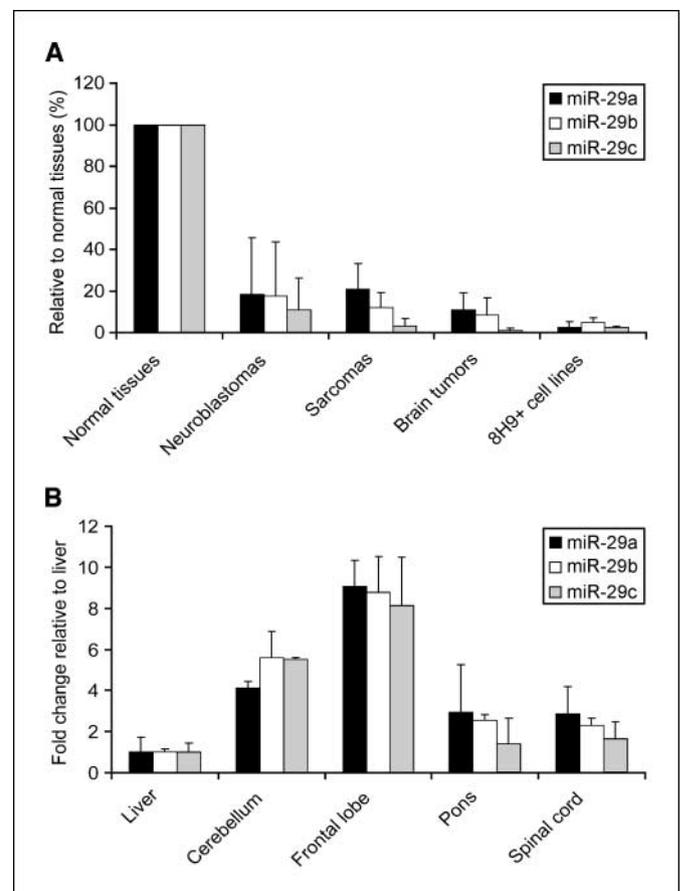

Figure 5. Inverse correlation between B7-H3 protein and miR-29s expression among solid tumors and normal tissues. miR-29 (a, b, and c) levels were measured by quantitative RT-PCR. RNU48 level served as the endogenous control, and miR-29 levels were normalized to the mean of 18 normal tissues (*A*): 18 normal tissues (detailed in the Results), 40 neuroblastomas, 8 sarcomas, 8 brain tumors, and 8 8H9+ (positive) tumor cell lines. *B*, normal CNS tissues were normalized to liver.
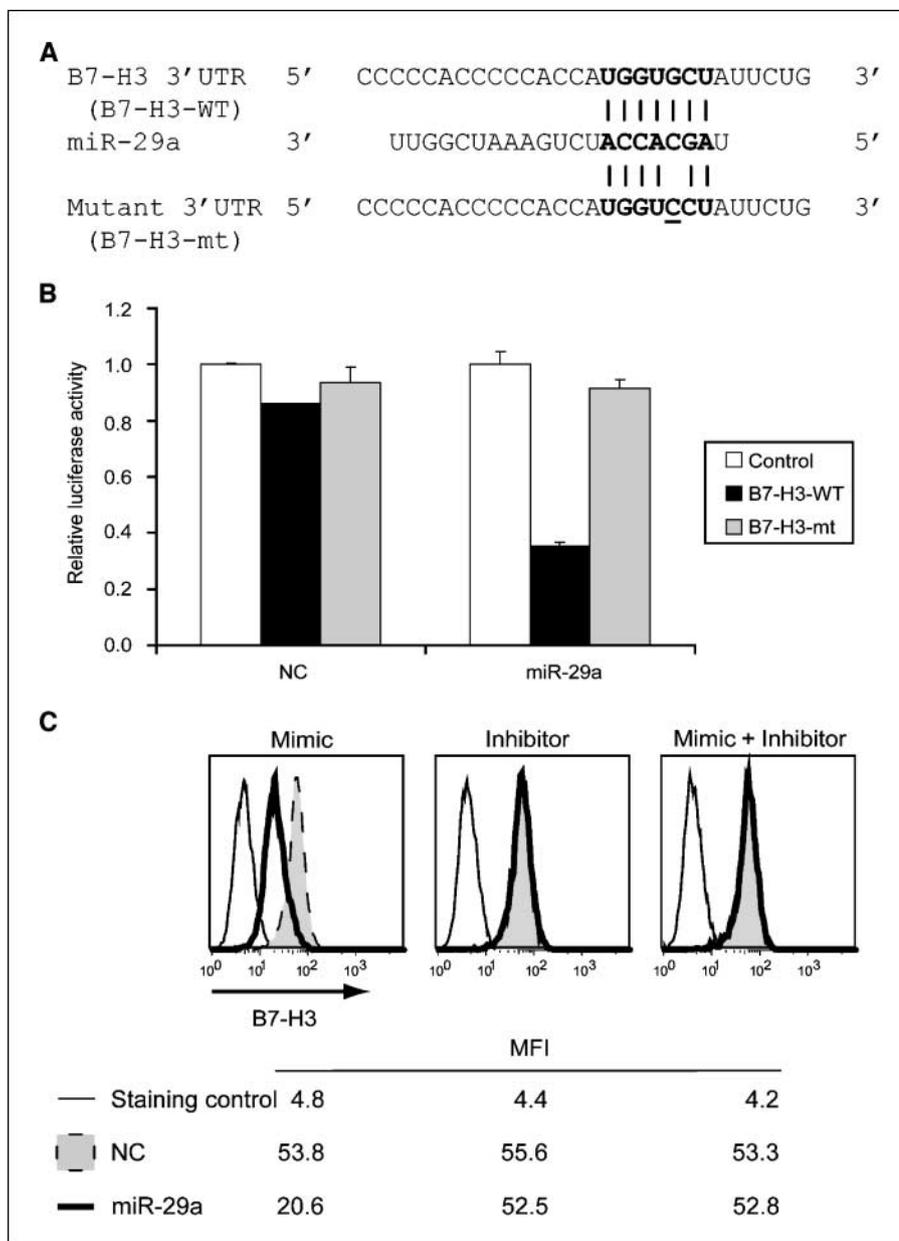
Figure 6. miR-29a directly targets B7-H3 3′UTR and down-regulates B7-H3 protein expression. *A,* alignment of miR-29a with the target site derived from B7-H3 3′UTR. Note the seed complementarity at the 5′ end of miR-29a (*boldfaced*). A single-base mutant (*underlined*) was also synthesized. *B,* luciferase activity in HeLa cells transiently cotransfected with firefly luciferase constructs and miRNA mimics. Luciferase vectors were parental (*Control*), with the B7-H3 3′UTR insert (*B7-H3-WT*), or with the mutant insert (*B7-H3-mt*). miRNA mimics were miR-29a or negative control (*NC*). Luciferase values were normalized to Control independently for negative control and miR-29a because each of these miRNAs differentially influenced the values of the renilla luciferase transfection control. *Columns,* mean; *bars,* SD. *C,* B7-H3 expression in NB1691 cells transiently transfected with miRNA mimics, inhibitors, or mimics plus inhibitors. miRNAs were miR-29a or negative control. Protein expression was measured by 8H9 immunofluorescence staining and analyzed by FACS. Representative FACS histograms with mean fluorescence intensity from three independent experiments; SD of the mean of the triplicates was <5%. A nonspecific mAb TIB114 served as the staining control.

the immune surveillance. Blockade of the inhibitory effects using mAbs like 8H9 or suppression of B7-H3 protein expression using miRNAs like miR-29 (discussed below) could potentially enhance immune response to tumors.

The discrepancy between the ubiquitous expression at the mRNA level (Fig. 3) versus the differential expression (tumor compared with normal tissues) at the protein level (Fig. 4) suggests a posttranscriptional control. The regulation of protein expression is complex. Protein turnover through the ubiquitination pathway is a classic mechanism for protein modulation. However, after binding to 8H9, B7-H3 was not significantly modulated even after 48 hours of incubation at 37°C (18). Aberrant glycosylation of cell-surface antigens cannot be the reason because the peptide-specific (not conformation dependent) antibody MAB1027 still detected the large differential between tumor and normal tissues, which did not change after *N*-glycanase treatment (data not shown).

An emerging paradigm for protein regulation is through miRNA (14). miR-29, although not been studied as extensively as some other miRNAs, is known to be down-regulated in chronic lymphocytic leukemia, cholangiocarcinoma, lung cancer, and rhabdomyosarcoma (23–26). Our study extended these findings of the down-regulation of miR-29 family to a broad spectrum of solid tumors, including neuroblastoma. Moreover, this down-regulation was inversely correlated with high protein expression of B7-H3, an immune modulator that seems to be overexpressed in many human solid tumors including neuroblastoma, sarcomas, brain tumors, and many carcinomas including breast cancer, non–small-cell lung cancer, prostate cancer, clear cell renal cell carcinoma, and urothelial cell carcinoma (6–10, 18, 27). The interaction between miR-29 and B7-H3 is direct, although only about half of the protein expression was repressed by miR-29 overexpression in our *in vitro* assays (Fig. 6B and C). This is probably due to the fact that only a single conserved miR-29 binding site is in B7-H3 3′UTR

(predicted by the software), and multiple miRNA binding sites are probably required for efficient translational repression in most cases (28). TargetScan software also predicted dozens of poorly conserved sites for the conserved miRNA families, plus sites for poorly conserved miRNA families in the human B7-H3 3′UTR. At this point, we cannot rule out the possibility of involvement of other miRNA families in the regulation of B7-H3 expression, alone or in cooperation with miR-29 family. The fact that there was near absence of B7-H3 protein (e.g., in spinal cord; Fig. 4) when only a small increase in miR-29 expression was found (Fig. 5*B*) implicates the involvement of other miRNAs and/or regulatory factors in the control of B7-H3 expression, possibly in a tissue-specific manner. One can speculate that these other miRNAs are also aberrant in tumor cells when compared with normal tissues. In fact, other miRNA families (miR-9, 34a, 125a/b, and 184) have been shown to be involved in the control of proliferation, differentiation, and apoptosis of human neuroblastoma (29–31). However, it does seem that because miR-29 binding site on B7-H3 is conserved in evolution, it probably plays a critical role in determining the differential protein expression of B7-H3 in tumor tissues versus normal tissues. Recent studies of NF-κB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma (26) suggest that miR-29 may act as a tumor suppressor through its promyogenic function. An additional role of miR-29 as "tumor suppressor" *in vivo* may be modulation of B7-H3 protein expression and engaging tumor surveillance by NK cells and T cells.

In summary, it is worth noting that this report is the first demonstration of miR-29 modulation of an immunomodulatory molecule. Whether up-regulation of miR-29 can sensitize B7-H3–overexpressing tumor cells to NK/T-cell killing will need to be investigated. Nevertheless, modulating B7-H3 protein expression by miR-29 should improve the therapeutic potential of mAbs like 8H9. Although most normal tissues were negative for 8H9 staining, we did see positive staining in the liver. In patient imaging studies, moderate uptake of 8H9 in the liver was observed (NCT00582608).[2] Our *in vitro* studies suggest that suppressing B7-H3 protein expression in the liver may be possible by increasing endogenous miR-29 or by administering miR-29 mimics, thereby improving the therapeutic index of mAb targeting to B7-H3.

Disclosure of Potential Conflicts of Interest

Acknowledgments

[2] http://clinicaltrials.gov/ct2/show/NCT00582608?term=8H9&rank=1

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