Frequent Overexpression of HMGA2 in Human Atypical Teratoid/Rhabdoid Tumor and Its Correlation with let-7a3/let-7b miRNA

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Abstract

Purpose: Atypical teratoid/rhabdoid tumors (AT/RT) are highly aggressive pediatric malignancies characterized by biallelic inactivation of the SMARCB1 tumor suppressor gene. We searched for novel genomic aberrations by investigating the copy number and expression alterations of let-7a3/let-7b microRNA (miRNA) and correlated these with expression of high-mobility group A-type hook 2 (HMGA2) oncoprotein, a target of let-7 miRNA family, in 18 AT/RT samples to elucidate potential roles of HMGA2 in the pathogenesis of AT/RT.

Experimental Design: Genomic aberrations, let-7a3/let-7b miRNA and HMGA2 expression in AT/RT tissues were identified using quantitative PCR, reverse transcription PCR (RT-PCR), and immunohistochemistry. The impact of let-7b miRNA on HMGA2 expression and the malignant potential of human rhabdoid tumor cell G401 (SMARCB1−/−) were investigated by antisense inhibition and ectopic overexpression studies.

Results: The copy number of let-7a3/let-7b miRNA was substantially decreased in 4 of 11 AT/RT samples. A significantly inverse correlation between let-7a3/let-7b miRNA expression and HMGA2 mRNA expression was observed in AT/RT tissues (R = −0.34; P < 0.05). Immunohistochemistry analysis demonstrated that HMGA2 was highly overexpressed in 83.3% (15 of 18) of AT/RT tissues. Restoration of let-7 mRNA or knockdown of HMGA2 oncoprotein significantly suppressed proliferation and colony formation, and almost abolished the invasive potential of G401 cells.

Conclusion: Reduction of let-7a3/let-7b miRNA may be one of mechanisms leading to overexpression of HMGA2 in AT/RT tissues. HMGA2 oncoprotein plays critical roles in the pathogenesis of AT/RT development; and reconstitution of let-7 miRNA or knockdown of HMGA2 oncoprotein may provide a novel therapeutic strategy for the treatment of patients with AT/RT.

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Translational Relevance

Atypical teratoid/rhabdoid tumors (AT/RT) are highly aggressive pediatric malignancies characterized by biallelic inactivation of the SMARCB1 tumor suppressor. Additional pathogenic mechanisms underlying AT/RT development for targeted therapeutics are poorly understood. Here, we report that besides inactivation of SMARCB1 tumor suppressor the frequent overexpression of high-mobility group AT-hook 2 (HMGA2) oncoprotein in AT/RT tissues, which may be partially caused by loss of let-7a3 and let-7b tumor suppressor miRNA, also plays a critical role in the pathogenesis of AT/RT development. HMGA2 oncoprotein plays essential roles in tumorigenesis and is generally regarded as a potential therapeutic target for various human cancers. Therefore, identification of the dysregulation of let-7 miRNA and overexpression of HMGA2 oncoprotein in AT/RT tissues may be beneficial in leading to novel targeted therapeutics against AT/RT.

Younger than 3 years was 11%; the overall survival rate was 17% (1, 8). Recently, several clinical studies demonstrated that an intensive multimodality regimen usually used in other CNS neoplasms significantly improved the prognosis of patients with AT/RT (9–12). For example, Chi and colleagues reported that the 2-year progression-free and overall survival rates for newly diagnosed patients with CNS AT/RT treated with the aggressive multimodality approach were about 53% and 70%, respectively (11). Despite favorable outcomes of aggressive treatment, most patients with AT/RT still suffered rapid disease recurrence and death owing to progression (11). Identification of additional genetic alterations will not only shed light on the pathogenetic mechanisms underlying AT/RT development, but also potentially lead to novel therapeutic approaches. Unfortunately, genome-wide analysis thus far has not uncovered consistent genetic alterations, and loss of SMARCB1 remains essentially the sole recurrent event (3, 13, 14). Therefore, efforts are still needed to develop novel therapeutic approaches for AT/RT treatment.

The role of let-7 tumor suppressor microRNA (miRNA) in cancer was first demonstrated by Johnson and colleagues, who found that the let-7 miRNA family negatively regulates let-60/RAS in C. elegans by binding to multiple let-7 complementary sites in the 3’ untranslated region (3’-UTR; ref. 15). It has been revealed that let-7 functions as a tumor suppressor through negatively regulating various proto-oncogenes, such as Ras and high-mobility mobility group AT-hook 2 (HMGA2) oncoprotein in a variety of human tumors (16, 17). Consistent with this, let-7 miRNA is rarely detectable in embryonic stages but increases in differentiated and mature tissues (18). In human cancers, significantly reduced expression of the let-7 miRNA family is associated with poor survival of patients (19). As an important regulator of cell growth, differentiation, apoptosis, and transformation, HMGA2 interacts with many different transcription factors such as E2F1 to influence the expression of numerous genes (20). Recently, we also found that HMGA2 may promote tumorigenesis through modulation of human telomerase reverse transcriptase (hTERT) transcriptional activity (21). Multiple studies have identified a negative correlation between reduced expression of the let-7 miRNA family and overexpression of Ras/HMGA2 in various human cancer tissues (22–24). Because HMGA2 plays an oncogenic role in neoplastic transformation, it has been generally considered as a potential target for anticancer therapeutics (19, 25).

In the present study, we initially investigated the whole exome of an AT/RT tumor sample from an adult patient. Using whole-exome sequencing by coupling Agilent whole exome to the Illumina DNA sequencing platform, we identified a copy number decrease in the genomic locus harboring let-7a3/let-7b miRNA. We further identified overexpression of HMGA2 oncoprotein in the AT/RT tissue. Deletion of the genomic locus harboring the let-7 miRNA family has been described as a frequent genetic alteration in various human cancers (26). For example, deletion of let-7a3/let-7b miRNA has been described in 44.1% of ovarian cancer specimens (27). Considering the critical roles of let-7 and HMGA2 oncoprotein in human cancers, we further characterized the status of let-7a3/let-7b miRNA and correlated this to HMGA2 oncoprotein expression in 18 AT/RT tissues. We have for the first time identified reduction of let-7a3/let-7b miRNA and overexpression of HMGA2 oncoprotein in AT/RT tissues, and our results suggest that reconstitution of let-7 expression may provide a novel therapeutic strategy for the treatment of patients with AT/RT.

Materials and Methods

Tumor specimens and DNA and RNA preparation

Four CNS AT/RT samples were from patients at the City of Hope National Medical Center (Duarte, CA), and 14 CNS AT/RT samples were from Taipei Veterans General Hospital, Taipei, Taiwan. Sixteen of the samples were from children younger than 6 years of age, only two were from adults. All clinically diagnosed AT/RT were further confirmed by negative SMARCB1 immunostaining. All tumor specimens were formalin-fixed, paraffin-embedded (FFPE) tissues. DNA was isolated from FFPE samples using the QIAamp FFPE DNA Mini Kit (Qiagen) according to the user manual. Saliva of 1 adult patient was collected into an Oragene DNA tube (DNA Genotek) for genomic DNA extraction to provide a normal control for exome sequencing.

Illumina Genome Analyzer (Solexa) library preparation, exome capture sequencing, alignment, various calling, and annotation

Three micrograms of genomic DNA was sheared by sonication using a Bioruptor (Diagenode). The resultant 150- to 250-bp fragmented DNA was end-repaired and ligated to Illumina adaptor oligonucleotides. Ligation products were purified and successfully ligated fragments were amplified...
with a 12-cycle PCR. The enriched PCR products were subject to the exome capture procedure using the SureSelect Human All Exon v4 Target Enrichment Kit (solution magnetic bead capture) according to manufacturer’s protocols (Agilent Technologies, Inc.). The captured products were then used for cluster generation and sequencing by synthesis using the Illumina Genome Analyzer Ix (Illumina). Image analysis and base calling were performed using Illumina’s default pipeline. The sequences were aligned to the human genome reference sequence (NCBI36) using Bowtie 0.12.7 (28). All subsequent analyses were done using customized R scripts. The average coverage of each exon defined in the University of California Santa Cruz (UCSC) consensus coding DNA sequence (CCDS) database was calculated based on the alignment result. The log2 coverage ratio of each exon was calculated for tumor versus normal tissue and median centered. Log ratios were smoothed by DNAcopy 25 using default values, and copy number variation (CNV) was detected by DNAcopy’s circular binary segmentation algorithm (29). Coverage difference between tumor and saliva for each exon was represented as the log2 ratio between their coverage with an offset of 1 (30). Only regions containing more than two consecutive exons and with log2 ratio of more than or less than 0.7 were considered significant. The individual single-nucleotide polymorphisms (SNP) were obtained through subtraction of the single nucleotide polymorphism database (dbSNP) and 1000 genomes first. SNPs present in saliva were further filtered out from SNPs in the tumor genome, and the remaining SNPs were assessed as mutations of AT/RT tissue.

Quantitative real-time PCR and quantitative reverse transcription PCR

Total RNAs from cells were extracted using TRizol total RNA isolation reagent (Invitrogen) according to the manufacturer’s protocol. To extract total RNA/miRNA from FFPE tissues, the QIAamp FFPE miRNA Mini Kit (Qiagen) was used, following the manufacturer’s instructions. cDNA was synthesized from 1.0 μg of total RNA using the SuperScript III first-strand cDNA synthesis Kit (Invitrogen). cDNA for small nucleolar and miRNA let-7a3 (Tm-378) and let-7b (Tm-378) was synthesized from 100 ng of total RNA using gene-specific primers according to the TaqMan miRNA Reverse Transcription Kit protocol (Applied Biosystems). Quantitative real-time PCR (qPCR) was carried out in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix to measure copy number of HMGA2, SMARCB1, let-7a3 and let-7b, and using TaqMan Real-Time PCR Master Mix (Applied Biosystems) to quantitatively measure mRNA and miRNA expression. β-globin and PEG6B genes were used as internal control for genomic analysis, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and small nuclear RNA RNU-48 were used as internal controls for mRNA and miRNA expression, respectively. Each qPCR experiment was performed three times. Data were presented as the relative quantity of targets, normalized with respect to internal control, or relative to a calibrator sample.

Mutation analysis

Oligonucleotide primers for exons 1–9 were designed from the intron/exon boundary sequence of the SMARCB1 gene (GenBank accession Number. NG_009303). Primer sequences are available upon request. The DNA samples extracted from tumors and adjacent normal sections producing a PCR product for individual exons were analyzed by direct sequencing. The sequences of individual exons were compared with the reference sequences in Hg18.

FISH assay

Dual-color FISH was performed using the FISH-mapped confirmed the bacterial artificial chromosome (BAC) probes; RP11-124F9 (chromosome 22q11.23) for SMARCB1 and RP11-462A13 (chromosome 12q13.4) for HMGA2 labeled in spectrum orange (Abbott Laboratories); and CEP12, CEP22 for chromosome 12, 22 centromere labeled in spectrum green (Abbott Laboratories) were selected for FISH assays. FISH assays were done on unstained FFPE slides from AT/RT tissues according to the method we previously reported (31). Two red and two green signals were considered normal in a diploid genome. Due to truncation and overlapping cells, at least 20% of 200 interphase cells must show an abnormal pattern to be considered abnormal.

Immunohistochemistry and Western blot

The antibodies against GAPDH and SMARCB1 were obtained from Sigma-Aldrich and Cell Signaling Technology, Inc., respectively. The anti-HMGA2 antibody was kindly provided by Dr. H. Helen Lin at City of Hope Medical Center. Immunohistochemical studies of SMARCB1 were done on FFPE tissues using the avidin–biotin–peroxidase method. After deparaffinization and blocking, the antigen–antibody reaction was carried out overnight at 4°C. The LSAB2 Kit (Dako) was applied to detect the signal of the SMARCB1 and HMGA2 antigen–antibody reaction (31). Western blot was performed routinely using ECL enhancer reagents.

Cell culture, proliferation assay, and soft agar assay

The human rhabdoid tumor of the kidney G401 cell line (ATCC CRL-1441) purchased from American Type Culture Collection (ATCC) was cultured in McCoy’s 5a Medium (ATCC) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cell line was newly DNA fingerprinted to confirm identity as previously described (32). Cells were seeded in 96-well plates in 10 replicates at densities of 5 × 10³ cells per well. Cell proliferation was monitored at 24, 48, and 72 hours using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (CellTiter 96 Aqueous Assay; Promega). For colony formation assays, 1 × 10³ cells were mixed in 0.35% top agar diluted in 2 × McCoy’s 5a Medium containing 20% FBS before plating onto 0.8% bottom agar in 6-multiwell plates. After 15 to 20 days, cells were stained (4–6 hours, 37°C) with 0.2 mg/mL of MTT (Sigma-Aldrich). Colonies > 0.2 mm in diameter were counted. The number of colonies was determined by analyzing 5 fields per well from 3 wells. All cultures were performed in triplicate.
miRNA/siRNA transient transfection and stable short hairpin RNA transfection

Individual miRNA, siRNA, or control small RNA (smRNA, random 22-mer RNA purchased from Invitrogen) and anti-mir-let-7 miRNA inhibitor (a chemically modified, single-stranded oligonucleotide) was used at a concentration of 60 pmol/well for 1 x 10^4/well cells in a 6-well plate with Lipofectamine RNAiMAX (Invitrogen). After transfection, cells were harvested and analyzed at the indicated times. Human let-7 miRNA (Cat. # 4464066) and HMGA2 siRNA (Cat. # 4392420) were purchased from Life Technologies, and their efficacy of HMGA2 knockdown was tested by Western blot analysis. Human let-7 miRNA (LentiMiRa-GFP-hsa-let-7 Vector, mh10004) and HMGA2 short hairpin RNA (shRNA) in pLenti-siRNA-GFP (1009810b) were purchased from Applied Biological Materials Inc.. Lentiviral production in plLenti-siRNA-GFP (I009810b) were purchased from Invitrogen. For stable infection, 8 human embryonic kidney (HEK) 293T cells was as we previously described (21). For stable infection, 8 x 10^4 cells were plated in each well of 6-well plates. After overnight incubation, 50 µL of concentrated lentiviral particles were added with 12 µg/mL polybrene to each well. Forty-eight hours later, fresh medium containing 2 µg/mL puromycin was added to each well. Fresh medium containing puromycin was replaced every 3 to 4 days. Single colonies were obtained after 2 weeks of puromycin selection. Substantial knockdown of HMGA2 protein was tested by Western blot analysis compared with the control lentivirus transfected G401 cells.

Matrigel invasion assay

Test cells (5 x 10^4) in serum-free medium were plated in the Matrigel-coated inserts of a 24-well plate (8-µm pores; BD) and medium with 10% FBS was added into the bottom in accordance with the manufacturer’s protocol. After 24 hours, cells that migrated through and attached to the bottom membrane were fixed and stained with the Diff-Quick kit (Fisher Scientific). Invading cells in three different fields were counted under a light microscope of x100 magnification. Three replicate experiments were performed.

Statistical analysis

Data were collected using an MS Excel spreadsheet. Group comparisons for continuous data were done with the Student t test for independent means or two-way ANOVA. Statistical significance was set at P < 0.05.

Results

Identification of genetic abnormalities of an AT/RT genomic DNA by whole-exome sequencing

We initially adopted whole-exome sequencing to investigate genetic alterations in an AT/RT tissue of a 19-year-old female. Due to the limited amount of genomic DNA from adjacent nontumor partial sections, genomic DNA from saliva was used as reference. Data analysis demonstrated that the sequencing quality for genomic DNA from saliva and FFPE AT/RT tissue was highly comparable (Supplementary Figs. S1 and S2). CNV analysis revealed that copy number of two medium-sized DNA regions on chromosome 22 was dramatically decreased (log2 ratio of less than 0.7; Fig. 1A). The first region located on chromosome 22q11 contains the featured SMARCB1 gene, and the second region includes let-7a3 and let-7b tumor suppressor miRNA. The detailed CNVs of chromosome 22 and locations of SMARCB1 and let-7a3 and let-7b are shown in Fig. 1B. We selected several genes with different locations for qPCR validation using genomic DNA of adjacent nontumor tissue in the section as reference material to get more accurate data. As shown in Fig. 1B, only the copy number of the genes, which are located at the first region (SMARCB1 and IGLL1) or at the second region (let-7a3, let-7b, LIF, and PPARG), and not those of genes outside the two regions (PDE6B located on chromosome 4, CP1BB, MYH9, GGT1, and NAGA on chromosome 22) were dramatically decreased. Results of gene copy number analysis indicated that the AT/RT tissue harbored a homozygous deletion of region 1, and a heterozygous deletion of region 2.

Tumor-specific mutations may provide essential translational genetic information for personalized cancer therapy. We did not find mutations or alterations in copy number of the common, broadly mutated genes such as p53, PIK3CA, K-Ras, and B-Raf in the AT/RT tissue. Similarly, a recent study showed the absence of mutations in canonical pathways critical for development and progression of adult cancers (33). By Sanger DNA sequencing, novel nonsynonymous point mutations including cancer-related genes such as DBF4, PMS1, RASSF4, Rap2b, and Tie2 were identified (Supplementary Fig. S3).

Inactivation of SMARCB1 tumor suppressor in AT/RT tissues

Loss of SMARCB1 protein is generally accepted as the diagnostic characteristic of AT/RT, and may be the result of varied genetic alterations in AT/RT (3). We further investigated genetic alterations of SMARCB1 gene by FISH and exon sequencing in 18 AT/RT tissues. FISH analysis showed that 7 AT/RT tissues harbored homozygous deletion of SMARCB1, and 5 AT/RT tissues contained heterozygous deletion of SMARCB1, whereas large deletions of regions of chromosome 22, including loss of the entire chromosome 22 in one case was discovered in three cases. Information about patients’ age and SMARCB1 FISH and immunohistochemistry (IHC) is shown in Supplementary Table S1. Genetic alterations leading to loss of SMARCB1 protein in another three AT/RT cases were not identified by FISH or exome sequencing.

Four typical AT/RT tissues (labeled as AT/RT-1, -2, -3, -4) are shown in Fig. 2. The first AT/RT tissue (AT/RT-1) was from a 19-year-old female, and other three were from children less than 10 years old. Hematoxylin/eosin staining revealed that tumor cells were morphologically highly heterogeneous (Fig. 2A). Double-color interphase FISH showed that AT/RT-1 harbored an intratumor homogeneous homozygous loss of SMARCB1 signal (0 red signal of SMARCB1 with 2 green signals of chromosome 22 centromere; Fig. 2B). AT/RT-2 harbored a heterozygous loss...
Let-7 miRNA and HMGA2 in Atypical Teratoid/Rhabdoid Tumor

Figure 1. Identification of deletion of SMARCB1 and let-7a3/let-7b miRNA in an adult AT/RT by whole-exome DNA sequencing. A, relative CNV plot of chromosome 22. SMARCB1, let-7a3, and let-7b were located at the first and second medium-sized deletions, respectively; arrows indicate their location. B, qPCR analysis to validate the loss of SMARCB1, miRNA let-7a3, and let-7b in the genome of tumor tissue. The copy number of each selected gene was first normalized to β-globin (on chromosome 11) and results presented as the ratio to the adjacent normal tissue copy number. SMARCB1-E2 and SMARCB1-E6 represent PCR amplicons for exon-2 and exon-6 of SMARCB1, IGLL1-E3, and IGLL1-E5 for exon 3 and 5 of IGLL1 (IGLL1; immunoglobulin λ-like polypeptide 1) gene. Let-7a3, let-7b, LIF (leukemia inhibitory factor), and PPARa (peroxisome proliferation activated receptor α) were selected as representatives of the second deletion. The copy number of HMGA2 of chromosome 12 and PDE6B (phosphodiesterase 6B) of chromosome 4 (unchanged-1) and genes outside the two deletions of chromosome 22 (unchanged-2); GP1BB (platelet glycoprotein 1b, β polypeptide), MYH9 (nonmuscle myosin heavy chain 9), GGT1 (γ glutamyltransferase 1), and NAGA (N-acetyl-N-galactosaminidase) were unchanged in the AT/RT tissue.

of SMARCB1 (one red with two green; Fig. 2B), whereas AT/RT-3 harbored a deletion of large DNA region containing SMARCB1 gene on chromosome 22 or monoallelic chromosomal 22 (one red with one green; Fig. 2B). However, AT/RT-4 displayed normal diploid SMARCB1 (Fig. 2B). For AT/RT-2 tissue with heterozygous deletion of SMARCB1, exon sequencing identified a nonsense mutation of c.197G>A, p.S66X in exon 2 (Fig. 2C), resulting in a truncated nonfunctional protein. For AT/RT-3, exon sequencing found an insertion of c.531_532insG, p.H177fs in exon 5 (Fig. 2C). However, no mutations were found in the AT/RT-4, and its genetic alterations are still unknown. It is also worthwhile pointing out that the technologies we used in the study would miss single/multiple exon deletions and copy number neutral LOH. Loss of nuclear immunostaining of SMARCB1 protein was found in all 4 AT/RT tissues, and the endothelial cells (AT/RT-1) and infiltrating lymphoid cells (AT/RT-2) were positively stained for the SMARCB1 protein (Fig. 2D).

Frequent overexpression of HMGA2 oncprotein and its correlation with let-7a3/let-7b in AT/RT tissues

The miRNA let-7 family is reported to negatively regulate HMGA2 expression in a variety of human cancers (16, 17). To reveal the correlation of let-7a3/let-7b with HMGA2 expression, we first examined HMGA2 oncprotein expression in the 18 AT/RT tissues. Based on the percentage of HMGA2-stained tumor cells, the levels of HMGA2 oncprotein in AT/RT tissues were scored on a scale of 0 to 3 (0 for <5% tumor cells; 1 for 5%–25% tumor cells; 2 for 25%–50% tumor cells; 3 for >50% tumor cells with positive HMGA2 nuclear staining). Figure 3A shows four levels of HMGA2 oncprotein expression in AT/RT tissues that were scored as 0 to 3, respectively. Quantitative analysis showed that HMGA2 oncprotein expression in 16.7% (3/18), 16.7% (3/18), and 50% (9/18) of examined AT/RT tissues was scored as 0, 1, 2, and 3, respectively (Fig. 3B). The expression score of HMGA2 oncprotein in each AT/RT tissue is also presented in Supplementary Table S1. The results indicate that overexpression of HMGA2 oncprotein may be a frequent event in AT/RT tissues.

To further investigate copy number alteration of let-7a3/let-7b miRNA; and whether overexpression of HMGA2 was related to gene amplification, we measured the gene copy number of let-7a3/let-7b and HMGA2 using qPCR analysis on genomic DNA extracted from 11 AT/RT tissues that had paired nontumor partial sections. The qPCR analysis showed that the copy number of let-7a3/let-7b decreased by at least 50% in 4 of 11 AT/RT tissues examined (Fig. 3C), whereas the average HMGA2 gene copy number did not
change (Fig. 3C). Consistent with this, FISH analysis further confirmed that HMGA2 was not amplified in any of the AT/RT tissues. IHC showed overexpression of HMGA2 oncoprotein (Fig. 3D, left); however, FISH analysis displayed normal diploid HMGA2 (Fig. 3D, right) in two representative AT/RT tissues. By quantitative reverse transcription PCR (qRT-PCR) analyses, HMGA2 mRNA was significantly higher, whereas let-7a3/let-7b was significantly lower, in AT/RT tissues than in adjacent normal tissues (Fig. 3E). Compared with let-7a3, the abundance of let-7b was much higher in AT/RT tissues. A significant inverse correlation between the expression of let-7b and HMGA2 in AT/RT tissues (N = 11) was demonstrated using the Pearson correlation coefficient analysis ($R = -0.34; P < 0.05$; Fig. 3F). In all, the above data indicated that HMGA2 overexpression in AT/RT tissues may be partially associated with the reduction in let-7a3/let-7b miRNA.

Overexpression of HMGA2 oncoprotein is not associated with inactivation of SMARCB1 tumor suppressor

The SMARCB1 complex regulates expression of many genes at the transcriptional level (7). We further questioned whether overexpression of HMGA2 was associated with the loss of SMARCB1, and whether SMARCB1 regulates the expression of HMGA2. siRNA knockdown of SMARCB1 did not affect HMGA2 expression at both the mRNA and protein levels in human fibroblast 293-T cells (Fig. 4A and B). Moreover, exogenous expression of SMARCB1 in human kidney rhabdoid tumor G401 cells harboring a homozygous SMARCB1 deletion did not suppress either HMGA2 mRNA or protein levels (Fig. 4C and D). These results indicate that the inactivation of SMARCB1 may not directly induce the overexpression of HMGA2 oncoprotein in the AT/RT tissues, and instead may be two independent pathologic events in AT/RT development.

Overexpression of let-7b or knockdown of HMGA2 significantly suppressed proliferation, and colony formation of rhabdoid tumor cell line G401

To assess the biologic significance of let-7/HMGA2 in AT/RT development, we examined the impact of overexpression of let-7b or knockdown of HMGA2 oncoprotein on cellular proliferation and colony formation of rhabdoid tumor G401 cells. Both let-7b miRNA and HMGA2-specific siRNA efficiently decreased HMGA2 mRNA (Fig. 5A) and HMGA2 protein (Fig. 5B) in G401 cells at 72 hours after transfection. Compared with control smRNA, let-7b miRNA and HMGA2 siRNA significantly suppressed proliferation of G401 cells. Suppression of cell proliferation was slightly stronger with let-7b miRNA than with HMGA2 siRNA, but not significantly different, indicating let-7b miRNA may function largely
through repression of HMGA2 expression (Fig. 5C). Consistent with this, we also observed that let-7 inhibitor significantly promoted proliferation of G401 cells, accompanied by upregulation of HMGA2 protein (Supplementary Fig. S4A). Importantly, compared with G401 cells transduced with control lentivirus, the number and size of colonies formed by G401 cells transduced with lentivirus expressing let-7 miRNA or HMGA2 shRNA was dramatically lower (Fig. 5D). Conversely, overexpression of HMGA2 enhanced colony formation by G401 cells (Supplementary Fig. S4B), supporting the notion that HMGA2 oncoprotein plays a critical role in the anchorage-independent growth of G401 cells.

Overexpression of let-7b or knockdown of HMGA2 significantly decreased invasive potential of rhabdoid tumor cell line G401

We also examined the impact of let-7 and HMGA2 on the invasive potential of G401 cells. G401 rhabdoid tumor cells were first transfected with control smRNA, let-7b miRNA, or
HMGA2 siRNA 24 hours before subjecting equal numbers of each modified cells to Matrigel invasion assay. Figure 6A shows representative photomicrographs of cells that invaded the Matrigel membrane. Quantitative analysis of invaded cells revealed that overexpression of let-7b or knockdown of HMGA2 almost abolished the invasive potential of G401 cells (Fig. 6B), implying that HMGA2 also plays an indispensable role in the invasion of rhabdoid tumor cells.

Discussion

Due to the highly aggressive nature of the tumor and high rate of dissemination at diagnosis, intensive multimodality treatment including surgery, radiation, and chemotherapy has been used to treat this disease, and proven to result in more favorable outcomes (9–12). However, most children die of progressive disseminated disease, with or without local recurrence, despite aggressive therapy, and the general outcome for patients with AT/RT is still dismal (8, 12). Mean survival with surgical intervention alone is 3 months and with adjuvant chemotherapy and radiotherapy is about 8 months (8, 11). Therefore, more effective therapies are desperately needed.

In the present study, we originally aimed at interrogating the genomic abnormalities in an adult AT/RT tissue, due to its extreme rarity, using state-of-the-art whole-exome DNA sequencing technology. We unexpectedly identified a copy number decrease in the genomic locus harboring let-7a3/let-7b miRNA. Because the 3’-UTR of HMGA2 has seven conserved sites complementary to the let-7 miRNA family (16, 17), we then extended our studies to examine the status and correlation of let-7a3/let-7b with HMGA2 in 2 adult and 16 pediatric AT/RT tissues, and report herein the identification of HMGA2 oncoprotein overexpression in both adult and pediatric AT/RT tissues.

The oncogenic properties of HMGA2 are known to be involved in tumor cell differentiation, transformation (16, 17), aggressive tumor growth (24, 34), early metastasis (35, 36), and DNA damage response (37). HMGA2 has also been found to participate in the epithelial-to-mesenchymal transition, an essential process important to both normal development and tumor progression (38, 39). Most recently, a study of genetically engineered mouse models of human lung adenocarcinoma showed that tumors are initiated synchronously by deletion of p53; however, only a subset with overexpressed HMGA2 due to inactivation of NK2-related homeobox transcription factor NKX2-1 became malignant and metastatic (40). These authors also observed that the expression of HMGA2 was dominant in the more widely and poorly differentiated human adenocarcinoma groups, supporting our notion that HMGA2 participates in dedifferentiation and disease progression of AT/RT. Because

Figure 4. Lack of association between overexpression of HMGA2 and inactivation of SMARCB1. A, HMGA2 mRNA expression assessed by qRT-PCR in G401 cells transfected with SMARCB1 or control siRNA. As expected SMARCB1 mRNA was inhibited by SMARCB1 siRNA (*, P < 0.01, compared with control). B, Western blot of protein levels in human fibroblast 293T cells transfected with either control or SMARCB1 siRNA. Reconstitution of SMARCB1 did not suppress HMGA2 expression at both the mRNA (qRT-PCR; #, >1,000) and protein (D) levels (Western blot) in G401 cells transfected with control and SMARCB1 expression plasmids.

Figure 5. Impact of let-7 miRNA overexpression and HMGA2 downregulation on cellular proliferation and colony formation in G401 cells. Let-7b miRNA and HMGA2 siRNA significantly decreased HMGA2 expression at both the mRNA A and protein B levels in G401 cells, HMGA2 mRNA was measured by qRT-PCR assay at 24, 48, and 72 hours after transfection; HMGA2 protein was extracted at 72 hours after transfection. C, cell proliferation assay of G401 cells transfected with equal amounts of either control smRNA, or let-7 miRNA, or HMGA2 siRNA. Shown are the cell proliferation index for each measured by MTS assay at 24, 48, and 72 hours after transfection (#, P < 0.05, compared with control smRNA). D, colony formation assay of G401 cells in soft agar. Let-7b miRNA overexpression or HMGA2 downregulation significantly decreased colony formation (**, P < 0.01, colony number of HMGA2 siRNA/let-7 miRNA compared with that of control).
brain invasion and widespread dissemination along cerebrospinal fluid pathways is a hallmark of CNS malignant rhabdoid tumors (41), our results also suggest HMGA2 a potential therapeutic target to suppress invasion of AT/RT.

Furthermore, we identified that HMGA2 mRNA was inversely correlated with let-7 miRNA in AT/RT tissues. Therefore, downregulation of let-7 miRNA may partially be ascribed to the decrease in let-7a3/let-7b miRNA copy number in at least a portion of AT/RT samples. HMGA2 is derepressed upon inhibition of let-7 in cells with high levels of the miRNA (22–24). miRNA deregulation, including miRNA gene deletion has been reported in several tumor types including ovarian cancer, breast cancer, and melanoma (26, 42). In epithelial ovarian cancer, deletion of miRNA let-7a3/let-7b was found in 44.1% of cases, suggesting the locus may be a fragile site (26, 42). Several studies point to the importance of the HMGA2/let-7 miRNA axis in tumor biology and the promise HMGA2 has as a therapeutic target (18, 19, 25). Consistent with these results, we showed that overexpression of let-7 miRNA or knockdown of HMGA2 expression significantly suppressed the in vitro growth of G401 rhabdoid tumor cells. Of note, let-7 miRNAs have many other targets besides HMGA2, and overexpression of let-7 miRNA could potentially have effects on other targets that also contribute to the antiproliferative effects of let-7 miRNA.

Identification of additional genetic alterations will further our understanding of the biology of AT/RT, also potentially lead to novel therapeutic strategies. However, several studies of genome-wide analysis thus far have yielded no consistent genetic alterations besides inactivation of SMARCB1 (3, 13, 14). Another recent study also demonstrated that besides a single activating mutation in N-RAS in a case of AT/RT, no other mutations in canonical pathways critical for development and progression of adult cancers were identified in a total of 25 pediatric patients with AT/RT, further implicating that distinct mechanisms might drive these highly malignant pediatric tumors (14, 33). Our whole-exome sequencing analysis also failed to identify mutations in the usual cancer-related genes, such as p53, PIK3CA, K-Ras, and B-Raf in the adult patient with AT/RT. However, mutations of DNA repair pathway genes such as DBF4 and PMS1 were found in this AT/RT tissue, and these genes may deserve further study to determine whether they are involved in AT/RT development. Although in silico analysis was not performed on these mutations identified in the AT/RT tissue, it is highly desirable to use the method to more systematically and accurately analyze and interpret these genetic alterations, after more data are collected in future. It is also important to note that genetic alterations in adult AT/RTs are likely to be different than in pediatric tumors, because somatic alterations can accumulate over time in late-stage adult AT/RTs.

In summary, we have uncovered a high frequency of upregulation of HMGA2 oncoprotein in AT/RT tissues, which may be partially contributed to by deletion of let-7a3 and let-7b miRNA. A significant inverse correlation

Figure 6. Impact of let-7 miRNA overexpression and HMGA2 downregulation on cellular invasive potential of G401 cells. G401 cells were transfected with either control smRNA, let-7b miRNA, or HMGA2 siRNA 24 hours before subjecting the same number of each modified cells to Matrigel invasion assay for 24 hours. A, representative photomicrographs. B, quantitative analysis of invaded cells presented as mean ± SD of 3 replicates per cell type (**; P < 0.01, compared with control smRNA).
between HMGA2 mRNA and let-7b miRNA was observed. Moreover, we have demonstrated that overexpression of let-7 miRNA or knockdown of HMGA2 can significantly reduce rhabdoid tumor cell proliferation, colony formation, and invasion. Our results suggest that reconstitution of let-7 expression or knockdown of HMGA2 may provide a novel therapeutic strategy for the treatment of patients with AT/RT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Frequent Overexpression of *HMGA2* in Human Atypical Teratoid/Rhabdoid Tumor and Its Correlation with *let-7a3/let-7b* miRNA

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