

RESEARCH ARTICLE

Aberrant DNA Methylation and Epigenetic Inactivation of *hMSH2* Decrease Overall Survival of Acute Lymphoblastic Leukemia Patients via Modulating Cell Cycle and Apoptosis

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Abstract

Objective: Altered regulation of many transcription factors has been shown to play important roles in the development of leukemia. *hMSH2* can modulate the activity of some important transcription factors and is known to be a regulator of hematopoietic differentiation. Herein, we investigated epigenetic regulation of *hMSH2* and its influence on cell growth and overall survival of acute lymphoblastic leukemia (ALL) patients. **Methods:** *hMSH2* promoter methylation status was assessed by COBRA and pyrosequencing in 60 ALL patients and 30 healthy volunteers. mRNA and protein expression levels of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax* were determined by real time PCR and Western blotting, respectively. The influence of *hMSH2* on cell proliferation and survival was assessed in transient and stable expression systems. **Results:** mRNA and protein expression of *hMSH2* and *Bcl-2* was decreased, and that of *PCNA*, *CyclinD1* and *Bax* was increased in ALL patients as compared to healthy volunteers ($P < 0.05$). *hMSH2* was inactivated in ALL patients through promoter hypermethylation. Furthermore, *hMSH2* hypermethylation was found in relapsed ALL patients (85.7% of all cases). The median survival of patients with *hMSH2* methylation was shorter than that of patients without *hMSH2* methylation (log-rank test, $P = 0.0035$). Over-expression of *hMSH2* in cell lines resulted in a significant reduction in growth and induction of apoptosis. **Conclusions:** This study suggests that aberrant DNA methylation and epigenetic inactivation of *hMSH2* play an important role in the development of ALL through altering cell growth and survival.

Keywords: Acute lymphoblastic leukemia - *hMSH2* - DNA methylation - survival - cell apoptosis

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of leukemia in childhood and adults. While the survival rate of ALL patients has been improved obviously, nearly one quarter of children with ALL will die (Gaynon, 2005). ALL in adults affects a comparatively young population and has proved to be refractory, with the 5-year survival rate of around 40% (Plasschaert et al., 2004). ALL originates from malignant transformation of lymphocyte progenitor cells into leukemic cells of the B-cell and T-cell lineages (Pui et al., 2011). However, one of the known genetic aberrations in ALL are insufficient to induce this disease (Pui et al., 2004), suggesting that there are other genetic or epigenetic alterations in the leukemic transformation.

Mismatch repair (MMR) system is involved in the DNA damage recognition and repair. Human mutS homolog 2 (*hMSH2*) functions as a core MMR protein and usually forms hetero-dimers with protein homologs *hMSH3* or *hMSH6* (Fishel, 2001). The hetero-dimer is

fundamental for the DNA damage recognition and crucial for the stability of MMR protein homologs (Marsischky et al., 1996). Defects in MMR proteins have been found to be associated with reduced or absent benefit from 5-FU adjuvant chemotherapy in clinical trials (Ribic et al., 2003). MMR impairment appears to cause reduced incorporation of 5-FU metabolites into DNA, leading to reduced G2/M arrest and apoptosis after 5-FU treatment (Meyers et al., 2005; Hewish et al., 2010). The deficiencies of MMR genes are also associated with MNNG, 6-thioguanine and cisplatin (Fiumicino et al., 2000; Meyers et al., 2001; Loo et al., 2005). Altered expression of key regulatory transcription factors has been shown to play a critical role in the leukemia development (O'Neil and Look, 2007). Molecular biological studies have shown that *hMSH2* may be functionally relevant in the lymphoid cells and potentially ALL.

It is now clear that epigenetic alterations are as important as genetic changes in the development of cancer (Costello and Plass, 2001). Many well established tumor suppressor genes have been shown to be inactivated predominantly

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Table 1. Characteristics of ALL Samples

	BCP ALL	T-ALL	Total
Number of patients (%)	39 (65.0)	21(35.0)	60
Median age (yr)	32.2	32.5	32.4
Sex ratio (M:F)	21:18	11:10	32:28
Median WBC (10 ⁹ /L)	42.7	28.3	53.9
t(12;21)-positive (%)	16(41.0)	0(0.0)	16
Diagnostic (%)	29(74.4)	17(80.9)	46
Relapsed ALL (%)	10(25.6)	4(19.4)	14
Median follow-up (range) (months)	41(11-60)	42(12-60)	41.4(11-60)

by promoter hypermethylation and many of them linked to leukemia development act as epigenetic regulators (Slany, 2005). *hMSH2* has been found to be implicated in the DNA mismatch repair system. Inactivation of this gene due to promoter hypermethylation has been reported to be associated with some human cancers (Deguchi et al., 2003). It is reported that *hMSH2* expression correlates with allelic imbalance on the chromosome 3p in the non-small cell lung cancer (Xinarianos et al., 2000). This study aimed to investigate the epigenetic regulation of *hMSH2* on the cell growth and survival. Our results showed that *hMSH2* was inactivated by promoter hypermethylation in most of ALL. Over-expression of *hMSH2* in ALL cell lines resulted in dramatic reduction in cell growth and induced apoptosis. Overall, our findings suggest that the epigenetic inactivation of *hMSH2* plays an important role in the occurrence of ALL, probably through altering cell growth and survival.

Materials and Methods

Patients and clinical information

Bone marrow or peripheral blood samples were collected from 60 ALL adults who were included in the present study including 32 males and 28 females, with a median age of 32.4 years. All the 60 samples were taken at initial diagnosis and 14 samples at relapse. Clinical information was collected from each patient by medical record reviewing. All patients received treatment in our hospital from 2005 to 2011. The clinical characteristics of these patients are listed in Table 1. Findings in follow up were retrieved from Department of Internal Medicine, Guangzhou First People's Hospital, Guangzhou Medical University, and the last follow-up was done in October 2012. The median follow-up duration for survived patients was 41.4 months (range, 11-60 months). Bone marrow or peripheral blood was also collected from thirty healthy volunteers as controls.

All the patients were diagnosed by pathological examination of bone marrow. Results showed very high white cell counts and >95% BCR/ABL positive cells. For patients with blast crisis, peripheral blood was obtained, and leucocytes were directly separated. Assay showed 80-99% blasts.

Blood was collected into heparinized tubes before treatment and processed for assay within 24-36 h. Leukemic cells were isolated by 1.077 g/mL Ficoll-Isopaque (Pharmacia) density gradient centrifugation. The proportion of leukemic cells was estimated under a light

Table 2. Primers and Amplicon Sizes for Selected Human Genes with Quantitative Real-time PCR

Genes	Primers	Size (bp)
<i>hMSH2</i>	Forward: 5'-AAGCCAGGATGCCATTG-3' Reverse: 5'-CATTTGACACGTGAGCAAAGC-3'	126
PCNA	Forward: 5'-GCCCTCAAAGACCTCATCAA-3' Reverse: 5'-TCTGGGATCCAAGTTGCTC-3'	338
<i>CyclinD1</i>	Forward: 5'-GCACAACGCACCTTTCTTTCTTTCCA-3' Reverse: 5'-CGCAGGCTTACTCCAGAAG-3'	97
<i>Bcl2</i>	Forward: 5'-ATGGGGTGAAGTGGGGGATTG-3' Reverse: 5'-TTCCGAATTTGTTTGGGGCAGGTC-3'	328
<i>Bax</i>	Forward: 5'-GGGTGGTTGCCCTTTTCTACT-3' Reverse: 5'-CCCGGAGGAAGTCCAGTGTC-3'	110
β -actin	forward: 5'-ACACTGTGCCCATCTACGAGG-3' Reverse: 5'-AGGGGCCGGACTCGTCATACT-3'	250

microscope following May-Grünwald-Giemsa staining. The samples used for analysis contained at least 90% lymphoblasts after separation. Pellets of 2-10 million cells were immediately frozen and stored at -70 °C.

Ethical approval

This study was in line with generally accepted ethical principles and approved by the Research Ethic Committee of Affiliated Guangzhou First People's Hospital of Guangzhou Medical University. All the procedures were performed in accordance with the principals of the Declaration of Helsinki. Written informed consent was obtained from all the patients before study.

DNA extraction and bisulfite modification

DNA was extracted from paraffin-embedded bone marrow. Samples were deparaffinized using xylene and ethanol, followed by digestion with proteinase K. After conventional DNA extraction, DNA was modified with sodium bisulfite. The bisulfite treatment of DNA may convert the unmethylated CpG to UpG without methylation. In brief, DNA was denatured in 0.2 N NaOH at 37°C for 10 min and incubated with 3 M sodium bisulfite at 50°C for 16 h. DNA was then purified using the Wizard cleaning system (Promega, Madison, WI) and desulfonated with 0.3 N NaOH at 25°C for 5 min. DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol, dried, and re-suspended in H₂O.

Detection of mRNA expression of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax*

Total RNA was isolated using the TRIzol reagent. Reverse transcription was performed using a TITANIUM real-time PCR (RT-PCR) kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The mRNA expression was quantified using a fluorescence-based RT-PCR according to manufacturer's instructions (Bio-Rad Laboratories). The primers used for RT-PCR of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax* are shown in Table 2.

Western blot assay

At the pre-designed time points, cells were washed twice with 5 ml of ice-cold CMF-PBS followed by addition of 0.5 ml of lysis buffer containing additional phosphatase (cocktails I and II) and protease inhibitor (Calbiochem). Cells were harvested, treated with lysis

buffer and then kept on ice. After storage at -80°C , all the extracts were homogenized by sonication, followed by centrifugation ($16,000\times g$, 10 min, 4°C) to remove insoluble materials. The resulting supernatant (cell extract) was gently removed, and total proteins were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Western blot assay for the selected proteins was performed according to previously described (Yu and Hong, 2008). Gels were transferred to polyvinylidene fluoride membranes (Millipore) using a vertical transfer apparatus. Membranes were rinsed briefly in Tris-buffered saline (TBS; pH 7.6), blocked with 5% non-fat-dried milk in TBS containing 0.1% Tweens-20 (TTBS) for 20 min and rinsed again with T-TBS. Membranes were then incubated overnight with primary antibody for 1.5 h and then with a secondary antibody. Following antibody incubation, the membrane was washed 4 times for 5 min with T-TBS. The primary antibodies included mouse antibodies against human *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2*, *Bax* and β -Actin (Abcam, Cambridge, MA, USA). β -Actin was used as an internal reference. After hybridization with anti-mouse secondary antibodies (Amersham, NJ, USA) conjugated to horseradish peroxidase, the immunocomplex was detected with the ECL detection reagent (Amersham Pharmacia Biotech) and exposed to X-ray films. Quantification of band intensities was achieved using the NIH ImageJ (<http://rsb.info.nih.gov/ij/index.html>).

Detection of *hMSH2* promoter methylation

The methylation of *hMSH2* promoter CpG island was determined by COBRA (combined bisulfite and restriction analysis) and bisulfite sequencing. In brief, 200 ng of genomic DNA was modified with sodium bisulfite using the Methylamp™ One-Step DNA Modification Kit (Epigentek, Brooklyn, NY, USA) according to the manufacturer's instructions. All samples were re-suspended in 15 μl of TE, and 1 μl of this mixture was used for subsequent PCR. Amplification was done with 25 μl containing 1X manufacturer's buffer, 1 U of FastStart taq DNA polymerase (Roche, Welwyn Garden City, UK), 2 mM MgCl_2 , 10 mM dNTPs, and 75 ng of each primer. Following amplification, the PCR products were digested with restriction endonucleases (TaqI and BsiEI [New England Biolabs, Hitchin, UK]), which were specific to the methylated sequence after sodium bisulfite modification. Following digestion, products were separated on 2% agarose gel and visualized by ethidium bromide staining. In vitro methylated (IVM) DNA (Millipore, Watford, UK) was diluted to produce standards (100%, 66%, 33% and 0%) of known methylation status for all COBRA assays. Table 3 lists the primers and PCR conditions.

To confirm the methylation profiles obtained by COBRA, bisulfite sequencing of *hMSH2* promoter region was performed. For bisulfite sequencing, nested PCR was performed in 25 μl of PCR mixture containing 12.5 μl of HotStarTaq Master Mix kit and PCR primers at appropriate concentrations. PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced on an ABI 3100-Avant DNA sequencing system.

Cell lines and transfections

The human ALL cell lines used in this study were Nalm-6 cells (B-cell precursor, ALL) which were kindly provided by the American Type Culture Collection and Reh cells which were obtained from DSMZ (Braunschweig, Germany). Both cell lines have been previously characterized as B-precursor ALL cells. The early B-lymphoid antigen CD19 is expressed on > 95% of cells.

ALL cell lines were maintained in RPMI containing 2 mM glutamine and 10% fetal calf serum (FBS) in an environment with 95% O_2 /5% CO_2 at 37°C . For *hMSH2* over-expression study, Nalm6 cells were treated with 1 μM 2'-deoxy-5-azacytidine (Sigma) for 24 h and then collected for qRT-PCR five days later. For transfection, the *hMSH2* cDNA was cloned into the pIRES2-eGFP vector to produce the pIRES-*hMSH2*-eGFP vector. This allows the expression of *hMSH2* and eGFP in a single transcript, but two proteins are translated separately due to the presence of IRES sequence between *hMSH2* and eGFP. Transfections were carried out using the Nucleofector system (Amaxa, Koeln, Germany) according to the manufacturer's instructions using 5×10^6 cells and 2 μg of DNA. Cells were transfected with either pIRES-eGFP or pIRES-*hMSH2*-eGFP. Transfected cells were either used for transient transfections or treated with 800 $\mu\text{g}/\text{ml}$ G418 following transfection to select the stably transfected cells. The G418 resistant cells were collected, and the GFP positive cells were determined by flow cytometry. Then, the GFP positive cells were sorted using a FACSA cell sorter (BD Biosciences, Oxford, UK), to acquire a cell population containing transfectants at a high level. *hMSH2* expression in this population was confirmed by qRT-PCR. These bulk cultures, as opposed to single clones, were used for subsequent experiments to avoid any potential influence of site of integration on downstream analysis.

Growth assays

The effect of *hMSH2* on the growth of ALL cells was assessed using the GFP positive Nalm6 cells and Reh cells. Stably transfected cells were grown for approximately 7 days, and the GFP expression was determined. Only the cells with high GFP positivity (>80% for Nalm6 and 70-80% for Reh) were used for further assays. Cells were counted using the Vi-CELL System (Beckman Coulter, High Wycombe, UK), and then 20,000 viable cells without transfection, transfected with blank vector and transfected with *hMSH2* vector were seeded in triplicate in 12-well plates. Samples were harvested at 4 and 7 days and counted using the Vi-CELL system. Data shown were the averages from 4 independent experiments.

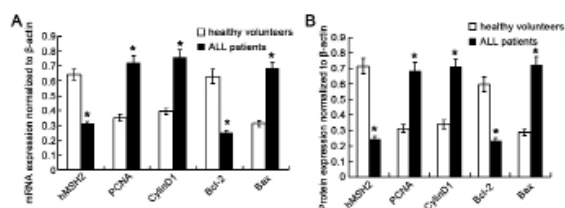
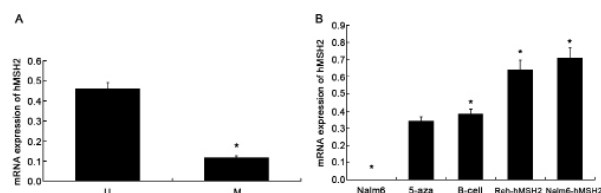
Growth assays were also carried out following dexamethasone treatment. Following cell counting with the Vi-CELL system, 30,000 transfected Nalm6 cells (either blank vector or *hMSH2* vector) were plated in triplicate in 12-well plates. These cells were treated with 0, 1 and 5 nM dexamethasone. Cells were harvested at 4 and 7 days for counting using the Vi-CELL system. Data shown were averages from 3 independent experiments.

Detection of apoptosis

Apoptotic cells were measured by flow cytometry

Table 3. Primers and PCR Conditions for *hMSH2* COBRA and Bisulfite Sequencing

Locus	Primer Sequence	Annealing temp (restriction enzyme)	Product Size (of PCR cycles)
Combined bisulfite restriction analysis (COBRA)			
<i>hMSH2</i> (-73bp)	F:5'GGGAAGTTGGGTYGYGTTTGT3' R:5'CRACCCACACCCACTAACTATT3'	156 (TaqI)	55 (7), 53 (38)
<i>hMSH2</i> (+91bp)	F:5'GAGGYGGGAAATAGTTTAGTG3' R:5'ACTTCTCCRACATACCCTAAAAA3'	158 (HpyCH4IV)	55 (7), 53 (38)
Bisulfite Sequencing of <i>MSH2</i>			
1 st	F:5'GGGAAGTTGGGTYGYGTTTGT3' R:5'CRAAACCTCCTCACCTCCTAA3'	191	55(7), 53 (30)
2 nd	F:5'GGGAAGTTGGGTCGCGTTTGT3'	191	55 (35)

**Figure 1. The mRNA (A) and Protein (B) Expression of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax* in Healthy Volunteers and ALL Patients. * $P < 0.05$ vs control group****Figure 2. Hypermethylation of *hMSH2* was Associated with Loss of Gene Expression.** (A) Gene expression was detected in ALL using qPCR. Relative expression is shown in unmethylated (U) and hypermethylated (M) samples. Methylation was significantly associated with loss of gene expression. * $P < 0.05$ vs. unmethylated (U) samples. (B) Nalm6 cells were either untreated (Nalm6) or treated with 1 μ M 2'-deoxy-5-azacytidine (5-aza) for 48 h and then the *hMSH2* expression was detected by qPCR 5 days later. Loss of *hMSH2* methylation was found to be associated with gene over-expression. * $P < 0.05$ 5-aza treated Nalm6 cells

after Annexin V staining using the Annexin V apoptosis detection kit I (BD Biosciences) according to the manufacturer's instructions. PE-conjugated Annexin V was used to allow differentiation from the green signal derived from GFP expression. In the detection of apoptosis in transiently transfected cells, Nalm6 cells and Reh cells transfected with either blank vector or *hMSH2* vector were determined at 48 h post transfection specifically in the GFP positive (i.e. transfected) population. The background was measured in the non-transfected GFP negative cells, and subtracted from that of GFP positive cells. For assays using cytotoxic agents, transfected Nalm6 cells were treated with either daunorubicin or etoposide (Sigma) at 0, 0.1 and 0.3 μ M. At 24 h after initial treatment, cells were collected and processed for apoptosis detection as described above. Data shown were the averages from 3-4 independent experiments.

Statistical analysis

Statistical analysis was done with SPSS version 13.0

software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean \pm standard deviation. Differences between groups were analyzed with t-test or analysis of variance. Cell count was expressed as rates and the differences were compared with chi-square test. The survival of *hMSH2* methylated cells and unmethylated cells in ALL patients were expressed as median and comparisons were carried out using the Kaplan-Meier method (Log-rank). A value of $P < 0.05$ was considered statistically different.

Results

Expression of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax* in ALL

To investigate the effect of ALL on the expression of *hMSH2*, *PCNA*, *CyclinD1*, *Bcl-2* and *Bax*, the mRNA and protein expression of these genes were determined by qPCR and western blot assay, respectively. Both the mRNA and protein expression of *hMSH2* and *Bcl-2* reduced in mononuclear cells of ALL patients, while that of *PCNA*, *CyclinD1* and *Bax* increased in these cells. Furthermore, the expression of these genes in ALL patients was significantly different from that in healthy volunteers ($P < 0.05$) (Figure 1A, B). These suggested that there were aberrant mRNA and protein expression of genes related to DNA repair, cell cycle, and cell apoptosis in ALL.

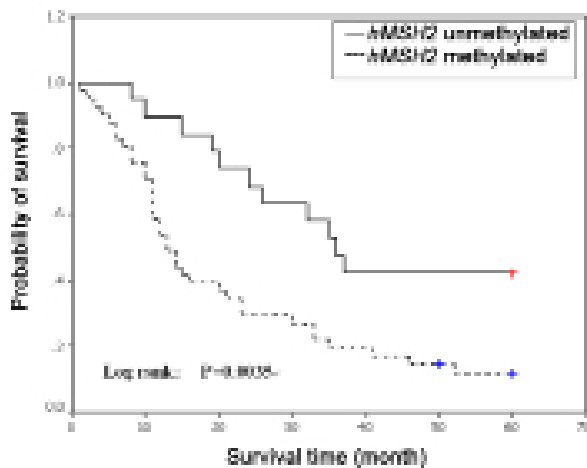
hMSH2 as a target for epigenetic inactivation in ALL

To determine the potential role of *hMSH2* promoter hypermethylation in the leukemia, the methylation status of genes was quantitatively analyzed in ALL by COBRA assay. No methylation was detectable in normal peripheral blood, but *hMSH2* hypermethylation (>50% methylated DNA) was identified in the peripheral blood of most ALL patients. This was true for ALL (70% of cases exhibiting >50% methylation, Table 3). To further analyze the methylation status of *hMSH2*, a second quantitative methylation assay, pyrosequencing, was used to confirm the methylation level in a subset of ALL samples. Pyrosequencing demonstrated that *hMSH2* hypermethylation (>50%) were frequently observed in ALL samples, as shown in the COBRA analysis. Furthermore, samples with methylation identified by COBRA assay were consistent with those with methylation identified by pyrosequencing assay.

The *hMSH2* hypermethylation was detected in patients and healthy volunteers. Additional 6 ETV6- RUNX1

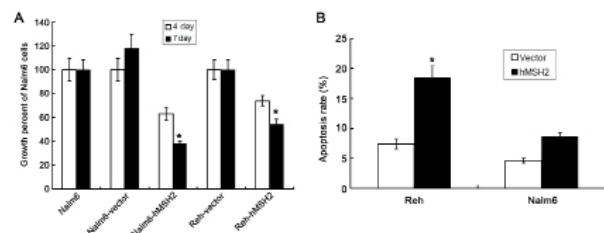
Table 4. Frequency of *hMSH2* Hypermethylation in Leukemia

Items	Total	Methylated, N (%)	Unmethylated, N (%)
Median age (yr)	32.4	31.2	33.3
Sex ratio	32:28:00	22:20	10:08
Median WBC ($\times 10^9/L$)	42.7	50.5	37.8
BCP-ALL (%)	39	24 (61.5)	15 (38.5)
T-ALL (%)	12	7(58.3)	5(41.7)
Diagnostic (%)	46	33(71.7)	13(28.3)
Relapsed ALL* (%)	14	12(85.7)	2(14.3)
t(12:21)-positive (%)	16	12(75.0)	4(25.0)
t(12:21)-negative (%)	40	17(42.5)	32(57.5)
HeH (%)	15	5(33.3)	10(66.7)
Total (%)	60	42(70.0)	18(30.0)

**Figure 3. Overall Survival Curves for *hMSH2* Methylated/unmethylated in ALL Patients.** Kaplan-Meier survival analysis of *hMSH2* methylated group (dotted line) and *hMSH2* unmethylated group (solid line) showed a significant difference in survival (log-rank test, $P=0.0035$)

positive ALL samples were obtained and assessed for *hMSH2* methylation, and cytogenetic data were obtained from previously examined samples. *hMSH2* hypermethylation was more common in ETV6-RUNX1 positive ALL samples than that in ALL samples without this fusion gene (75.0% [12/16] vs. 42.5% [17/40], $P=0.028$, [chi-square test]). *hMSH2* methylation did not significantly correlate with age, white blood cells, sex, immunophenotype and any other cytogenetic subgroup (Table 4).

To further assess the role of *hMSH2* methylation in ALL, 14 samples were collected from adults with relapsed ALL and assessed for *hMSH2* methylation. Results showed that almost 12 relapse patients (85.7%) exhibited the hypermethylation of *hMSH2* gene, consistent with the possibility that *hMSH2* plays a role in chemosensitivity, as seen in vitro experiments. Subsequently, the corresponding diagnostic samples were collected from relapsed patients after treatment, and the *hMSH2* methylation was determined. Results demonstrated that *hMSH2* methylation significantly increased in the relapse patients when compared with the corresponding diagnostic sample ($P=0.01$). However, the average increase in methylation was relatively small (66% vs. 74%) and this

**Figure 4. Over-expression of *hMSH2* in ALL Cells is Associated with Reduced Growth and Increased Apoptosis.** (A) *hMSH2* expression was associated with a dramatic reduction in Nalm6 cells growth ($P=0.002$, t-test) and Reh cell ($P=0.0006$, t-test). Data were the averages from 4 (Nalm6) or 6 (Reh) independent experiments. $*P < 0.05$ vs 4 days. (B) Apoptosis (determined after Annexin V staining) significantly increased in Reh cells in the presence of *hMSH2* expression, but not in Nalm6 cells. Data were averages from 3 independent experiments. $*P < 0.05$ vs cells transfected with blank vector

increase was confined to samples with lower methylation levels at diagnosis (samples with $<70\%$ methylation at diagnosis showed an average increase of 14% at relapse, $P=0.001$). It is suggested that a combination of high *hMSH2* methylation at diagnosis and increased *hMSH2* methylation at relapse (especially for samples lacking very high methylation at diagnosis) may result in the extremely high level of *hMSH2* methylation seen in relapse samples.

Hypermethylation of *hMSH2*

hMSH2 expression was assessed using qPCR in 60 samples including 18 unmethylated and 42 hypermethylated samples. Expression was detectable in almost all unmethylated samples, but at low level or absent in methylated samples. The *hMSH2* mRNA expression in unmethylated samples was significantly different from that in the methylated samples ($P=0.003$, t-test) (Figure 2A). To further explore the importance of *hMSH2* methylation, methylated *hMSH2* expression was examined in ALL cell lines. The hypermethylation of *hMSH2* was examined in both ALL cell lines, and results showed an absence of methylated *hMSH2* expression in both Reh and Nalm6 cell lines which were used for subsequent functional assays. The Nalm6 cells treated with 2'-deoxy-5-azacytidine reduced methylation of the *hMSH2* promoter and re-expression of *hMSH2* mRNA, demonstrating that DNA methylation of the gene was required for the suppression of expression (Figure 2B).

Prognostic significance of *hMSH2* methylation in ALL patients

To test the prognostic value of *hMSH2* hypermethylation in ALL patients, the associations between *hMSH2* methylation and overall survival was evaluated in ALL patients. In brief, 60 ALL patients were separated into *hMSH2* methylation group and unmethylation group. The median survival of patients in *hMSH2* methylation group was 22 months, whereas it was 39 months in *hMSH2* unmethylation group. Kaplan-Meier survival analysis showed a significant difference between them (log-rank test, $P=0.0035$; Figure 3), indicating that methylation may have both clinical and biologic effects in ALL patients.

Restoration of *hMSH2* expression in ALL cells inhibits cell growth and induces apoptosis in Reh cells

To assess the functional significance of *hMSH2* in ALL cells, the *hMSH2* gene was transfected into Nalm6 cells, in which *hMSH2* was epigenetically silenced. As transfection of leukemia cells was generally inefficient, this was done using the pIRES2-eGFP vector, which also expresses eGFP. Following G418 selection, eGFP expressing cells (and thus *hMSH2* expressing cells) were subsequently isolated by flow cytometry and eGFP/*hMSH2* positive cells (>80% positive) with high purity were collected. *hMSH2* expression in this population was confirmed by qPCR. The growth of these cells was then measured within 7 days. As shown in Figure 4A, the *hMSH2* expressing cells exhibited a significant decrease in cell growth when compared with untransfected Nalm6 cells or Nalm6 cells transfected with blank vector. Significant inhibition of proliferation was also seen in Reh cells (expressing RUNX1-ETV6 fusion gene) after *hMSH2* transfection. It was noted that continued growth in culture of both cell lines resulted in a decline in the fraction of *hMSH2* positive cells, presumably due to their lower proliferation rates. This effect was more obvious in Reh cells (levels typically dropped from 70-80% to <50% within 7 days in Reh cells, whereas 3-4 weeks in Nalm6 cells). To determine whether the increased selection against *hMSH2* expression was due to the toxicity of *hMSH2* in Reh cells, the apoptosis was measured in Nalm6 cells and Reh cells following transient transfection. As shown in Figure 4B, transfection of Nalm6 cells with *hMSH2* resulted in only a minor increase in apoptosis as compared to cells transfected with blank vector. In contrast, over-expression of *hMSH2* in Reh cells resulted in a significant induction of apoptosis. In addition to negatively regulating cell growth, *hMSH2* is shown to negatively influence the survival of ALL cells, but this effect is dependent on the genetic background.

Discussion

Epigenetic inactivation of genes is crucial in the development of leukemia and has dramatic effects on the biological and clinical behaviors of these diseases. DNA methylation is an epigenetic modification that plays an important role in the control of gene expression in mammalian cells, and aberrant DNA methylation plays an important role in the tumor progression (Calvisi et al., 2007; Choi et al., 2007; Shimizu et al., 2007). Many studies have shown that alterations at the genomic levels and regional changes in the patterns of DNA methylation are commonly observed in the neoplasia at early stages (Baylin et al., 1998; Pogribny and James, 2002). Studies have shown that there are highly significant correlation between gene methylation and negative protein expression for mismatch repair *hMSH2* (Ward et al., 2003; Ligtenberg et al., 2009; Takeshi et al., 2010; Zhiheng et al., 2012). Herein, our findings showed that the *hMSH2* gene was hypermethylated in over half of ALL cases. *hMSH2* has been shown to be expressed in normal B lymphocytes. In the present study, our results revealed that hypermethylation of *hMSH2* suppressed its expression in both primary cancer and cell lines. Treatment

with 2'-deoxy-5-azacytidine resulted in over-expression in Nalm6 cells, demonstrating that the DNA methylation is required for the suppression of *hMSH2* expression. Furthermore, functional detection indicated that *hMSH2* exhibited multiple important biological effects on ALL cells, including control of cell proliferation and survival.

In early studies, findings revealed that aberrant DNA methylation and epigenetic inactivation of some genes were significantly correlated with the prognosis of ALL (Kuang et al., 2008; Shaoqing et al., 2010). Some studies show that CpG island methylation of *hMSH2* is closely related to the prognosis of colorectal cancers (Takeshi et al., 2010; KyungHwa et al., 2011). Few studies have been conducted to investigate the *hMSH2* promoter methylation in ALL. Some investigators propose that there's low *hMSH2* expression and high *hMSH2* methylation in ALL which is consistent with our previous findings (Caixia et al., 2011). However, no study has been conducted to study the prognostic value of *hMSH2* methylation in ALL. Herein, our results showed that aberrant DNA methylation and epigenetic inactivation of *hMSH2* decreased overall survival of ALL patients. This means that the early diagnosis of *hMSH2* methylation in ALL patients may have prognostic implication, a concept that mandates further exploration in the future.

Over-expression of *hMSH2*, but not GFP alone, in ALL cells resulted in a dramatic inhibition of cell growth, indicating that *hMSH2* can regulate the growth and survival of ALL cells, and *hMSH2* has functions similar to those of a tumor suppressor gene. These effects may be due to loss of regulation of RUNX1 or through yet to be identified *hMSH2* target proteins. The mechanism by which *hMSH2* inhibits cell growth is not yet clear. However, RUNX1 would represent a potential candidate mediator of this effect. Several previous studies have demonstrated that *hMSH2* can bind to and inactivate RUNX1 in osteoblasts and myeloid cells. Furthermore, RUNX1 is known to be able to facilitate proliferation of hematopoietic cells and enhance B-cell survival (Cameron and Neil, 2004; Blyth et al., 2009). Consistent with the hypothesis that RUNX1 is a key target for *hMSH2*, our findings showed that loss of *hMSH2* expression in primary ALL was more common in patients with leukemia expressing RUNX1-ETV6 fusion gene. It is likely that *hMSH2* binds to the product of this fusion gene, because it retains the *hMSH2* binding runt domain (Zelent et al., 2004). The ability of *hMSH2* to induce apoptosis was found in the RUNX1-ETV6 positive Reh cells but not in the Nalm6 cells (having no fusion gene, but highly expressing WT RUNX1), suggesting a heightened role for *hMSH2* in RUNX1-ETV6 positive leukemia. However, there are likely to be multiple genetic differences between these cell lines and thus the increased apoptosis in Reh cells is not directly linked to the presence of RUNX1-ETV6. We also attempted to confirm the association between loss of *hMSH2* expression and presence of RUNX1-ETV6 in ALL by examining publicly available expression database. However, *hMSH2* proved to be absent in most array formats used and thus *hMSH2* expression cannot be determined. It has been reported that *hMSH2* was significantly related to *Bcl-2* (Youn et al., 2005). *Bcl-2* can suppress DNA mismatch

repair (*hMSH2*-*hMSH6*) with promotion of mutagenesis, which potentially contributes to the genetic instability and carcinogenesis (Yongzhong et al., 2007). In addition, a significant role of *hMSH2* in ALL without *RUNX1-ETV6* is also observed. Firstly, since over-expression of *hMSH2* still produces a very clear inhibition of cell growth in Nalm6 cells, and secondly, *hMSH2* hypermethylation is still seen in 42.5% of ALL cases negative for *RUNX1-ETV6* (Mrozek et al., 2004). Further studies are required to compare its roles in *RUNX1-ETV6* positive and *RUNX1-ETV6* negative ALL.

Some investigators proposed a relationship between the expression and methylation of *hMSH2* and drug resistance or the response to therapy (chemotherapy and radiotherapy) in cancer patients (Brooks et al., 2003). Hypermethylation of *hMSH2* was extremely common in relapsed adult ALL (85.7% of samples), suggesting that treatment preserves either cells with increased CpG island methylation in general or cells with increased *hMSH2* methylation in particular. It is also suggested that the pathways regulated by *hMSH2* may become targets to reverse the chemoresistance in *hMSH2* deficient cells. This may be more important for relapsed ALL adults, who usually exhibit *hMSH2* hypermethylation and whose outcome is extremely poor.

In conclusion, our results show relatively frequent aberrant methylation of *hMSH2* gene promoter in ALL patients. The aberrant DNA methylation and epigenetic inactivation of *hMSH2* play an important role in the occurrence and development of ALL, which is likely related to the alteration of growth and survival of cancer cells.

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