

## ABERRANT PROMOTER HYPERMETHYLATION OF SELECTED APOPTOTIC GENES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA AMONG NORTH INDIAN POPULATION

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Promoter hypermethylation mediates gene silencing in many neoplasms. Acute leukemia has been reported to harbor multiple genes aberrantly silenced by hypermethylation. **Aim:** In present study, we investigated the prevalence of hypermethylation of *cas-pase-8 (CASP8)*, *TMS1* and *DAPK* genes in correlation with clinicopathological factors in childhood acute lymphoblastic leukemia (ALL). **Materials and Methods:** A case-control study has been conducted based on bone marrow and peripheral blood samples from 125 ALL patients and 100 sex-age matched healthy controls. Methylation specific polymerase chain reaction (PCR) and bisulfite sequencing PCR was performed to analyze the methylation status of these genes. Reverse transcription PCR and real time PCR was carried out to determine changes in the mRNA expression level of the genes due to hypermethylation. **Results:** Hypermethylation of the 5' CpG islands of the *CASP8*, *TMS1* and *DAPK* gene promoters was found in 3.2, 6.4, and 13.6% of 125 childhood ALL samples from north Indian population, respectively. There were significant differences in pattern of hypermethylation of *TMS1* ( $p = 0.045$ ) and *DAPK* ( $p < 0.001$ ) between patients and healthy controls. Down-regulation of mRNA expression was found in cases in which *CASP8*, *TMS1* and *DAPK* were hypermethylated. **Conclusions:** The present study indicated the impact of hypermethylation-mediated inactivation of *CASP8*, *TMS1* and *DAPK* genes, which is associated with risk of childhood ALL. This abnormality occurs in leukemogenesis and it may be used as a biomarker and for predicting the prognosis of ALL.

**Key Words:** acute lymphoblastic leukemia, epigenetics, hypermethylation, apoptotic genes.

Childhood acute lymphoblastic leukemia (ALL), clinically known as heterogeneous disorder, is caused by uncontrolled proliferation of immature B or T lymphoblasts. It was estimated to be the seventh most prevalent type of cancer accounting for 3% of the new cancer cases and also accounts for the ninth rank in causes of death among children. Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence. In higher eukaryotes, DNA is methylated only at cytosines adjacent to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands (CGI) located in the promoter regions of many genes [1]. Hypermethylation of the CGI of gene promoter of tumor suppressor genes is one of the earliest and most frequent alterations leading to cancer. The specific patterns of CGI

hypermethylation may provide a useful signature for tumor diagnosis and prognosis [2]. Aberrant promoter hypermethylation of tumor suppressor genes (without any structural alteration) and its association with transcriptional inactivation is considered as an epigenetic mechanism in leukemia pathogenesis [3–5].

Death-associated protein kinase 1 (DAPK) is a 160 kDa Ca<sup>2+</sup>/calmodulin dependent, cytoskeletal-associated protein kinase. It has been found that DAPK exerts an apoptotic effect by inside-out inactivation of integrin, thereby suppressing the matrix survival signal and activating a p53-dependent apoptosis pathway [6, 7]. Several studies have suggested that loss of DAPK expression or methylation of CGI in *DAPK* promoter region may be characteristic of highly invasive or metastatic tumors [8]. More than 100 reports from different laboratories were published on *DAPK* gene methylation in various human tumors. Also based on other report several B-cell lymphoma cell lines have decreased or absent expression of DAPK RNA or protein [9]. This finding suggests that abnormal loss of DAPK expression could be associated with aberrant promoter region methylation, serving as an alternative to the genetic loss of a tumor-suppressor gene function by deletion or mutation [10, 11].

TMS1 (target of methylation-mediated silencing, also known as ASC or PYCARD), is commonly silenced

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**Abbreviations used:** ALL – acute lymphoblastic leukemia; BM – bone marrow; CGI – CpG islands; CI – confidence interval; DAPK – death-associated protein kinase 1; MSP-PCR – methylation specific PCR; OR – odds ratio; PCR – polymerase chain reaction; RT – reverse transcription; TMS – target of methylation-mediated silencing.

by methylation in human cancer cells. TMS1 was identified in a screen for downstream targets of methylation-mediated gene silencing induced by overexpression of human DNA methyltransferase-1 [12]. Methylation pattern of *TMS1* at the TMS1 locus as well as the expression of TMS1 in many cancer cell lines especially epithelial cancer cell lines has been shown by many research groups [12–16]. TMS1-induced apoptosis could be blocked with a dominant-negative caspase-9, but not a dominant-negative caspase-8, which suggested that it was a part of intrinsic mitochondrial signaling [17].

Caspase-8 gene (*CASP8*) located at human chromosome 2q33-q34 [18] is involved in the regulation of apoptosis and also in several non-apoptotic processes such as proliferation, differentiation, and NF- $\kappa$ B activation [19]. Caspase-8 expression or function can be impaired in cancers by mutation, deletion, epigenetic silencing, alternative splicing or posttranscriptional mechanisms such as phosphorylation. A high methylation rate and thereby gene silencing for *CASP8* in medulloblastoma has been reported recently [20, 21].

So, the aim of present study was to investigate the prevalence of hypermethylation of *CASP8*, *TMS1* and *DAPK* in correlation with clinicopathological factors in childhood ALL. Our results indicate that hypermethylation-mediated inactivation of caspase-8, TMS1 and DAPK is associated with the risk of childhood ALL and it could be used as a biomarker and prognostic marker in these patients.

## MATERIALS AND METHODS

**Sample collection.** The bone marrow (BM) aspirate was collected from patients admitted to Hematology-Oncology ward, Advanced Pediatric Center, PGIMER, Chandigarh, India, at the time of initial diagnosis and prior to any treatment regimen. The study was approved by Institute Ethical Committee and informed consent was obtained from the guardians of all the patients recruited in the study. Diagnosis was based on standard hematological protocol according to WHO classification. For statistical analysis, children were grouped according to National Cancer Research Institute (NCRI) risk classification; the clinical characteristics of the leukemic patients and regional distribution among northern states of India are shown in Table 1 and 2, respectively. For controls, blood samples were obtained from healthy donors with respect to median and mean of sex and age of patients. Mononuclear cells were obtained from BM aspirate and blood samples by centrifugation on Ficoll-Histopaque (Sigma-Aldrich, St. Louis, Missouri, USA) density gradient and were used for DNA and RNA extraction.

**DNA isolation.** DNA was extracted from mononuclear cells of BM and blood using standard proteinase K (Sigma-Aldrich, St. Louis, Missouri, USA) digestion followed by phenol chloroform extraction and ethanol precipitation. The DNA concentration was determined by spectrophotometry and its integrity was checked by 1.5% agarose gel electrophoresis.

**Table 1.** Demographic and clinical characteristics of study population

	Patients (N = 125)	Control group (N = 100)
	n (%)	n (%)
Gender		
Male	97 (77.6)	77 (77.0)
Female	28 (22.4)	23 (23.0)
Mean age, years ( $\pm$ SD)	6.46 $\pm$ 3.34	6.55 $\pm$ 3.87
Age < 2	12 (9.6)	11 (11.0)
2 < age < 5	43 (34.4)	33 (33.0)
age > 5	70 (56.0)	56 (56.0)
Cell lineage		
B-lineage ALL	118 (94.4)	–
T-lineage ALL	7 (5.6)	–
NCRI risk group		
Standard	103 (82.4)	–
High	22 (17.6)	–

**Table 2.** Distribution of cases on the basis of regional distribution

State	Patients (n = 125), %
Punjab	55
Haryana	34
Uttar Pradesh	16
Himachal Pradesh	10
Chandigarh	5
Jammu & Kashmir	5

**Sodium bisulfite modification of DNA.** Sodium bisulfite modification of genomic DNA was performed as described previously, with little modifications [1]. A sample of genomic DNA (2  $\mu$ g) was denatured with NaOH (final concentration 0.2 M) followed by incubation at 37 °C for 10 min. Then, DNA was modified by incubation with 30  $\mu$ l of 10 mM hydroquinone (Sigma-Aldrich, St. Louis, Missouri, USA). 520  $\mu$ l of freshly prepared 3 M sodium bisulfite (Sigma-Aldrich, St. Louis, Missouri, USA) (pH = 5) was added and the mixture was incubated at 50 °C for 16 h. After incubation, modified DNA was purified using DNA Wizard Clean-up (Promega, Madison, Wisconsin, USA) according to manufacturer's procedure and eluted into 50  $\mu$ l of sterile water followed by desulfonation in 0.3 N NaOH at 25 °C for 5 min. Desulfonated DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol and re-suspended in 20  $\mu$ l of sterile water.

**Methylation specific polymerase chain reaction (MSP-PCR).** MSP-PCR was used to study the methylation status of the CGI in promoter regions of *CASP8*, *TMS1* and *DAPK*. The reaction mixture (50  $\mu$ l) contained 2  $\mu$ l of above bisulfite-treated DNA, 0.2 mM of dNTP (each at concentration 10 mM) (Fermentas Life Science, Waltham, Massachusetts, USA), 10  $\mu$ M of each primer (synthesized by Sigma), 5  $\mu$ l of BSA (10 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA), 10X PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween [20], 2.5 mM/I MgCl<sub>2</sub>, and 1 unit of thermostable Hot Start Taq DNA polymerase (Qiagen, Hilden, Germany). The primer sequence and PCR conditions are listed in Table 3. MSP-PCR products were analyzed by using 2% agarose gel electrophoresis followed by ethidium-bromide staining and visualized under UV transillumination.

**Bisulfite sequencing.** MSP-PCR products were gel purified (Sigma-Aldrich, St. Louis, Missouri, USA), sequenced by using ABI prism Big-Dye terminator (Applied Biosystems, Foster City, California, USA) and analyzed on an automated DNA sequence analyzer (3700 ABI Prism, PE Biosystem) to determine the

methylation status of CpG sites in the amplified regions. Treatment of genomic DNA with sodium bisulfate induces deamination of nonmethylated cytosine and converts it to uracil and finally to thymidine, but has no effect on methylated cytosine. This modification allows for differentiation of methylated and nonmethylated CGI in promoter region of tumor suppressor genes by MSP-PCR or sequencing. For this purpose, 5 µl of the bisulfite-treated DNA amplified by MSP-PCR was run on a 2% agarose gel and desired band was gel purified using Promega SV gel purification kit (Promega, Madison, Wisconsin, USA) and sent for sequencing using the ABI prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an automated DNA sequence analyzer (3700 ABI Prism, PE Biosystem).

**Table 3.** Primer sequences, annealing temperatures and PCR product size used for MSP-PCR and reverse transcription PCR (RT-PCR)

	Sequence 5'→3'	Product size	Annealing temperature (°C)	Ref
CASP8 methylated	Forward: TAGGGGATTCGGAGATTGCCG Reverse: CGTATATCTACATTCGAAACGA	320	50	[24]
CASP8 nonmethylated	Forward: TAGGGGATTTGGAGATTGTGA Reverse: CCATATATCTACATTCAAAACAA'	321	53	[24]
TMS1 methylated	Forward: CGATTTTGGCGTTTTTCGACGGTT Reverse: CCGCTCACCCCGCTACAACCG C	207	55	[33]
TMS1 nonmethylated	Forward: TTGTTGGAGGGTAATGGATT Reverse: CCCACAAAATACACCCATA	272	58	[33]
DAPK methylated	Forward: GGATAGTCGGATCGAGTTAACGTC Reverse: CCTCCCAAACGCCGA	321	63	[2]
DAPK nonmethylated	Forward: GGAGGATAGTTGGATTGAGTTAATGTT Reverse: CAAATCCCTCCAAACACCAA	321	63	[2]
CASP8 RT-PCR	Forward: AGAGAAGCAGCAGCCTTGAAGG Reverse: AGACAGTATCCCGAGGTTTGC'	160	55	[2]
TMS1 RT-PCR	Forward: GGACGCCTTGGCCCTCACCG' Reverse: GGC GCGGCTCCAGAGCCCTG'	148	65	[33]
DAPK RT-PCR	Forward: GATAGAAATGTCCCAAACCTCG' Reverse: TCTTCTTGGATCCTTGACCAGAA'	280	55	[2]
β-actin RT-PCR	Forward: CATGTACGTGCTATCCAGGC Reverse: CTCCTTAATGTACGCACGAT	254	58	[2]

Note: MSP-PCR conditions: 95 °C/5 min followed by 35 cycle 95 °C/45 s; annealing temp./30 s; 72 °C/45 s; 72 °C/10 min; RT-PCR conditions: 94 °C/5 min followed by 35 cycle 95 °C/45 s; annealing temp./30 s; 72 °C/45 s; 72 °C/7 min.

**RNA isolation.** Total RNA was extracted using the TRI reagent (Sigma-Aldrich, St. Louis, Missouri, USA), according to the manufacturer's instructions. The RNA pellets were reconstituted in DEPC (Sigma-Aldrich, St. Louis, Missouri, USA) treated water. The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels staining with ethidium bromide; the 18S and 28S RNA bands were visualized under transilluminator. The quality and quantity of total RNA was determined spectrophotometrically at 260 and 280 nm.

**RT-PCR.** Complementary DNAs (cDNAs) were reverse transcribed from 1–2 µg of total RNA by use of Revert Aid first strand cDNA synthesis kit (Fermentas Life Science, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Briefly, 1 µl (0.2 µg/µl) of random hexamer primers was added to 1.5 µg of total cellular RNA in a final volume of 12 µl followed by addition of supplied 1 µl MuLV reverse transcriptase. The reaction mixture was then

incubated for 1 h at 42 °C, followed by incubation at 70 °C for 10 min and subsequently stored at –20 °C.

Gel electrophoresis of the PCR products and graphs of melt curve and melt peak indicated that target genes could be specifically amplified using specific primers. The cDNA concentration was then normalized in series of PCR by using β-actin primers (see Table 3) and by carefully diluting cDNA until PCR products of different samples were similar to each other in band intensity using Scion analyzer software. The normalized cDNAs were subjected to amplification for detection of expression of *MTS2* and *MTS1* using gene specific primers for real-time RT-PCR.

**Real-time PCR.** Expression of genes was analyzed by quantitative real-time PCR using specific primer for each gene and SYBR Green Quantitative RT-PCR kit (Stratagene, Agilent Technology, USA). All samples were analyzed on the Stratagene Real Time Detector. β-Actin gene expression was used as invariant control to normalize input cDNA. Quantitative real-time RT-PCR was performed in a reaction volume of 20 µl including 1 µl of cDNA. The comparative Ct method was used to compute relative expression values.

**Statistical analysis.** The data were analyzed by computing odds ratio (OR) and 95% confidence interval (CI) using  $\chi^2$ -test, Fisher exact test and multivariate logistic regression with adjustment for age as a continuous variable to find out any difference between cases and healthy controls according to age, gender, immunophenotypes, and NCRI risk group's classification. The reported OR can be interpreted as age-adjusted estimates of the relative risk of developing ALL with methylation of studied genes. Analysis of continuous data like age was done by one way ANOVA. Statistical analysis was performed using SPSS (Version 18, SPSS Inc, Chicago, IL, USA). The Mann – Whitney *U* test was used to assess the significance of the difference in mRNA expression level of the individual genes and clinical stage. All *p* values < 0.05 were considered statistically significant.

## RESULTS

The relevant characteristics of the study subjects are summarized in Table 1. In this study, samples from 125 patients with ALL and 100 healthy controls were collected. The cases and the controls were well matched with respect to age, sex and place of living. The mean age was 6.50 ± 3.37 years for cases and 6.40 ± 3.33 years for controls (*p* = 0.9). 9.6% of patients were in the age-group of 0–2 years, 43.4% were in the age-group of 2–5 years and 56.0% in age group of more than 5 years old. The percent of males and females among cases and controls were 77.6:77.0 for males and 22.4:23.0 for females, respectively.

The cell lineages and NCRI risk groups of ALL patients are also presented in Table 1. 94.4% of patients were diagnosed with B-lineage ALL and 5.6% with T-lineage ALL. The patients were stratified into two risk groups according to the protocol of National Cancer Institute: (i) the high-risk category included

patients less than one year or more than 9 years old who had a white blood cells count of more than 50,000/ $\mu$ l at the time of diagnosis (ii) the standard-risk category included patients aged 1 to 9 years who had a white blood cells count of less than 50,000/ $\mu$ l at the time of diagnosis. All children with T-lineage ALL were considered at high risk regardless of age and initial white blood cells count. The distribution of ALL patients on the basis of NCRI risk groups were 82.4% in standard risk group and 17.6% in high risk group.

**Study of the methylation status of apoptotic genes.** Bisulfite-treated DNA from all 125 ALL patients was analyzed for *CASP8*, *TMS1* and *DAPK* promoter hypermethylation by standard MSP-PCR technique. Primers for this technique were designed to be located within the promoter region associated CGI and near the major MSP-PCR analysis of the *CASP8*, *TMS1* and *DAPK* promoters in samples using defined nonmethylated and methylated specific primers. The frequency of methylation pattern of *CASP8*, *TMS1* and *DAPK* genes has been depicted in Table 4. Methylation of *CASP8* was found in 3.2% of patients. Significant risk of ALL development was not observed in patients with hypermethylated *CASP8* promoter ( $p > 0.05$ ). Methylation of *TMS1* was found in 6.4% of patients. Statistically significant difference in methylation of *TMS1* ( $p = 0.045$ ) between patients and controls was observed. Risk of ALL increased by 1.64 fold with hypermethylated *TMS1* promoter (OR = 1.64, 95% CI = 1.26–2.13). Methylation of *DAPK* was found in 13.6% of patients. Statistically significant difference in methylation of *DAPK* ( $p < 0.001$ ) between patients and controls was observed. Significant risk of ALL development was evident in patients with hypermethylated *DAPK* promoter ( $p < 0.01$ ; OR = 1.93, 95% CI = 1.69–2.19) (see Table 4).

**Table 4.** Frequency of methylation with risk of ALL (patients vs healthy control)

Genes	Patients (N = 125), n (%)	Control (N = 100), n (%)	OR (95% CI)	$p$
Presence of methylation				
<i>CASP8</i>	4 (3.2)	0 (0.0)	1.83 (1.62–2.06)	0.13
<i>TMS1</i>	8 (6.4)	1 (1.0)	1.64 (1.26–2.13)	0.045
<i>DAPK</i>	17 (13.6)	0 (0.0)	1.93 (1.69–2.19)	0.001

The frequency of methylation of *CASP8*, *TMS1* and *DAPK* was further analyzed according to selected clinicopathological features in ALL patients (Table 5). Hypermethylation of *CASP8* and *DAPK* was observed in 4.1 and 12.4% and hypermethylation of *TMS1* was observed in 5.15% of male ALL patients. Statistically significant difference in methylation of *DAPK*

**Table 5.** Methylation status of genes with respect to clinicopathological features in childhood ALL patients from North India

	<i>CASP8</i>				<i>TMS1</i>				<i>DAPK</i>			
	Presence of methylation		OR (95% CI)	$p$	Presence of methylation		OR (95% CI)	$p$	Presence of methylation		OR (95% CI)	$p$
	Patients, n (%)	Control, n (%)			Patients, n (%)	Control, n (%)			Patients, n (%)	Control, n (%)		
Male	4 (4.1)	–	2.08 (1.79–2.40)	0.05	5 (5.2)	1 (1.2)	1.52 (1.04–2.23)	0.22	12 (12.4)	–	1.91 (1.65–2.21)	–
Female	–	–	–	–	3 (10.7)	–	1.92 (1.46–2.52)	0.24	5 (17.9)	–	2.00 (1.50–2.67)	0.05
Age < 2	–	–	–	–	1 (8.3)	–	2.00 (1.31–3.04)	1	2 (16.6)	–	2.10 (1.34–3.29)	0.47
2 < Age < 5	1 (2.3)	–	1.79 (1.46–2.18)	1	3 (7.0)	1 (3.0)	1.35 (0.74–2.47)	0.62	5 (11.6)	–	1.84 (1.50–2.32)	0.06
Age > 5	3 (4.3)	–	1.84 (1.56–2.16)	0.25	4 (5.7)	–	1.85 (1.57–2.18)	0.12	10 (14.3)	–	1.93 (1.62–2.30)	–

( $p < 0.01$ ) between male ALL patients and controls was evident. Hypermethylation of *CASP8* was observed in 4.8% of male patients, methylated promoter was not detected in female patients. Statistically significant difference in methylation of *CASP8* ( $p = 0.045$ ) between male ALL patients and controls was observed (OR = 2.08, 95% CI = 1.79–2.4). Hypermethylation of *TMS1* was detected in 5.15% of male patients and 10.7% of female patients. Methylation of *TMS1* gene in association with gender was not statistically significant among ALL patients. Hypermethylation of *DAPK* was detected in 12.4% of male patients and 17.9% of female patients. Methylation of *DAPK* gene was statistically significant in male and female ALL patients as compared to controls. The risk of ALL due to hypermethylation of *DAPK* increased around 1.9 fold in male patients and 2.00 fold in female patients (OR = 1.91, 95% CI = 1.65–2.21 for male and OR = 2.00, 95% CI = 1.50–2.67 for female patients) (Table 5).

The methylation of *CASP8*, *TMS1* and *DAPK* in association with cell lineage of ALL patients is presented in Table 6. Statistically significant relation was not evident among these two groups.

**Table 6.** The frequency and percentage of hypermethylation in promoter of genes with B- or T-lineage ALL

Genes	B-lineage ALL (N = 118), n (%)	T-lineage ALL (N = 7), n (%)	OR (95% CI)	$p$
Presence of methylation				
<i>CASP8</i>	4 (3.3)	–	1.06 (1.02–1.11)	1
<i>TMS1</i>	6 (5.1)	2 (28.57)	0.80 (0.52–1.17)	0.06
<i>DAPK</i>	16 (13.6)	1 (14.2)	1.00 (0.88–1.13)	1

The frequency of methylation of these genes in patients with stratified age groups, namely Age < 2 and 2 < Age  $\leq$  5, Age > 5 are presented in Table 5. Hypermethylation of *CASP8* was not observed among patients with age < 2 years. For age group 2 < Age  $\leq$  5 hypermethylation was 2.3% and for Age > 5 it was 4.28%. Methylation of *CASP8* was not statistically significant in patients with any of these age groups ( $p > 0.05$ ). The percentage of methylated *TMS1* was 8.3% in patients of Age < 2, 7% in patients of 2 < Age  $\leq$  5 and 5.7% in patients of Age > 5. Methylation of *TMS1* was not statistically significant in patients of all of these age groups. The percentage of methylated *DAPK* was 16.6% in patients of Age < 2 years, 11.63% in patients of 2 < Age  $\leq$  5, and 14.28% in patients of Age > 5. Statistically significant association between methylation of *DAPK* gene and development of ALL in patients of age > 5 years as compared to healthy controls was evident (OR = 1.93, 95% CI = 1.62–2.30) (see Table 5).

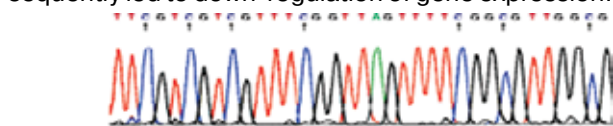
The frequency of methylation of *CASP8*, *TMS1* and *DAPK* in relation to NCRI risk is shown in Table 7. Hy-

permethylation of *CASP8*, *TMS1* and *DAPK* was not found to be statistically significant in patients of standard and high risk groups ( $p > 0.05$ ).

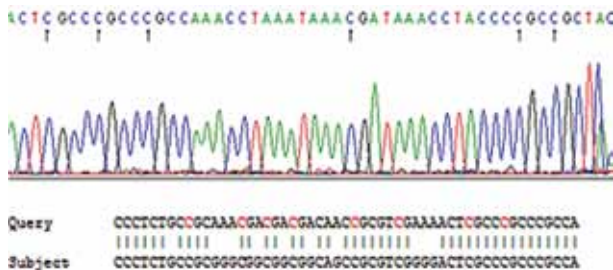
**Table 7.** The frequency of methylation of genes and NCRI risk groups of ALL patients

Genes	Standard (N = 103), n (%)	High (N = 22), n (%)	OR (95% CI)	$p$
	Presence of methylation			
<i>CASP8</i>	3 (2.9)	1 (4.5)	0.91 (0.51–1.61)	0.5
<i>TMS1</i>	6 (5.6)	2 (9.0)	0.90 (0.60–1.36)	0.06
<i>DAPK</i>	13 (12.6)	4 (18.1)	0.92 (0.70–1.21)	0.49

**Bisulfite sequencing analysis.** Treatment of genomic DNA with sodium bisulfate induces deamination of nonmethylated cytosine, and converting it first to uracil and finally to thymidine, but has no effect on methylated cytosine. This modification allows differentiation of methylated and non-methylated CGI in promoter region of tumor suppressor genes by MSP-PCR or sequencing (Fig. 1, 2). The bisulfite sequencing analysis of those samples, which were hypermethylated in their promoter region of these genes as compared with wild type (nonmethylated promoter region) revealed conversion of methylated cytosine, but not nonmethylated cytosine, which subsequently led to down-regulation of gene expression.



**Fig. 1.** Result of bisulfite sequence of *DAPK*



**Fig. 2.** Result of bisulfite sequence of *TMS1*

**Study on the effect of promoter methylation on gene expression by RT-PCR.** The differences in *CASP8*, *TMS1* and *DAPK* gene expression in ALL patients between methylated and nonmethylated studied genes are presented in Table 8. The transcription of these genes was significantly reduced in patients with methylated promoter ( $p < 0.001$ ) in comparison to patients with nonmethylated promoter. Methylation resulted in up to 7.5% down-regulation in transcription for *CASP8*, 22.08% for *TMS1* and 29.5% for *DAPK*. A significant reduction in the mRNA level in relation to patients with selected clinicopathological features in ALL patients ( $p < 0.05$ ) was observed.

**Quantitative RT-PCR.** The real time analysis of methylated and nonmethylated samples confirmed the result of RT-PCR. The hypermethylation of promoter region of *CASP8*, *TMS1* and *DAPK* resulted in their transcriptional down-regulation

by approximately 0.95-fold, 1.95-fold and 2.85-fold, respectively.

**Table 8.** The impact of promoter hypermethylation on expression genes in transcription level among patients of ALL

Genes	Nonmethylated, Mean ( $\pm$ SE)	Methylated, Mean ( $\pm$ SE)	Down-regulation, %	$p$
<i>CASP8</i>	0.748 ( $\pm$ 0.01)	0.691 ( $\pm$ 0.01)	7.58	0.03
<i>TMS1</i>	0.801 ( $\pm$ 0.02)	0.667 ( $\pm$ 0.01)	22.08	0.001
<i>DAPK</i>	0.866 ( $\pm$ 0.02)	0.610 ( $\pm$ 0.05)	29.50	0.001

## DISCUSSION

The present study was carried out to investigate epigenetic changes like promoter hypermethylation of tumor suppressor genes like *CASP8*, *TMS1* and *DAPK* with respect to different clinicopathological factors in childhood ALL within the population under study. *CASP8* encodes an initiator caspase that plays an important role in the Fas-Fas ligand pathway [22, 23]. Alterations of these genes have been described in several neoplasia, like neuroblastoma and medulloblastoma [19, 24]. Harada *et al.* [25] observed frequent methylation of *CASP8* in pediatric rhabdomyosarcomas, medulloblastomas, retinoblastomas and neuroblastomas. The frequency of hypermethylated *CASP8* was found to be low in Wilms tumors and they could not observe any aberrant methylation of *CASP8* in hepatoblastomas, acute leukemias, osteosarcomas and ganglioneuromas and also in healthy controls.

Harada *et al.* [25] reported the absence of methylation of *CASP8* in Ewing sarcoma tumors. In contrast, Fulda *et al.* [26] showed around 65% methylation of *CASP8* in Ewing sarcoma. *CASP8* as reported by other research group was methylated and inactivated in neuroendocrine lung carcinomas (small cell and bronchial carcinoids) but not in non-small cell carcinomas [27]. Also, hypermethylation-mediated down-regulation of caspase-8 has been reported by Wu *et al.* [28]. Structurally, the promoter region of *CASP8* has binding sites for p53, NF- $\kappa$ B, AP-1, SP-1, IRF-1, and Ets-like transcription factors [29]. Therefore, caspase-8 functions both as a pivotal molecule for death-receptor-induced apoptosis and as a selective signal transducer, such as for NF- $\kappa$ B activation [30]. Absence or down-regulation of caspase-8 could cause resistance to apoptosis and correlated with unfavorable disease outcome, such as in childhood medulloblastoma and neuroblastoma [31, 32].

Till now, very few investigations of hematological malignancies have focused on *CASP8* promoter hypermethylation. As caspase-8 mRNA was down-regulated or silenced in the other malignancies, the possibility of promoter hypermethylation induced caspase-8 transcriptional silencing was investigated in the present study. It was observed that the distribution pattern of methylation was different among male ALL patients as compared with healthy controls ( $p < 0.05$ ). These results revealed significant trend towards increasing risk of ALL with methylation of *CASP8* in male



patient samples as compared with healthy controls. To the best of our knowledge, there are no reports on correlation of *CASP8* hypermethylation in childhood ALL. Overall, the hypermethylation of *CASP8* in association with age of patient did not show any impact in development and increasing risk of childhood ALL in our population under study. The cell lineage of ALL does not have any impact on methylation pattern of *CASP8* in our population under study ( $p > 0.05$ ). The bisulfite sequencing results confirm the MSP-PCR data. Regarding the distribution pattern of methylated CGI, no significant pattern was found in this study. The low level of down-regulation of mRNA expression of caspase-8 (7.58%) was due to aberrant promoter methylation in childhood ALL. In the quantitative study of transcription with real time PCR, 0.95-fold down-regulation of caspase-8 was observed.

*TMS1* is commonly silenced by methylation in human cancer cells. *TMS1* down-regulation is induced by overexpression of human DNA methyltransferase-1 [12]. Methylation pattern of *TMS1* and its effect on the mRNA expression of *TMS1* in so many cancer cell lines especially, epithelial cancer cell lines have been shown by many research groups [12, 33]. Methylation of the promoter region of *TMS1/ASC* has been reported in small cell lung cancer and non-small cell lung cancer [34], glioblastoma [35], ovarian, colorectal and prostate cancer [33, 36, 37]. In the present study, hypermethylation of *TMS1* was found in 8 out of 125 (6.5%) ALL patients, which was marginally significant. Our result was contrary to the report of Roman-Gomez *et al.* [38] in ALL patients. Our data revealed no significant trend towards increasing risk of ALL with methylation of *TMS1* gene in both genders among the patient samples as compared with healthy controls ( $p > 0.05$ ). The hypermethylation of *TMS1* in association with the age of patients did not show any impact in development of childhood ALL in the north Indian population ( $p > 0.05$ ). This finding was in consistency with the report of Roman-Gomez *et al.* [38]. Our data indicated that cell lineage of ALL does not have any impact on methylation pattern of *TMS1* in the population under study ( $p > 0.05$ ). It might be assumed that the methylation pattern of *TMS1* in standard risk group and high risk group could not be used as a potential biomarker in risk of ALL. Interestingly, 22.8% down-regulation of *TMS1* mRNA expression was observed in our population under study. In the quantitative study of transcription using real time PCR, 1.95-fold down-regulation of *TMS1* was observed. The analysis of the results of semiquantitative and quantitative PCR showed the effect of hypermethylated promoter in down-regulation of *TMS1*. Furthermore, it is also involved in an intrinsic p53-dependent apoptotic checkpoint [39].

*DAPK* has been shown to be involved in normal cell differentiation and loss of the *DAPK* gene expression was reported in some malignancies as well as cancerous cell lines [8, 9]. Clinically, loss of *DAPK* expression, and therefore, absence of apoptosis in tumor

cells could be considered as a biomarker of disease progression [40]. The promoter region of the *DAPK* contains a CGI that has been shown to be hypermethylated in solid tumors like squamous cell carcinoma of neck in 67% in German population.

Apoptosis is one of the hallmarks of cancer [41]. *DAPK* is an important pro-apoptotic gene inactivated in many tumor types. Several studies of *DAPK* inactivation in lung cancers have been published [42–45] in which hypermethylation of the *DAPK* promoter was shown to be associated with loss of expression [45–47]. Although limited number of reports is available, attempts were made to find out the profile of *DAPK* promoter hypermethylation in childhood ALL in the present study. The results were shown to be completely in contrast to each other in different ethnic groups. It has been reported that *DAPK* promoter was approximately 17% hypermethylated in adult and ~10% in childhood ALL in Hispanic children [38]. In contrast, *DAPK* promoter was not hypermethylated in T-ALL samples of Dutch population [45]. Also, it has been reported from Japanese childhood ALL that in none of the samples *DAPK* was hypermethylated [48]. Aberrant *DAPK* transcription has been reported in leukemia and lymphoma [38]. It was demonstrated that the down-regulation of expression of *DAPK* gene resulting from aberrant gene promoter hypermethylation was associated with disease progression in hematological malignancies [49, 50].

In present work, an attempt has been made to study the involvement of *DAPK* in clinicopathological process of ALL. As *DAPK* transcription was down-regulated in some of the ALL samples analyzed in this study, the possibility of promoter hypermethylation induced *DAPK* transcriptional down-regulation was therefore investigated. It was observed that hypermethylation of *DAPK* was present in 17 out of 125 (13.6%) ALL patients. This is in consistency with the other reports of Roman-Gomez *et al.* and Chim *et al.* [38, 51]. Significant effect of *DAPK* hypermethylation was observed in their population of study. The hypermethylation of *DAPK* in both the genders increased the risk of childhood ALL, but no significant trend towards risk of ALL was observed in our population under study. No significant difference in aberrant methylation of *DAPK* promoter could be observed in all age groups ( $p > 0.05$ ). Also we assume that the methylation pattern of *DAPK* in standard risk group and high risk group did not have any impact towards the increasing risk of ALL in studied population. Unlike other groups which in their reports did not mention percentage of down-regulation for *DAPK* it was tried to find out the percentage of down-regulation of hypermethylated promoter of *DAPK* for each variable of study separately and this was done on the basis of statistical calculation to find out the correlation between each variable and expression of *DAPK* gene. In the present study, 19% down-regulation of *DAPK* was observed. In the quantitative study of transcription with real time PCR, 2.85-fold down-regulation of *DAPK* was observed.

The analysis of result of semiquantitative and quantitative RT-PCR showed the effect of hypermethylated promoter in down-regulation of *DAPK*. It was demonstrated that the down-regulation of mRNA expression of *DAPK* by its aberrant promoter hypermethylation could be considered as one of the factors affecting the *DAPK* transcription.

In this study, we tried to detect the relation among the apoptotic genes which have been selected for this study with respect to variables. In our set of ALL samples, the most common situation is the simultaneous absence of methylation in both promoters but after this, the possible scenarios (*CASP8* methylated alone, *TMS1* methylated alone, *DAPK* methylated alone, *CASP8*, *TMS1* both methylated or *CASP8*, *DAPK* both methylated and *CASP8* and *DAPK* both methylated) are similarly represented. In ALL, hypermethylation in the promoter of these genes was an independent event. In the present study, an attempt was made to detect the relation among the apoptotic genes which have been selected for this study with respect to variable of study of interaction between *CASP8* and *TMS1*. Association in the risk of ALL for each variable of study even when the both the genes were methylated was not detected. Also in interaction between *CASP8* and *DAPK*, no association was found even when both the genes were methylated simultaneously. In the study of interaction between *DAPK* and *TMS1* no association was observed. Significant correlation in interaction among this gene family was not observed. From these results it has been deduced that the hypermethylation in promoter region of these genes was independent to each other. The present findings need to be extended to a larger series.

To sum up, our results showed no significant role of caspase-8 as a tumor suppressor gene in pathogenesis of ALL in north Indian population. Aberrant promoter hypermethylation of *DAPK* and *TMS1* as tumor suppressor genes may support the role in pathogenesis and increase the risk of ALL in north Indian population. The methylation status of the *DAPK* and *TMS1* genes might be considered as a predictive factor in the clinical outcome of ALL.

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