Cancer Science
Japanese Cancer Association JCA
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Epigenetic modifications of splicing factor genes in myelodysplastic syndromes and acute myeloid leukemia

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Key words

Acute myeloid leukemia, DNA hypermethylation, epigenetics, myelodysplastic syndromes, splicing factors

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Funding Information

Young Centenary Foundation; National Health and Medical Research Council of Australia; Cure the Future; Tour de Cure; New South Wales Cancer Council.

Received July 2, 2014; Revised August 28, 2014; Accepted September 1, 2014

Cancer Sci **105** (2014) 1457–1463

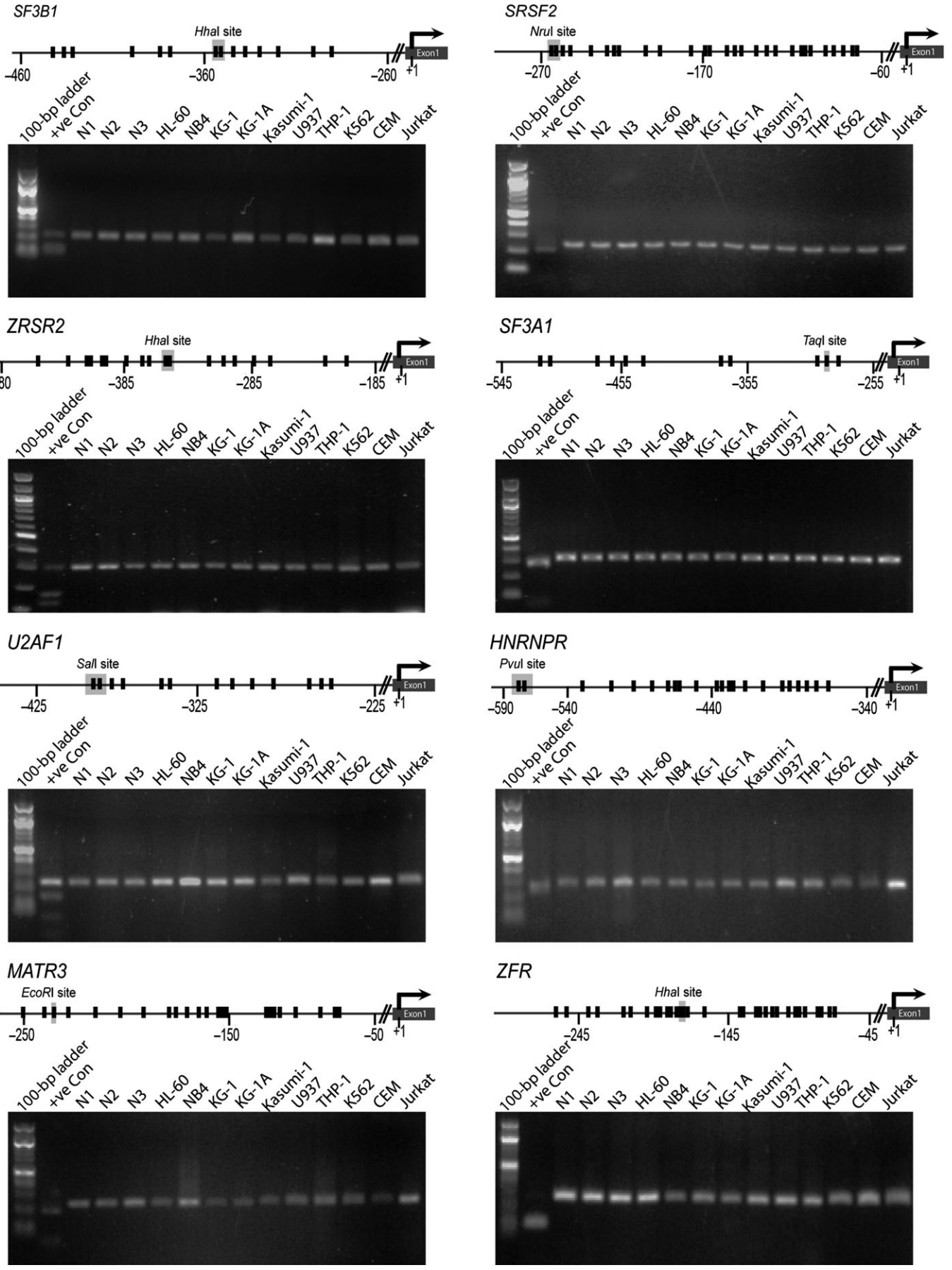
doi: 10.1111/cas.12532

Somatic mutations in splicing factor genes have frequently been reported in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Although aberrant epigenetic changes are frequently implicated in blood cancers, their direct role in suppressing one or both alleles of critical splicing factors has not been previously examined. Here, we examined promoter DNA hypermethylation of nine splicing factors, *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF3A1*, *HNRNPR*, *MATR3*, *ZFR*, and *YBX3* in 10 leukemic cell lines and 94 MDS or AML patient samples from the Australasian Leukemia and Lymphoma Group Tissue Bank. The only evidence of epigenetic effects was hypermethylation of the *YBX3* promoter in U937 cells in conjunction with an enrichment of histone marks associated with gene silencing. *In silico* analysis of DNA methylation data for 173 AML samples generated by the Cancer Genome Atlas Research Network revealed promoter hypermethylation of the gene encoding Y box binding protein 3, *YBX3*, in 11/173 (6.4%) AML cases, which was significantly associated with reduced mRNA expression ($P < 0.0001$). Hypermethylation of the *ZRSR2* promoter was also detected in 7/173 (4%) cases but was not associated with decreased mRNA expression ($P = 0.1204$). Hypermethylation was absent at the promoter of seven other splicing factor genes in all cell lines and patient samples examined. We conclude that DNA hypermethylation does not frequently silence splicing factors in MDS and AML. However, in the case of *YBX3*, promoter hypermethylation-induced downregulation may contribute to the pathogenesis or maintenance of AML.

Aberrant hypermethylation at the promoter region of genes, leading to their silencing, is a hallmark of human cancers including hematological malignancies. This epigenetic abnormality often affects tumor suppressor genes that can also be inactivated due to sequence mutations. In myeloid neoplasia, hypermethylation of tumor suppressor genes including *CDH1*, *CDKN2B*, and *HIC1* have been found in myelodysplastic syndromes (MDS) and are associated with poorer clinical outcomes.[1] Hypermethylation of *CDH1*, *CDKN2B*, and *CEBPA* have been described in acute myeloid leukemia (AML) by independent large cohort studies.[2,3] Promoter hypermethylation can be mono or biallelic; for example, the former acting as the "second hit" in silencing a gene when a mutation exists in one allele as the "first hit".[4]

Recent studies using massively parallel sequencing have identified mutations in genes encoding several components of the mRNA splicing machinery in hematological neoplasia.[5–7] Mutations of splicing factor encoding genes, including *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*, have been found in over 60% of individuals with MDS.[5,7–12] Splicing factor mutations are strongly associated with specific phenotypic features such as *SF3B1* in refractory anemia with ring sideroblasts and *SRSF2* with more advanced forms of MDS including secondary AML and refractory anemia with excess blasts.[7] Mutations of *SF3B1*, *U2AF1*, *HCFC1*, *SAP130*, *SRSF6*, *SON*, *U2AF26*, *LUC7L2*, *PRPF8*, and *SFPQ* have been identified in individuals with AML.[5,7,13,14] However, mutations of splicing factors detected to date are mostly heterozygous missense mutations leading to aberrant RNA splicing. It remains unknown if alterations in splicing factor genes can also be caused by epigenetic defects such as DNA hypermethylation, potentially affecting a second allele when mutation has already occurred in the first. Biallelic promoter hypermethylation may also affect splicing factor genes, as seen in the silencing of other cancer-causing genes.[2,3] Hence, there is a compelling reason to pursue the possibility of epigenetic silencing as a collaborating mechanism in the pathogenesis or maintenance of hematological neoplasms.

Here we have determined the promoter methylation signatures of splicing factor genes in leukemic cell lines and diagnostic bone marrow samples from individuals with MDS and AML. Histone modifications were also investigated in cell lines when promoter hypermethylation was present. We also analyzed publicly available DNA methylation data of 173 AML samples from the Cancer Genome Atlas Research Network (TCGA) for promoter hypermethylation affecting splicing factor genes.



Materials and Methods

Cell lines and patient samples. HL-60, KG-1, KG-1A, Kasumi-1, THP-1, CEM, and Jurkat cells were cultured in RPMI-1640 media containing 10% FCS. NB4, U937, and K562 were cultured in DMEM supplemented with 10% FCS. 5U/mL penicillin, 50 μg/mL streptomycin sulphate, and 2 mM L-glutamine were added to all growth media. DNA samples from MDS, AML, and acute promyelocytic leukemia patients (n = 94) were acquired from the Australasian Leukemia and Lymphoma Group (ALLG) Tissue Bank with ethics approval from the Human Research Ethics Committee of the Royal Prince Alfred Hospital (HREC/08/RPAH/222; Camperdown, NSW, Australia).

DNA methylation analysis. Genomic DNA extracted from cell lines and patient samples were bisulfite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. Combined bisulfite restriction analysis (COBRA) and clonal bisulfite sequencing were carried out as previously described.[15,16] For each candidate gene, bisulfite-converted DNA was amplified using primers that coamplify methylated and unmethylated template at an equal efficiency. Primer sequences are provided in Table S1. Following PCR, amplicons were either digested with a restriction enzyme that discriminates between methylated and unmethylated CpG, or cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA) and sequenced using standard Sanger Sequencing. Reversal of DNA hypermethylation was carried out using 500 nM 5-Aza-2′deoxycitidine for 72 h with drug replenished every 24 h.

Chromatin immunoprecipitation. HL-60 or U937 cells (5×10^6) were fixed in 0.1% formaldehyde. Chromatin immunoprecipitation was carried out using the Millipore Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Anti-H3K4me3, anti-H3K27me3 and IgG control antibodies (Millipore) were used. Following ChIP, quantitative RT-PCRs were carried out using primers designed against the promoter region of *YBX3* (Table S1).

Analyses of DNA methylation and mRNA expression data from the TCGA AML cohort. DNA methylation levels determined using Illumina's Infinium 450 K array were downloaded as β-values. We chose a cut-off of 10% to distinguish between methylated and unmethylated loci, as previously published.[17] All promoter methylation probes located 1 kb upstream of the transcriptional start site were considered for each gene. For genes with multiple promoter methylation probes, analyses were carried out for probes least correlated with mRNA expression to minimize false positive results. mRNA expression was downloaded as RNA-Seq by Expectation Maximization values[18] from the cBioPortal freeware (http://www.cbioportal.org/public-portal.[19,20] Only samples with matched DNA methylation and mRNA expression data were included in this analysis (n = 173).

Analyses of clinicopathological and molecular features. Clinical data for the AML cohort were downloaded from the TCGA repository website (http://cancergenome.nih.gov/). Clinicopathological and molecular features including age, sex, white blood cell count at diagnosis, blast percentage, subtypes, cytogenetic risks, cytoplasm-dislocalized leukemic nucleophosmin protein status, and mutations of *FLT3*, *IDH1*, *RAS* and *DNMT3A* were compared with the methylation status of *YBX3* and *ZRSR2* in AML samples (n = 173).

Statistical analyses. The significance of fold change in mRNA expression and histone mark enrichment was analyzed using Student's t-test. Categorical variables were compared using the Chi-square test or Fisher's exact test. The Mann–Whitney U-test was used to compare continuous variables that were not normally distributed. Analyses were carried out using GraphPad Prism version 5 (La Jolla, CA, USA). P-values < 0.05 were considered significant.

Results

Epigenetic modifications of splicing factor genes in leukemic cell lines. In order to explore the role of promoter DNA hypermethylation in the regulation of splicing factor expression in leukemia, we used COBRA and Clonal Bisulfite Sequencing to determine the promoter methylation signatures of splicing factor genes in eight myeloid: HL-60, NB4, KG-1, KG-1A, Kasumi-1, U937, THP-1, K562, and two lymphoid leukemia cell lines, CEM and Jurkat. We examined a total of nine splicing factors, five of which were previously reported to be mutated in MDS and AML, namely *SF3B1*, *SRSF2, U2AF1*, *ZRSR2*, and *SF3A1*.[5,7–14] The other four, *HNRNPR*, *MATR3*, *ZFR*, and *YBX3*, have been reported to be associated with the spliceosome.[21,22] They have also been found to be aberrantly hypermethylated in AML samples compared to normal reference.[23] Nonetheless, it was not previously reported whether promoter hypermethylation induced their silencing, and how these factors may contribute to the development and/or maintenance of AML.

Our COBRA analysis indicated that methylation was absent at the promoter regions of *SF3B1*, *SRSF2, U2AF1, ZRSR2, SF3A1, HNRNPR*, *MATR3*, and *ZFR* in cell lines (Fig. 1). The *YBX3* promoter was hypermethylated only in the monocytic leukemia cell line U937 (Fig. 2a–c). Hypermethylation of *YBX3* in U937 cells was associated with its reduced mRNA expression. Inhibition of DNA methylation using the DNA methyltransferase inhibitor 5-Aza-2′deoxycytidine increased the expression of *YBX3* following reversal of CpG methylation (Fig. 2d,e), indicating that promoter DNA hypermethylation was associated with the regulation of its expression. DNA methylation also occurred in conjunction with altered histone modifications at the promoter of this gene. We found >100-fold enrichment of the histone mark associated with active transcription, H3K4me3, at the *YBX3* promoter in *YBX3*-expressing HL-60 cells (Fig. 2f). Enrichment of H3K4me3 was not present at the *YBX3* promoter in U937 cells, which lacks *YBX3* expression (Fig. 2f). The silencing mark, H3K27me3 was enriched twofold at the promoter of *YBX3* in U937 but not HL-60 cells (Fig. 2g). Thus, appropriate histone modifications occur concomitantly with promoter DNA methylation to induce silencing of *YBX3* in U937 cells.

Fig. 1. Absence of promoter DNA hypermethylation of splicing factor genes in leukemic cell lines. Gene promoter maps show CpG sites (vertical black lines) and their distance from the transcriptional start site (+1) for each splicing factor gene. Recognition sites for respective restriction enzymes used in combined bisulfite restriction analysis (COBRA) assays are shown on the maps. For each splicing factor, the gel electrophoretogram of COBRA is shown below its respective gene promoter map. +ve Con, positive control, completely methylated human genomic DNA; N1, N2, N3, negative controls, peripheral blood DNA from three healthy individuals.

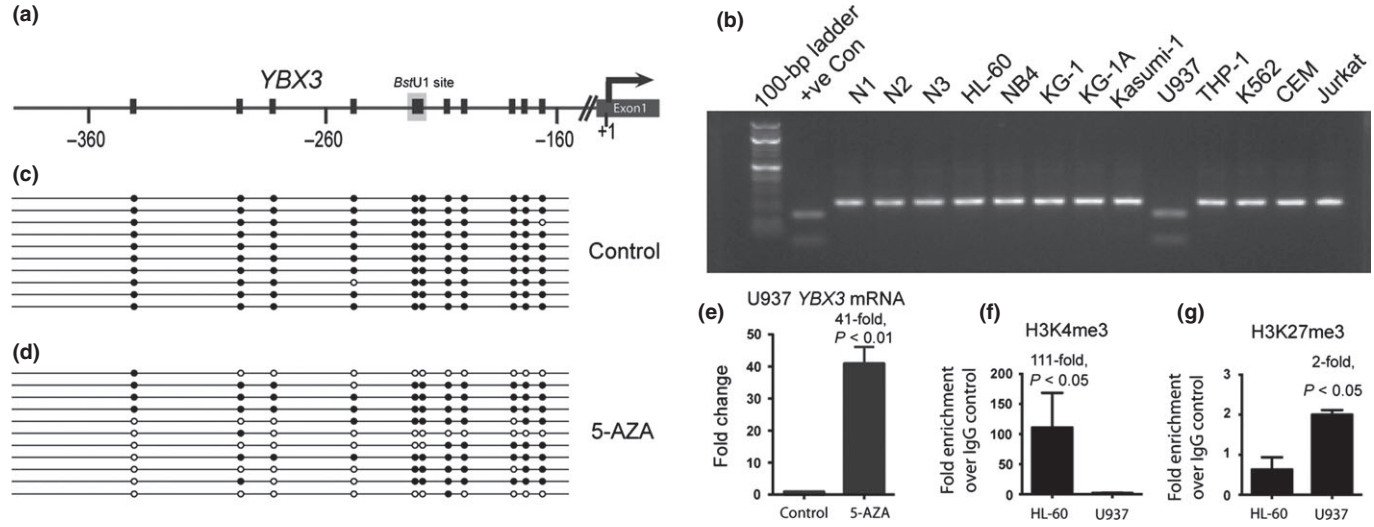


Fig. 2. Silencing of *YBX3* in U937 cells is associated with promoter DNA hypermethylation and altered histone modifications. (a) *YBX3* promoter showing individual CpGs (vertical black line) and their distance from the transcriptional start site (+1). Recognition site for *Bst*U1 used in combined bisulfite restriction analysis (COBRA) assays for *YBX3* is shown on the map. (b) Gel electrophoretogram of COBRA showing PCR amplicons of the *YBX3* promoter following bisulfite conversion and digestion with *Bst*U1 of genomic DNA from leukemic cell lines and controls. (c) Clonal bisulfite sequencing confirming hypermethylation at the *YBX3* promoter in U937 cells. Each row represents a single cloned PCR amplicon aligned to the map shown in (a). Black and white circles denote methylated and unmethylated CpGs, respectively. (d) Reversal of DNA hypermethylation in 5-Aza-2′deoxycytidine (5-AZA)-treated U937 cells as detected by clonal bisulfite sequencing. (e) Quantitative RT-PCR showing increased expression of *YBX3* following treatment of U937 cells with 5-AZA. (f) Fold enrichment of *YBX3* promoter sequence bound to H3K4me3 (activation mark) and (g) H3K27me3 (silencing mark) in HL-60 and U937 cells normalized to IgG control. +ve Con, positive control, completely methylated human genomic DNA; N1, N2, N3, negative controls, peripheral blood DNA from three healthy individuals.

(a)

Splicing factor gene promoter	No. methylated (n = 173)	%
SF3B1	0	0.0
SRSF2	0	0.0
U2AF1	0	0.0
ZRSR2	7	4.0
SF3A1	0	0.0
HNRNPR	0	0.0
MATR3	0	0.0
ZFR	0	0.0
YBX3	11	6.4

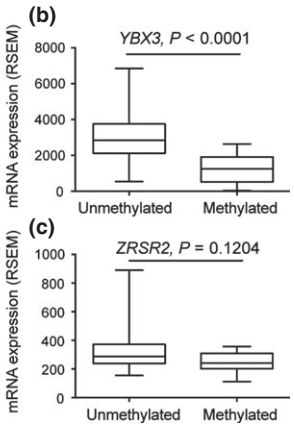


Fig. 3. Hypermethylation-induced downregulation of *YBX3* in the Cancer Genome Atlas Research Network acute myeloid leukemia cohort. (a) *ZRSR2* and *YBX3* promoters were hypermethylated in 7/173 (4.0%) and 11/173 (6.4%) acute myeloid leukemia cases, respectively. Promoter hypermethylation was not detected in other splicing factors. (b) *YBX3* promoter hypermethylation was significantly associated with the loss of *YBX3* mRNA expression ($P < 0.0001$). (c) Promoter hypermethylation of *ZRSR2* was not associated with expression levels of *ZRSR2* mRNA ($P = 0.1204$). RSEM, RNA-Seq by Expectation Maximization.

Promoter hypermethylation of splicing factor genes in primary MDS and AML samples. We next determined the frequency of promoter DNA hypermethylation affecting splicing factor genes in the diagnostic bone marrow of 32 individuals with MDS, 50 with AML, and 12 with acute promyelocytic leukemia (n = 94), from the ALLG Tissue Bank. The clinical characteristics of patients are summarized in Table S2. For MDS samples, the male to female ratio and mean age were comparable to published large cohort studies.[24,25] The AML samples were representative of a broad range of major and minor cytogenetic AML subgroups.

Consistent with our findings in cell lines, we did not observe hypermethylation at the promoter of any of the eight splicing factor genes, *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF3A1*, *HNRNPR*, *MATR3*, and *ZFR* in MDS and AML diagnostic samples (Table S3). The *YBX3* promoter was also not hypermethylated in these samples (Table S3). One caveat in our study is the absence of certain subtypes of MDS and AML, such as refractory anemia with ring sideroblasts associated with the highest levels of *SF3B1* mutations. Promoter hypermethylation of other splicing factors may be present in other MDS and AML subtypes. However, promoter hypermethylation of splicing factors would have been readily detected in our samples if they occur at a similar frequency as known sequence mutations (over 60% for MDS and 14% in AML).[5,7,8,14] We further analyzed publicly available data from TCGA to determine the presence of promoter methylation of these nine splicing factors in 173 AML samples.[14] With the exception of *YBX3* and *ZRSR2*, promoter methylation was not detected (Fig. 3a). The *YBX3* promoter was methylated in 11 samples (6.4%) (Fig. 3a). Importantly, there was a significant association between the presence of promoter

methylation and mRNA expression of this gene ($P < 0.0001$) in AML ($n = 173$) (Fig. 3b). The *ZRSR2* promoter was methylated in seven samples (4%). However, the presence of methylation was not associated with *ZRSR2* mRNA expression ($P = 0.1204$) (Fig. 3c).

We determined the clinicopathological features associated with *YBX3* or *ZRSR2* promoter methylation. The presence of methylation at the *YBX3* and *ZRSR2* promoters was significantly associated with age ($P = 0.0399$) and sex ($P = 0.0079$), respectively, but no other features analyzed (Table 1).

Discussion

Many studies have reported frequent mutations of splicing factors in MDS and AML. Importantly, mutations of specific splicing factors were strongly associated with particular phenotypic features, such as *SF3B1* in MDS with ring sideroblasts[5] and *SRSF2* with more advanced forms of MDS including secondary AML and refractory anemia with excess blasts.[7] Mutations of splicing factors may have prognostic significance, although the impact remains controversial. Several independent studies indicated improved overall survival for MDS patients with *SF3B1* mutations,[8,26,27] but others did not.[28,29] One study reported that mutations of *SRSF2* but not *SF3B1*, *U2AF1*, or *ZRSR2* were associated with poorer overall survival in MDS.[30] Regardless, there is a strong association between altered expression or mutations of splicing genes and splicing changes including exon skipping and intron retention.[31,32] Mutations of individual splicing factors also show particular patterns of splicing in specific gene subsets. For example,

Table 1. Clinicopathological and molecular associations of *YBX3* and *ZRSR2* promoter hypermethylation in the Cancer Genome Atlas Research Network acute myeloid leukemia (AML) cohort

	YBX3 hypermethylation			*ZRSR2* hypermethylation		
	Present ($n = 11$)	Absent ($n = 162$)	*P*-value	Present ($n = 7$)	Absent ($n = 166$)	*P*-value
Age, years						
Median (range)	39 (18–68)	59 (18–88)	**0.0399**	53 (23–66)	59 (18–88)	0.1316
Sex, *n* (%)						
Male	7 (64)	86 (53)	0.4971	7 (100)	86 (52)	**0.0079**
Female	4 (36)	76 (47)		0 (0)	80 (48)	
WBC, k/μL						
Median (range)	27 (3–203)	16 (1–297)	0.5248	22 (1–88)	18 (1–297)	0.8154
Blast, %						
Median (range)	62 (34–86)	72 (0–100)	0.2908	72 (35–86)	71 (0–100)	0.9025
Subtypes, *n* (%)						
M0	1 (10)	15 (9)	0.6555	0 (0)	16 (10)	0.8374
M1	2 (18)	40 (25)		1 (14)	41 (25)	
M2	5 (45)	34 (21)		3 (43)	36 (22)	
M3	0 (0)	16 (10)		1 (14)	15 (9)	
M4	3 (27)	32 (20)		2 (29)	33 (20)	
M5	0 (0)	18 (11)		0 (0)	18 (11)	
M6	0 (0)	2 (1)		0 (0)	2 (1)	
M7	0 (0)	3 (2)		0 (0)	3 (2)	
Not classified	0 (0)	2 (1)		0 (0)	2 (1)	
Cytogenetic risks, *n* (%)						
Low	0 (0)	32 (20)	0.2065	3 (43)	29 (18)	0.2468
Intermediate	9 (82)	94 (58)		3 (43)	100 (60)	
High	2 (18)	34 (21)		1 (14)	35 (21)	
Not available	0 (0)	2 (1)		0 (0)	2 (1)	
FLT3 mutation, *n* (%)						
Mutant	1 (9)	49 (30)	0.1763	2 (29)	48 (29)	0.3163
Wild-type	9 (82)	107 (66)		5 (71)	111 (67)	
Not determined	1 (9)	6 (4)		0 (9)	7 (4)	
RAS mutation, *n* (%)						
Mutant	0 (0)	9 (6)	1.0000	0 (0)	9 (5)	1.0000
Wild-type	11 (100)	150 (92)		7 (100)	154 (93)	
Not determined	0 (0)	3 (2)		0 (0)	3 (2)	
DNMT3A mutation, *n* (%)						
Mutant	2 (18)	40 (25)	1.0000	0 (0)	42 (25)	0.2027
Wild-type	9 (82)	122 (75)		7 (100)	124 (75)	
*NPM*c status, *n* (%)						
Positive	0 (0)	42 (26)	0.0708	1 (14)	41 (25)	1.0000
Negative	11 (100)	117 (72)		6 (86)	122 (73)	
Not determined	0 (0)	3 (2)		0 (0)	3 (2)	

Significant *P*-values are in bold. M0, AML with minimal differentiation; M1, AML without differentiation; M2, AML with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; M5, acute monoblastic and monocytic leukemia; M6/M7, acute erythroid leukemia/acute megakaryoblastic leukemia; *NPM*c, cytoplasm-dislocalized leukemic nucleophosmin protein.

U2AF1 mutations in myeloid neoplasms resulted in specific changes in splicing patterns affecting mainly cell cycle and RNA processing genes.[31] These gene ontology subsets were distinct from those associated with mutations of *SF3B1* and *SRSF2*.[31] These results indicate the potential role of individual splicing factor mutations in the pathogenesis and/or maintenance of MDS and AML through selective impairment of tumor-associated genes within different molecular pathways.

It is important to recognise that most mutations affecting splicing factors such as *SF3B1*, *U2AF1*, and *SRSF2* are more likely to cause gain of functions,[9,33] indicating that they are unlikely to function as tumor suppressors. Therefore, it is not surprising that haploinsufficiency of Sf3b1 alone was insufficient to cause MDS in mice.[34,35] Rather, mutations of these splicing factors may promote further splicing-related aberrancies affecting a series of genes, potentially in a multistep process. The exact sequence of such defects in the pathogenesis of myeloid neoplasm remains elusive and is an important subject of future studies.

In contrast to *SF3B1*, *U2AF1*, and *SRSF2*, mutations of *ZRSR2* have been associated with loss of functions as the majority of these lead to nonsense or frameshift changes.[9,33] Whether loss of *ZRSR2* expression is sufficient to cause MDS or AML awaits the results of *in vivo* experiments.

Irrespective of the consequences of splicing factor mutations in MDS and AML, it is unknown if splicing factors can be rendered defective by other mechanisms. Understanding whether expression of splicing factors can be affected by epigenetic changes is important to obtain a clearer insight into their role in MDS and AML. If promoter hypermethylation of splicing factors had been consistently identified, it would have suggested their role as tumor suppressor genes.

Our data indicate that promoter methylation leads to the silencing of the *YBX3* gene in the myelomonocytic cell line U937 and ~6% of AML samples in the TCGA cohort. It was surprising that *YBX3* methylation was significantly associated with age but no other clinicopathological or molecular features. Independent analysis in a larger cohort would be necessary to confirm this observation.

YBX3 was found to be associated with spliceosomes in large-scale spliceosome capture and mass spectrometry analyses and is therefore considered a splicing factor.[21,22] Nevertheless, its exact role in splicing is as yet undetermined. A previous study reported upregulation of *YBX3* mRNA levels during myeloid differentiation.[36] Therefore, downregulation of YBX3 may lead to perturbation of myeloid differentiation, which is a characteristic of AML. The exact role of promoter hypermethylation-induced downregulation of *YBX3* in AML pathogenesis is currently unclear and is worthy of future studies.

Although promoter methylation of *ZRSR2* was found in 4% of AML samples in the TCGA cohort, it is unlikely to exert an effect on *ZRSR2* expression. Promoter methylation levels for *ZRSR2* were also much lower (average β-value = 13.1%) than *YBX3* (average β-value = 33.1%), indicating that subtle promoter methylation levels affecting *ZRSR2* may not play a substantial role in its regulation.

Overall, our study showed that promoter hypermethylation-induced silencing of splicing factors occurs uncommonly in the subtypes of MDS and AML examined. Epigenetic modification is unlikely either to act as a second hit in the silencing of many splicing factors or to cause biallelic silencing of splicing factors in these types of myeloid neoplasia.

Acknowledgments

We thank Paula Marlton and Megan Ellis from the ALLG Tissue Bank for the provision of patients' data and samples, and Jane Gordon for karyotype risk analysis. We thank the TCGA Research Network for the availability of data from AML patients. This work was supported in part by a Young Centenary Foundation Grant (JJ-LW), National Health and Medical Research Council Project Grants (#1061906 and #1030830, to JEJR), Cure the Future (JEJR), Tour de Cure (JEJR), NSW Cancer Council Grant (#RG1409 to JEJR) and an anonymous foundation (JEJR). JJ-LW is a Cancer Institute of NSW Fellow.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Primer sequences used in this study.

Table S2. Characteristics of individuals with myelodysplastic syndromes or acute myeloid leukemia from the Australasian Leukemia and Lymphoma Group Tissue Bank.

Table S3. Absence of hypermethylation at the promoter of splicing factor genes in myelodysplastic syndrome and acute myeloid leukemia samples from the Australasian Leukemia and Lymphoma Group Tissue Bank.