

Methylation of TET2, CBL and CEBPA in Ph-negative myeloproliferative neoplasms

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ABSTRACT

A loss-of-function mutation of TET2, CBL and CEBPA has been implicated in the pathogenesis or leukaemic transformation of myeloproliferative neoplasm. As tumour suppressor genes may potentially be inactivated by promoter hypermethylation, the authors studied the methylation status of these genes in three cell lines and diagnostic marrow samples from 45 patients with myeloproliferative neoplasm (MPN) (essential thrombocythaemia, N=34; polycythaemia vera, N=7 and primary myelofibrosis, N=4) by methylation-specific PCR. TET2 was heterozygously methylated in MEG-01 and K562 but completely unmethylated in HEL. On the other hand, both CBL and CEBPA were completely unmethylated in all three cell lines. In the primary marrow samples, methylation of TET2 occurred in two (5.9%) patients with essential thrombocythaemia (4.4% of all patients), both without JAK2 V617 mutation, but not in polycythaemia vera or primary myelofibrosis. There was no association between TET2 methylation with the type of MPN ($p=0.713$). Hypermethylation of CBL or CEBPA was not detected in any patients. In summary, methylation of TET2, CBL and CEBPA is infrequent in MPN at diagnosis. The role of methylation of these genes at the time of leukaemic transformation warrants further study.

INTRODUCTION

Philadelphia-negative (Ph^{-ve}) myeloproliferative neoplasm (MPN) is a stem cell disease with proliferation of myeloid compartment, leading to development of distinct clinical entities including polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF).¹⁻³ JAK2 V617F mutation, resulting in constitutive activation of JAK-STAT signalling, occurs in about half of the patients with ET and PMF but in more than 90% of patients with PV.¹ The recent discovery of JAK2 V617F in Ph^{-ve} MPN is a major advance in the understanding of the pathogenesis of MPN, which is characterised by proliferation of the myeloid compartment resulting in distinct clinical entities.

Gene methylation is an alternative mechanism of gene inactivation, and various tumour suppressor genes regulating the cell cycle, apoptosis and cell signalling have been shown to be hypermethylated in haematological malignancies.⁴

Recently, inactivating mutations of several tumour suppressor genes have been detected in various myeloproliferative diseases or myeloid malignancies, including Casitas B lineage lymphoma (CBL), TET2 and CEBPA. CBL, which is localised to 11q23, possesses an E3 ubiquitinated ligase

responsible for termination of activated tyrosine kinases. The tumour suppressor role of CBL has been demonstrated in mouse models, in which *c-Cbl*^{-/-} mice displayed splenomegaly and an expanded haematopoietic progenitor pool, and accelerated blastic crisis when introduced into a BCR-ABL transgenic background.⁵ On the other hand, TET2 (TET oncogene family, member 2), localised to 4q24, is a putative tumour suppressor gene shown to be deleted in MDS/AML and was also shown to be mutated in 24 (12%) of 198 patients with MPN (with or without the JAK2 V617F mutation).⁶ Finally, CCAAT/enhancer binding protein (C/EBP), α , (CEBPA) which is localised to 19q13, is an important tumour suppressor gene regulating myeloid differentiation. Mutation, translocation and methylation of CEBPA have been demonstrated in AML.^{7,8} Moreover, knock-in mice with a targeted mutation in the CEBPA basic region, which specifically inhibits the CEBPA-E2F interaction, predisposed mice to a myeloproliferative disorder.⁹

As these three genes are putative tumour suppressors important in the pathogenesis of myeloid malignancies, we hypothesised that these genes might be inactivated by gene hypermethylation in patients with MPN.

MATERIALS AND METHODS

Cell lines

MEG-01 and K562 cells were kindly provided by Dr Yang Mo, Department of Paediatrics, Queen Mary

Table 1 Clinical demographics

	No of patients (%)
Myeloproliferative neoplasm type	45 (100%)
Polycythaemia vera	7 (15.6%)
Essential thrombocythaemia	34 (75.5%)
Primary myelofibrosis	4 (8.9%)
Gender	
Male	24 (53.3%)
Female	21 (46.7%)
Age (years)	
Median (range)	67.5 (28–89)
CBP	
Hb (g/dl)	13.3 (9–22)
Leucocyte count ($\times 10^9/l$)	14.4 (7–28)
Platelet count ($\times 10^9/l$)	848 (196–2275)
Symptoms at diagnosis*	
Asymptomatic	25 (62.5%)
Bleeding	4 (10%)
Erythromelalgia	4 (10%)
Stroke	2 (5%)
Abdominal pain	3 (7.5%)
Blurred vision	1 (2.5%)
Weight loss	1 (2.5%)

*Data available in 40 patients.
 CBP, complete blood picture.

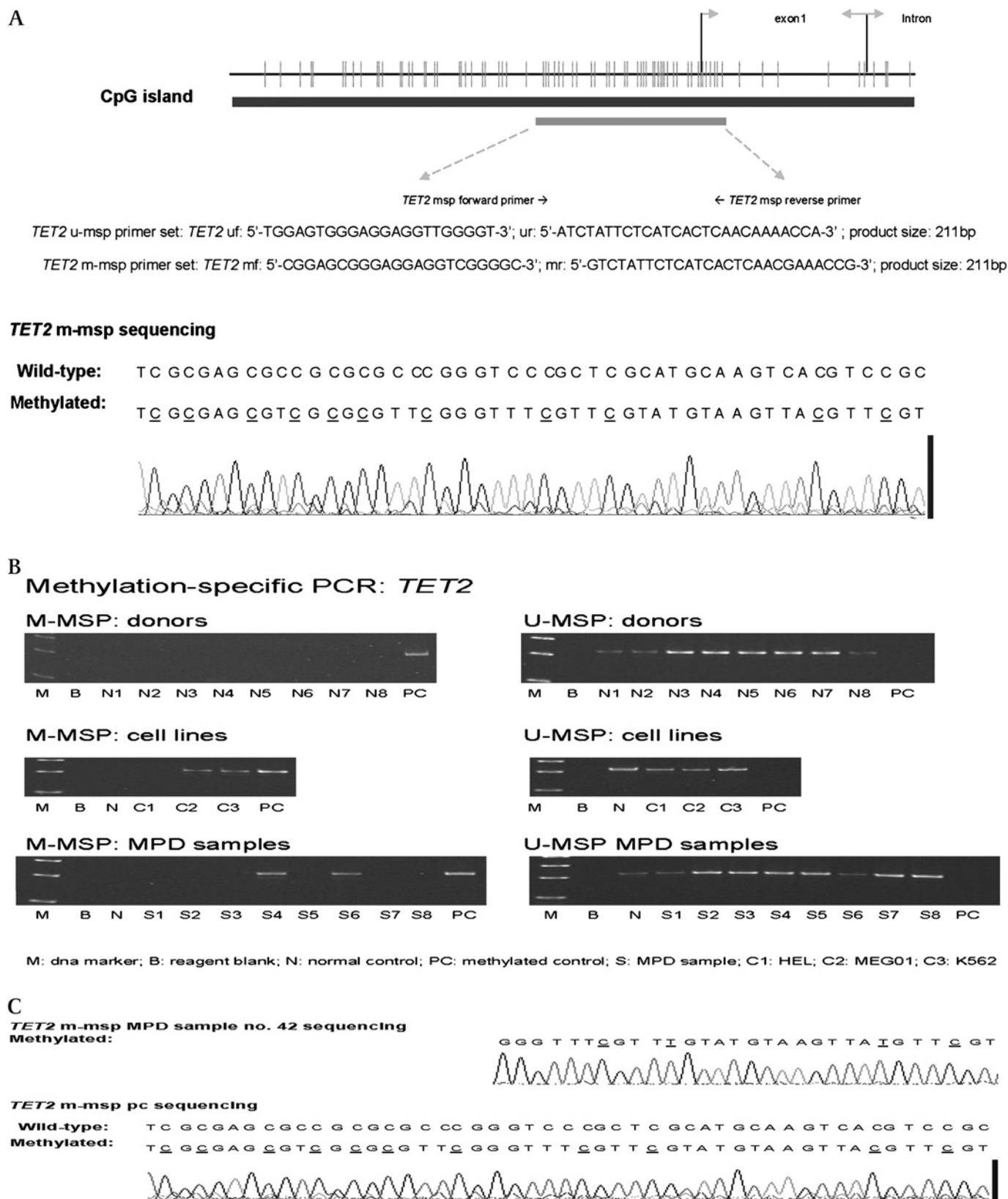


Figure 1 (A) CpG island of *TET2* gene with location and sequence of methylation-specific polymerase chain reaction (MSP) primers (mf, forward M-MSP primer; mr, reverse M-MSP primer; uf, forward U-MSP primer; ur, reverse U-MSP primer). (B) Direct sequencing of methylated positive control showing complete bisulfite conversion, and authenticity of MSP amplification. (C) M- and U-MSP of normal unmethylated controls (N1–N8), cell lines (C1–C3) and primary marrow samples (S1–S8). M, molecular weight marker; NC, normal unmethylated control; PC, methylated positive control.

Short report

Hospital, Hong Kong; and HEL from Dr Dong-Er Zhang, Department of Pathology and Molecular Biology, Moores Cancer Centre, University of California, San Diego, USA.

Patient and diagnosis

Diagnosis of MPN including ET, PV and PMF was based on WHO criteria.¹ The clinical demographics of the patients are illustrated in table 1.

Methylation-specific polymerase chain reaction (MSP)

DNA was extracted from cell lines and primary bone marrow samples at diagnosis using a standard method. Using computer software, the presence of CpG islands at the corresponding gene

promoters were determined, and unmethylated and methylated MSP primers were designed. Treatment of DNA with bisulfite for conversion of unmethylated cytosine to uracil (but unaffected methylated cytosine) was performed with a commercially available kit (CpGenome DNA modification kit, Chemicon, New York). Methylation-specific PCR (MSP) was then validated in methylated (positive) and unmethylated (negative or normal) controls (DNA from normal cells) with methylated and unmethylated MSP primers.

RESULTS

Presence of a CpG island at the 5' untranslated regions (UTRs) for the all three genes was confirmed (figures 1–3).

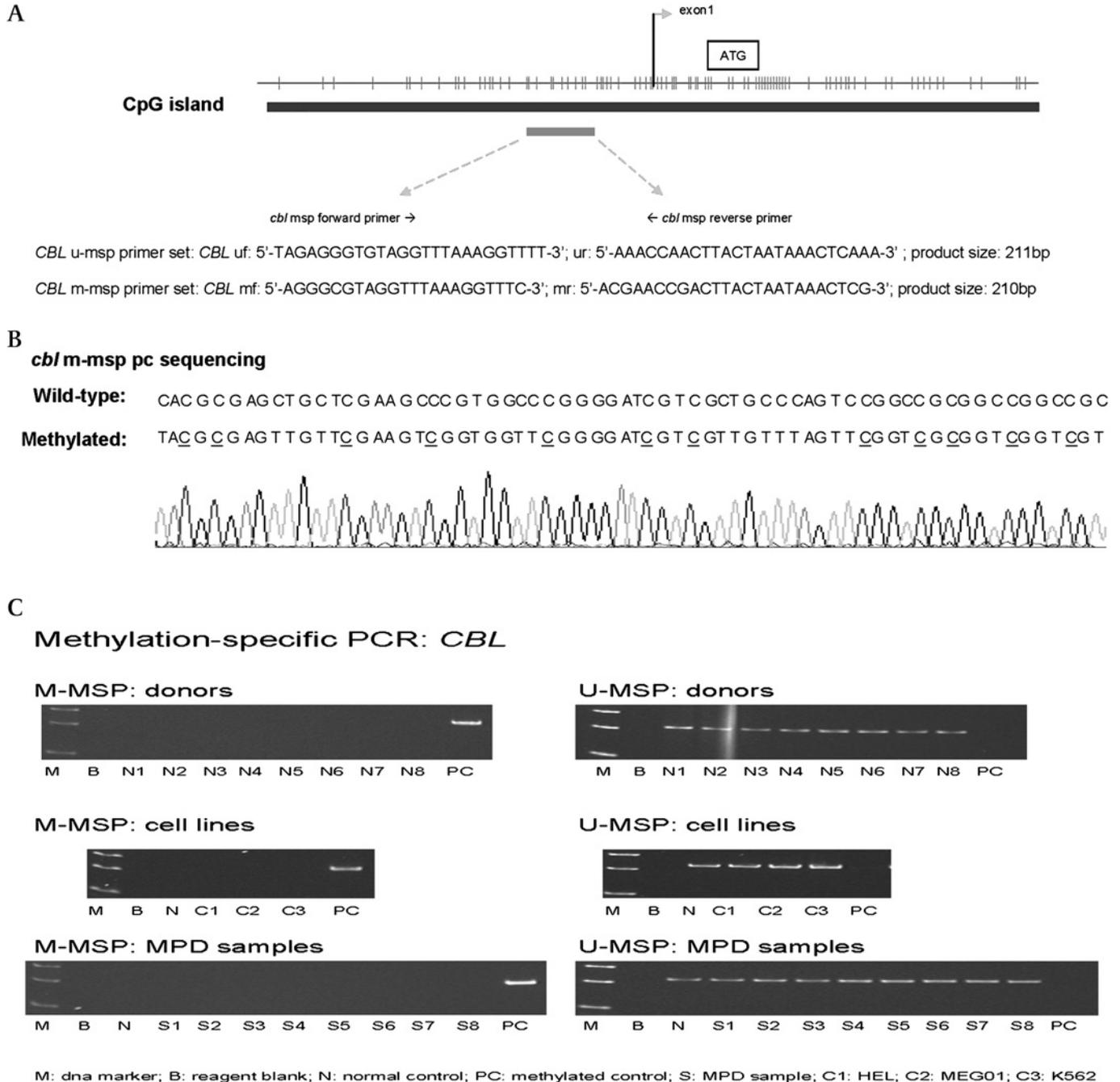
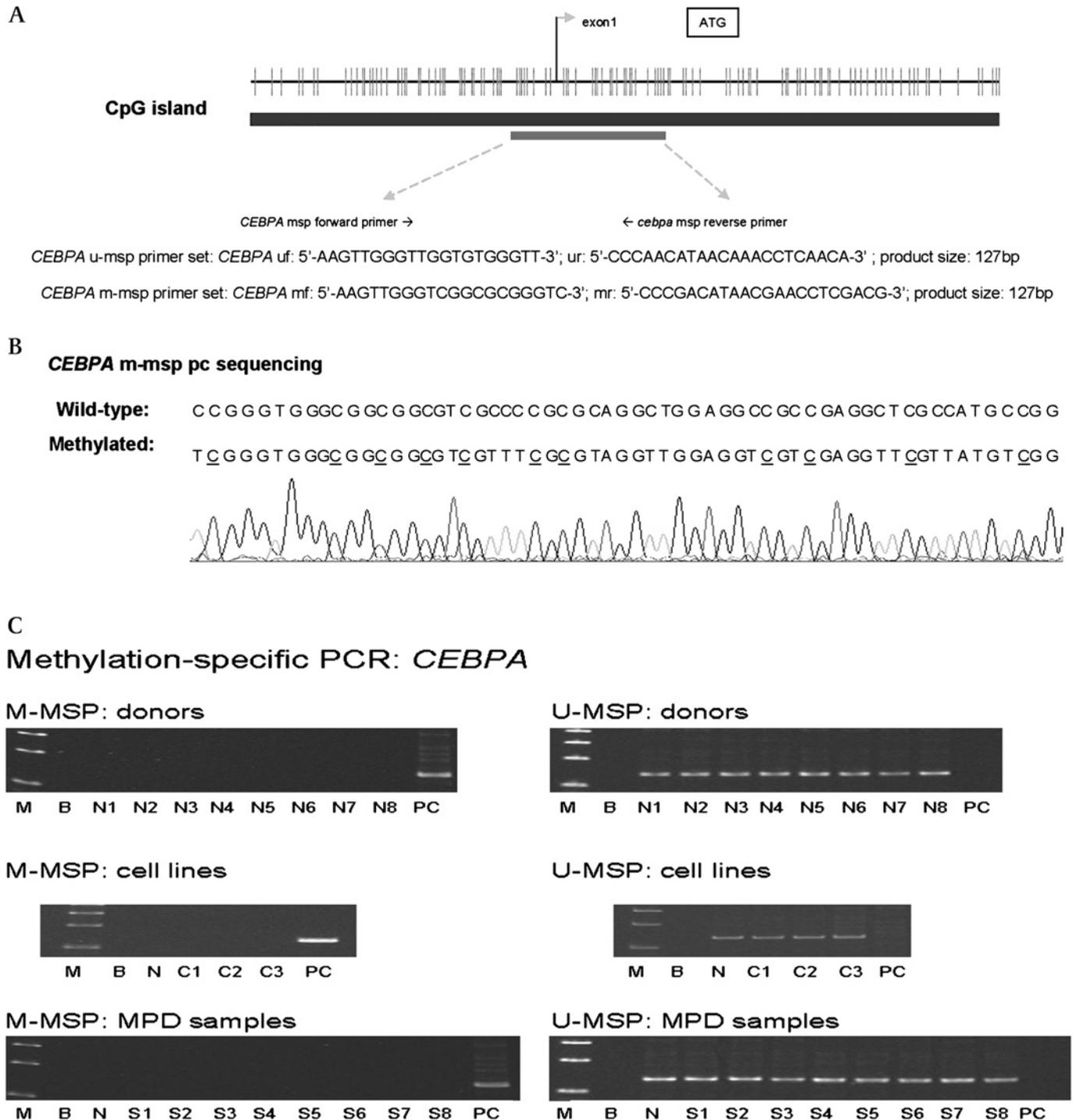


Figure 2 (A) CpG island of *CBL* gene with location and sequence of methylation-specific polymerase chain reaction (MSP) primers (mf, forward M-MSP primer; mr, reverse M-MSP primer; uf, forward U-MSP primer; ur, reverse U-MSP primer). (B) Direct sequencing of methylated positive control showing complete bisulfite conversion, and authenticity of MSP amplification. (C) M- and U-MSP of normal unmethylated controls (N1–N8), cell lines (C1–C3) and primary marrow samples (S1–S8). M, molecular weight marker; NC, normal unmethylated control; PC, methylated positive control.

Methylation-specific PCR (MSP) was then performed in methylated (positive) and unmethylated (negative or normal) controls (DNA from normal cells) with methylated and unmethylated MSP primers, which showed methylation of genes in the positive but not the normal DNA controls (figures 1–3). Direct sequencing of the methylated MSP of positive control confirmed specificity of the methylated amplification, in which

methylated cytosine remained as cytosine upon sequencing (underlined) while unmethylated cytosine appeared as thymidine (figures 1–3). MSP was performed on cells from these three cell lines, HEL, MEG-01 and K562. HEL carried JAK2 V617F mutation, and MEG-01 and K562 were derived from blastic transformation of patients with chronic myeloid leukaemia. TET2 was heterozygously methylated in MEG-01 and K562



M: dna marker; **B:** reagent blank; **N:** normal control; **PC:** methylated control; **S:** MPD sample; **C1:** HEL; **C2:** MEG01; **C3:** K562

Figure 3 (A) CpG island of CEBPA gene with location and sequence of methylation-specific polymerase chain reaction (MSP) primers (mf, forward M-MSP primer; mr, reverse M-MSP primer; uf, forward U-MSP primer; ur, reverse U-MSP primer). (B) Direct sequencing of methylated positive control showing complete bisulfite conversion, and authenticity of MSP amplification. (C) M- and U-MSP of normal unmethylated controls (N1–N8), cell lines (C1–C3) and primary marrow samples (S1–S8). M, molecular weight marker; NC, normal unmethylated control; PC, methylated positive control.

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Take-home messages

- ▶ Loss-of-function mutation of TET2, CBL and CEBPA has been implicated in the pathogenesis or leukaemic transformation of MPN.
- ▶ Methylation of TET2, CBL and CEBPA is infrequent in MPN at diagnosis.
- ▶ The role of methylation of these genes at the time of leukaemic transformation warrants further study.

but completely unmethylated in HEL (figure 1). On the other hand, both CBL and CEBPA were completely unmethylated in all three cell lines (figures 2, 3).

In the primary diagnostic marrow samples, methylation of TET2 occurred in two (5.9%) patients with ET (4.4% of all patients) (figure 1), and none of the patients carried hypermethylation of CBL or CEBPA (figures 2, 3). These two patients did not carry a JAK2 V617F mutation. However, there was no association between TET2 methylation with the type of MPN ($p=0.713$).

DISCUSSION

Recent studies strongly suggested that TET2 is a tumour suppressor gene as a TET2 mutation has been demonstrated to be acquired in 12–24% of myeloid neoplasms including AML, MDS, CMML and MPN. Moreover, while occasional patients possessed homozygous TET2 deletion, the majority showed a TET2 mutation together with gene deletion, thereby implicating a tumour suppressor role of TET2 in myeloid neoplasms. In particular, it is mutated in 12–13% of MPN including ET, PV and PMF.^{6–10} Therefore, we postulated that TET2 hypermethylation might occur. Indeed, it was demonstrated in two (5.9%) patients in this cohort, and hence might be a possible mechanism to result in biallelic gene inactivation, and hence fulfil Knudson's hypothesis, which proposed that both alleles of a tumour suppressor gene have to be inactivated, usually by deletion and/or mutation, for complete abrogation of its function.¹¹ The low frequency of TET2 gene hypermethylation was similar to the finding of absence of TET2 hypermethylation in another cohort of 354 MPN patients.¹² CBL is a tumour suppressor gene responsible for termination of receptor-associated tyrosine kinase signalling. The most common abnormality in myeloid neoplasms, in particular CMML, is the acquisition of uniparental disomy of a mutant allele of CBL, leading to prolonged activation of tyrosine kinases after cytokine stimulation.⁵ In a study of CBL mutation in 577 patients with various haematological myeloproliferative disorders including 151 patients with Ph^{-ve} MPN (74 PV, 53 PMF and 24 ET), the CBL mutation was detected in 26 patients. Three patients with PMF but none of those with PV or ET

(ie, 2% of the Ph^{-ve} MPN patients) were found to carry CBL mutation.¹³ Our study showed that there was no methylation of CBL in MPN. Therefore, CBL mutation or methylation appeared infrequent in Ph^{-ve} MPNs.

Finally, CEBPA methylation has been shown in up to 51% of AML patients,¹⁴ and hence might be important in myeloid malignancy. It is one of the first reports of the absence of methylation of this gene in MPN patients. However, as inactivation of CEBPA might be responsible for leukaemic transformation, it will be important to study CEBPA hypermethylation in patients with leukaemia transforming from underlying MPN.

In summary, methylation of TET2, CBL and CEBPA is infrequent in MPN at diagnosis. The role of methylation of these genes at the time of leukaemic transformation warrants further study.

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Institutional Review Board of Queen Mary Hospital.

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