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## Methylation of cyclin-dependent kinase inhibitors, XAF1, JUNB, CDH13 and soluble Wnt inhibitors in essential thrombocythaemia

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# Methylation of cyclin-dependent kinase inhibitors, XAF1, JUNB, CDH13 and soluble Wnt inhibitors in essential thrombocythaemia

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## ABSTRACT

**Background** Methylation of genes regulating cell-cycle check-point (INK4 cyclin-dependent kinase inhibitors), apoptosis (XAF1), adhesion (CDH13), JUNB and Wnt signalling (soluble Wnt inhibitors) has been implicated in pathogenesis of haematological and epithelial cancers.

**Method** The authors studied the methylation status of CDKN2A, CDKN2B, XAF1, CDH13, JUNB and a panel of soluble Wnt inhibitors including *WIF1*, *DKK3*, *APC*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* by methylation-specific PCR in 31 bone marrow and 21 peripheral blood samples of patients with essential thrombocythaemia.

**Results and discussion** There was no evidence of hypermethylation of all these genes in both the BM and PB samples. Therefore, in contrast to myeloid leukaemias, methylation of these genes regulating the cell cycle, apoptosis, adhesion and Wnt signalling does not play an important role in the pathogenesis of myeloproliferative diseases. Whether differential methylation may occur in the progenitor or mature blood cell compartments remains to be verified. Our study contributes to the literature on methylation in chronic myeloproliferative diseases.

## INTRODUCTION

ET is a stem cell disease with proliferation of the myeloid compartment, in particular the megakaryocytic lineage, leading to thrombocytosis.<sup>1 2</sup> About half of the patients carry the JAK2 V617F mutation with constitutive activation of JAK-STAT signalling.<sup>2</sup> On the other hand, pathogenesis in those without JAK2 V617F mutation remains unknown. Moreover, while inactivation of tumour suppressor genes negatively regulating cell-cycle progression, apoptosis,<sup>3</sup> adhesion and Wnt signalling contributes to carcinogenesis, the role of these regulators in ET is rarely reported.

Aberrant promoter DNA methylation (gene hypermethylation) results in gene silencing, and has been implicated in the pathogenesis of epithelial and haematopoietic cancers.<sup>4</sup> Deregulation of the cell cycle, apoptosis and Wnt signalling, by virtue of methylation, and hence silencing, of cyclin-dependent kinase inhibitors, pro-apoptotic factors and soluble Wnt inhibitors has been reported in a wide array of haematopoietic cancers.<sup>4–7</sup>

The cell cycle is tightly regulated at the G<sub>1</sub>S check-point by the INK4 and CIP/KIP families of CKI.<sup>3</sup> The Wnt signalling pathway, important in haematopoiesis, has also been implicated in leukaemogenesis.<sup>8</sup> In the canonical Wnt pathway, binding of Wnt to Frizzled (Wnt receptor) leads to inhibition of GSK3 $\beta$ , resulting in hypophosphor-

ylation of  $\beta$ -catenin, and hence activation of transcription of target genes.

The canonical Wnt pathway is negatively regulated by a multitude of soluble factors in the extracellular milieu. These comprise: (1) Wnt inhibitory factor 1 (*WIF1*), secreted Frizzled related proteins (SFRPs) and (3) the Dickkopf (Dkk) family of secreted proteins. Both *WIF1* and SFRP bind to Wnt ligands and thus inhibit their activities. Besides, SFRPs may also downregulate Wnt signalling by the formation of inhibitory complex with Frizzled receptors. On the other hand, DKKs acts by depleting Frizzled co-receptors, LRP receptors, from the surface, thereby resulting in failure of Frizzled activation even in the presence of Wnt ligands.<sup>8</sup> XAF1, which binds to XIAP, is emerging as an important tumour suppressive pro-apoptotic factor recently shown to be hypermethylated in gastric cancer.<sup>9</sup> Finally, CDH13 and JUNB, were frequently hypermethylated in another myeloproliferative disease, chronic myeloid leukaemia (CML).<sup>10 11</sup> Moreover, an MPD-like phenotype has been demonstrated in JUN-B knock-out mice.<sup>12</sup>

Herein, we studied the role of hypermethylation of CKIs, XAF1, CDH13, JUNB and a panel of seven soluble Wnt antagonists including *WIF1*, *DKK3*, *APC*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* in ET. Moreover, as mature blood cells in the peripheral blood are derived from cellular differentiation of the bone marrow progenitors, we postulated that there might be a differential methylation pattern in the bone marrow and peripheral blood cell compartments.

## MATERIALS AND METHOD

### Patient diagnosis

Sixteen (51.6%) female and 15 male (48.4%) patients with a median age of 60 years (range: 28–89) were studied. Diagnosis of ET was based on the 2001 WHO criteria,<sup>13</sup> including a persistent thrombocytosis of  $>600 \times 10^9/l$ , and exclusion of reactive causes, MDS, CML (by the absence of t(9;22)(q34;q11) on conventional karyotyping, fluorescence in situ hybridisation or BCR/ABL rearrangement on reverse transcription polymerase chain reaction, RT-PCR), and other myeloproliferative diseases (MPD) by a haemoglobin level  $<16.8$  g/dl with normal marrow iron store and serum ferritin level, absent or minimal marrow fibrosis. The study has been approved by the IRB of Queen Mary Hospital with informed consent. JAK2V617F mutation, by allele-specific PCR, was present in 18 (58.1%) of these ET patients (data not shown).

### Methylation-specific polymerase chain reaction (MSP)

DNA was extracted from 31 bone marrow and 21 peripheral blood samples at diagnosis by the standard method. Of the 21 patients with peripheral blood (PB) buffy coat DNA, concomitant bone marrow (BM) samples were available. MSP for aberrant gene promoter methylation was performed as previously described.<sup>5,6</sup> 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). The methylation status of *Wif1*, *DKK3*, *APC*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* was tested. Primers for the methylated (M-MSP) and unmethylated (U-MSP) promoters were previously reported,<sup>5,7</sup> and those for XAF1, CDH13 and JUNB were listed in table 1.<sup>9–11</sup> DNA from eight normal bone marrow donors was used as a negative control, while methylated control DNA (CpGenome Universal Methylated DNA, Chemicon) was used as a positive control in all the experiments. MSP was performed in a thermal cycler (9700, ABI Biosystems, Foster City, California) with the following cycling conditions: 95°C for 4 min, 35 cycles of 95°C for 45 s, specific annealing temperature for 30 s, 72°C for 30 s and a final extension of 10 min at 72°C. The MSP mixture contained 50 ng of bisulfite-treated DNA, 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub>, 10 pmol of each primer, 1×PCR buffer and 2.5 units of AmpliTaq Gold DNA Polymerase (ABI Biosystems) in a final volume of 50 µl. Ten microlitres of PCR products was loaded onto 6% non-denaturing polyacrylamide gels, electrophoresed and visualised under ultraviolet light after staining with ethidium bromide. *SFRP3* was not studied because of the absence of a CpG island. The study complied with the Declaration of Helsinki and has been approved by the Institutional Review Board of Queen Mary Hospital with informed consent.

### RESULTS

The DNA sequence of methylated control DNA for CDH13, XAF1 and JUNB was aligned and compared with the germline sequence of the wild-type DNA (WT) (figure 1). Methylated cytosine residues in CpG dinucleotide remained as 'C,' whereas unmethylated cytosine were read as 'T' after bisulfite conversion. None of the eight normal control marrows showed aberrant methylation of *APC*, *DKK3*, *Wif1*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* (figure 1). The positive and negative controls showed the expected MSP results (normal DNA: U-MSP positive/M-MSP negative; methylated DNA: U-MSP negative/M-MSP positive). There was no methylation of CKIs (*CDKN2A*, *CDKN2B*), XAF1, CDH13, JUNB and all the seven soluble Wnt inhibitors in both the peripheral blood and bone marrow samples.

### DISCUSSION

The essence of gene hypermethylation is that it serves as an alternative mechanism of gene inactivation. Therefore, it will be important if gene silencing could be demonstrated in case a tumour suppressor gene is shown to be hypermethylated. However, in this study, none of the genes were hypermethylated. Moreover, it is unclear if all the marrow cells in MPD belong to the neoplastic clone, or if there is residual haematopoiesis. If so, the expression of unmethylated alleles in the normal haematopoietic cells will confound the expression study.

There are a few points worthy of discussion. First, while *CDKN2B* (alias *p15*) is frequently methylated in AML including myelodysplastic syndrome (MDS),<sup>6</sup> there is an absence of methylation of all CKIs in all the ET samples. Despite the fact that ET is a clonal myeloproliferative disease, in which myeloid transformations including AML may occur, none of the cases here showed methylation of the panel of CKIs including *CDKN2B*. However, it would be interesting to determine whether the methylation status of *CDKN2A* and *CDKN2B* may be acquired during leukaemic transformation from an underlying MPD.<sup>1</sup>

Second, in previous studies of the methylation of the same panel of soluble Wnt inhibitors in other forms of haematological malignancies,<sup>7</sup> we have shown that multiple Wnt inhibitors may be hypermethylated in the same sample. Therefore, to avoid missing methylation in any one of these soluble inhibitors, the methylation status of the whole panel of seven inhibitors was studied. Despite this comprehensive approach, none of the ET samples displayed methylation of the soluble Wnt inhibitors.

Third, we postulated that the pattern of methylation may be different in myeloid progenitors and peripheral blood granulocytes. If so, methylated genes in the myeloid progenitors will show methylated signal in the bone marrow sample, while methylated genes in the granulocytes will result in methylated signals in both the BM and PB compartments. This is particularly relevant, as Wnt signalling is important in haematopoiesis.<sup>8</sup> However, none of the samples showed any methylation of any of these seven soluble Wnt inhibitors, suggesting that methylation of these soluble Wnt inhibitors is unimportant in the pathogenesis of ET. Moreover, as none of these Wnt inhibitors is hypermethylated in either the bone marrow or the peripheral blood cellular components, the hypothesis of differential methylation in the progenitor and mature blood cell compartments could not be verified.

Finally, the methylation of various tumour suppressors has been extensively studied in leukaemias and lymphoma but not myeloproliferative disease. Only recently, there were a few reports of methylation of the negative regulators of the

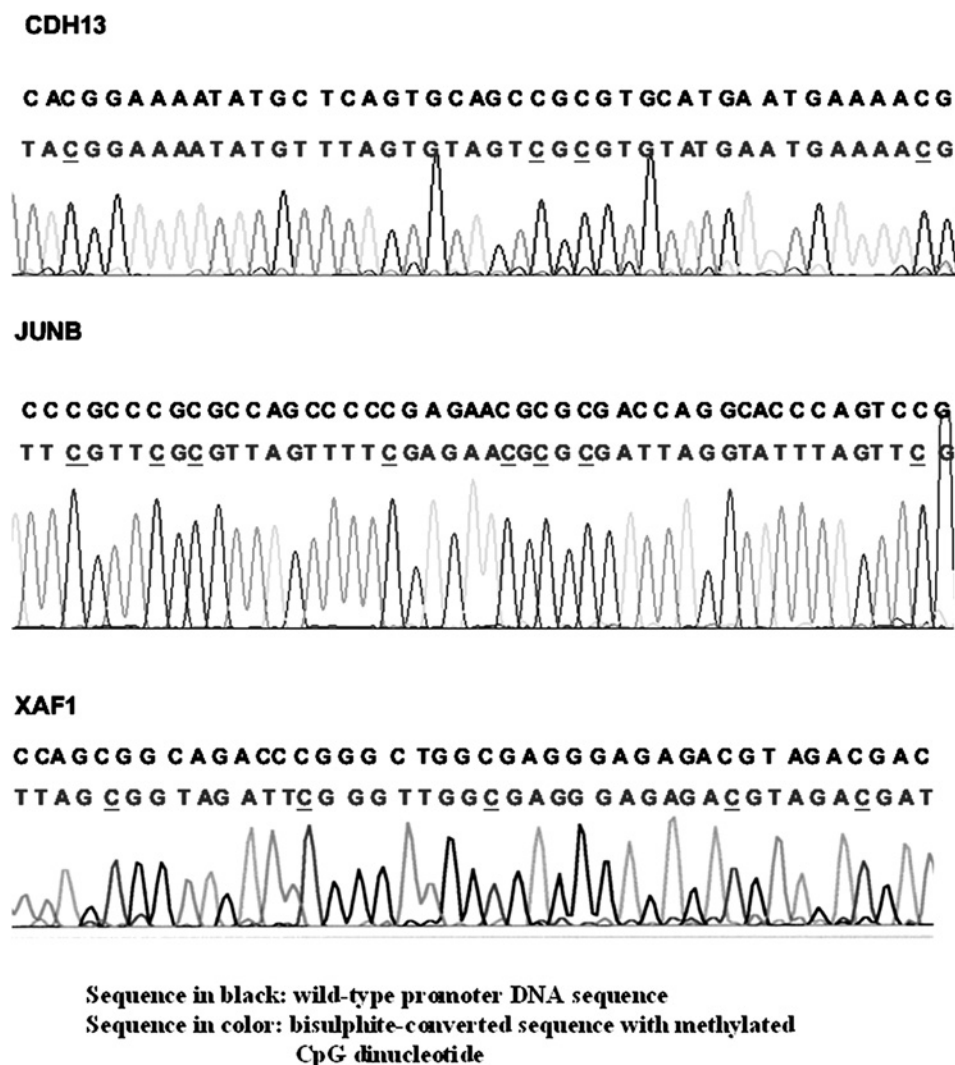
**Table 1** Methylation-specific polymerase (MSP) chain reaction: primer sequences and reaction conditions

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Reference
<i>XAF1</i>			Zou <i>et al</i> <sup>9</sup>
M-MSP	TTGTAAGA AACGAAATTTAATCGA	CCTACCCTTAAAACCCAGAT	
U-MSP	TTGTAAGAAATGAAATTTAATTGA	CTCCTACCCTTAAAACCCACAAT	
<i>CDH13</i>			Roman-Gomez <i>et al</i> <sup>10</sup>
M-MSP	TCGCGGGGTTTCGTTTTTCGC	GACGTTTTTCATTACATACACGCG	
U-MSP	TTGTGGGGTTGTTTTTGT	AACTTTTCATTACATACACA	
<i>JUNB</i>			Yang <i>et al</i> <sup>11</sup>
M-MSP	GACGTTAGGAAAGTTATCGC	CGAACTAAATACCTAATCGCG	
U-MSP	TTGGGGGAAATGA	ACTACAACAAACACAAA	
	TGTTAGGAAAGTTATTGT	CTCTCCACTACA	

Primer sequences kindly offered by S Baylin.<sup>7</sup>

M-MSP, MSP chain reaction for the methylated allele; Tm, annealing temperature; U-MSP, MSP chain reaction for the unmethylated allele.

**Figure 1** Direct bisulfite sequencing of methylated control showing conversion of unmethylated cytosine to thymidine but conservation of methylated cytosine in methylated CpG dinucleotides.



JAK-STAT signalling pathway, SOCS1 and SOCS3, in various CMPD regardless of the JAK2 V617F mutation status.<sup>13 14</sup> These reports are interesting, as they confirmed our previous finding that SOCS1 exon 2 is hypermethylated, even in normal controls, and hence is not suitable for methylation study.<sup>6</sup> Moreover, these studies suggest a redundant role of SOCS methylation in those patients carrying JAK2 V617F mutation, which would have rendered constitutive activation of JAK/STAT signalling in the affected myeloid cells, resulting in myeloid proliferation and hence clinical CMPD.

In conclusion, our study showed that methylation of the putative tumour suppressor genes negatively regulating the cell cycle, apoptosis or Wnt signalling is not important in the

pathogenesis of ET. These data contribute to the literature on methylation in chronic MPDs.

**Competing interests** None.

**Patient consent** Obtained.

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

**Contributors** CSC: patient care, study design, supervision of experiment, writing of manuscript, manuscript approval. TKF: laboratory study of the samples. RL: patient care, supervision of experiment, manuscript approval.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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## Take home messages

In contrast to acute leukaemias, genes regulating cell cycle, apoptosis and Wnt signalling are not hypermethylated in essential thrombocythaemia. Moreover, although a MPD-like phenotype has been demonstrated in JUN-B knock-out mice, JUN-B is not hypermethylated. Therefore, methylation of other tumour suppressor genes should be studied.

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