Distinct association between aberrant methylation of Wnt inhibitors and genetic alterations in acute myeloid leukaemia

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BACKGROUND: Aberrant activation of Wnt signalling through hypermethylation of Wnt inhibitor genes is involved in several human malignancies, including acute myeloid leukaemia (AML). It remains unclear whether hypermethylation of Wnt inhibitors is associated with molecular gene mutations in the development of AML.

METHODS: We investigated the association of hypermethylation of six Wnt inhibitors (Wif-1, SFRP1, SFRP2, SFRP4, SFRP5, and DKK1) with gene aberrations in the leukaemogenesis of 269 AML patients.

RESULTS: In total, 166 patients (61.7%) had hypermethylation of at least one Wnt inhibitor. The majority (68.5%) of patients with Wnt inhibitor hypermethylation had concurrent Class II gene mutations that affect transcription factors or cofactors. There was a close association of Wif-1 hypermethylation with t(15;17) (P = 0.0005) and CEBPA mutation (P < 0.0001), DKK1 hypermethylation with t(8;21) (P < 0.0001) and ASXL1 mutation (P = 0.0078), SFRP-1 hypermethylation with t(8;21) (P < 0.0001), SFRP-2 hypermethylation with AML1/RUNX1 mutation (P = 0.0012), and SFRP-5 hypermethylation with MLL/PTD (P = 0.0505). On the other side, hypermethylation of Wnt inhibitors was always negatively associated with NPM1 mutation and FLT3/ITD.

CONCLUSION: There was distinct association between hypermethylation of individual Wnt inhibitors and specific gene aberrations, especially Class II mutations. The Wnt inhibitor hypermethylation might interact with genetic alterations in the leukaemogenesis.

Keywords: Wnt pathway inhibitors; methylation; genetic alteration; acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a heterogeneous group of haematological malignancies with great variability in the pathogenesis and clinical course. A two-hit model proposes that the development of AML requires cooperation between at least two classes of gene mutations; Class I mutations, such as FLT3, RAS, JAK2, PTPN11, and KIT mutations activate genes in the kinase signalling pathways conferring proliferation and/or survival advantage to haematopoietic cells and Class II mutations, such as RUNX1/ RUNX1T1, PML/RARα, GFBF/MYH11, MLL/PTD, AML1/RUNX1, and CEBPA mutations, affect transcription factors impairing haematopoietic differentiation (Gilliland, 2002; McCormack et al., 2008; Renneville et al., 2008). Recently, inappropriate gene silencing through epigenetic modification, such as aberrant methylation in the promoter areas of suppressor genes, was also found to affect the development and progression of malignancies (Jones and Baylin, 2002). A link between genetic and epigenetic changes has been well demonstrated in colorectal cancer (Herman et al., 1998).

The Wnt pathway is highly conserved and instrumental in the embryogenesis and tissue maintenance (Reya et al., 2003; Willert et al., 2003). Activation of the Wnt/β-catenin pathway has been shown to be crucial for the establishment of normal and leukaemic stem cells (Austin et al., 1997; Reya et al., 2003; Wang et al., 2010). Dysregulation of Wnt signalling pathway is linked with several types of cancers, including AML (Korinek et al., 1997; Morin et al., 1997; Polakis, 2000; Chung et al., 2002; Clements et al., 2002; Reya and Clevers, 2005; Yesebaert et al., 2006). Chronic activation of Wnt pathway genes resulting from either aberrant overexpression of these genes or loss of natural Wnt inhibitors promotes uncontrolled cell growth and survival (Barker and Clevers, 2006). The Wnt signalling pathway is controlled by several natural Wnt inhibitors including Dickkopfs (DKK), secreted frizzled related proteins (SFRP), Wnt inhibitory factor-1 (WIF-1), human Dapper protein-1 (HDPR1), and so on. Abnormal activation of Wnt signalling by epigenetic silencing of these natural inhibitors was found in human cancers (Suzuki et al., 2004). Association between hypermethylation of some Wnt inhibitors and specific chromosomal translocations in AML was reported in a few studies (Chim et al., 2006; Suzuki et al., 2007; Jost et al., 2008), but not in others (Valencia et al., 2009). Furthermore, the association between hypermethylation of Wnt inhibitors and molecular gene alterations, especially the mutations recently detected, has not been reported. In this study, we aimed to investigate the role of hypermethylation of Wnt inhibitors in adult patients afflicted with AML. To the best of our knowledge, this is the first study to comprehensively address the association of hypermethylation of...
Wnt inhibitors with various genetic mutations in a large cohort of patients with de novo AML. We found distinct association between hypermethylation of certain Wnt inhibitors and specific genetic alterations, mostly Class II mutations, in the leukaemogenesis.

MATERIALS AND METHODS

Subjects

From April 1996 to June 2007, a total of 269 adult patients who were newly diagnosed as having de novo AML and had adequate samples for methylation analysis at the National Taiwan University Hospital (NTUH) were enrolled. Among 269 patients, 219 (81.4%) patients received intensive induction chemotherapy (Idarubicin 12 mg m\(^{-2}\) per day on days 1–3 and Cytarabine 100 mg m\(^{-2}\) per day on days 1–7) and then consolidation chemotherapy with 2–4 courses of high-dose Cytarabine (2000 mg m\(^{-2}\) q12h days 1–4, total eight doses), with or without an anthracycline if complete remission (CR) was achieved (Tang et al., 2009; Hou et al., 2010). The remaining 50 patients received low-dose chemotherapy and/or supportive care because of the poor performance status or the patients’ will. This study was approved by the Institutional Review Board of the NTUH, and written informed consent was obtained from all the participants in accordance with the Declaration of Helsinki Principles.

Methylation-specific polymerase chain reaction (MSP)

Bone marrow (BM) samples were obtained from all the patients at diagnosis. High-molecular-weight DNA was prepared from mononuclear cells as described previously (Tien et al., 1994). A CpG island is defined as a region with at least 200 bp and with a GC percentage greater than 50% and an observed/expected CpG ratio greater than 60% (Gardiner-Garden and Frommer, 1987). The primer sets for the analyses of Wnt inhibitor hypermethylation were designed by the help of the UCSC Genome Browser website (Kent et al., 2002), and the locations of these primers and the regions of the Wnt inhibitors analysed are shown in Supplementary Table 1. Methylation status of the CpG islands in the Wnt inhibitors SFRP1, SFRP2, SFRP4, SFRP5, HDPR1, Wif-1, and DKK1 gene promoters was determined by bisulfite treatment of genomic DNA followed by MSP as reported (Herman et al., 2002), and the statistical analyses were performed with the SPSS 16 software using StatXact-8 (Cytel Inc., Cambridge, MA, USA), and other statistical analyses were performed with the SPSS 16 software (SPSS Inc., Chicago, IL, USA) and Statsdirect (Altrincham, Cheshire, UK).

RESULTS

Methylation in promoters of Wnt inhibitors in AML patients

The frequencies of hypermethylation of Wnt inhibitors (in descending order) were as follows: 31.6% for SFRP1, 30.1% for DKK1, 26.0% for Wif-1, 19.3% for SFRP2, 12.6% for SFRP5, and 1.5% for SFRP4. Taken together, 166 AML patients (61.7%) had promoter hypermethylation of at least one Wnt inhibitor at diagnosis. A half (83 out of 166) of them had hypermethylation of two or more Wnt inhibitors. No abnormal methylation was found in HDPR1. Hypermethylation of at least one SFRP gene occurred in 40.1% (n = 108) of the AML patients. Aberrant methylation of SFRP1, SFRP2, and SFRP5 was closely associated with each other (all P < 0.0001). DKK1 hypermethylation frequently occurred concomitantly with hypermethylation of SFRP family (P < 0.0001), but not Wif-1 (P = 0.7645).

Clinical characteristics of patients with aberrant methylation of Wnt inhibitors

The comparison of clinical characteristics of patients with and without promoter hypermethylation of at least one Wnt inhibitor technique and karyotyped according to the International System for Human Cytogenetic Nomenclature.

Immunophenotype analysis

A panel of monoclonal antibodies to myeloid-associated antigens, including CD13, CD33, CD11b, CD15, CD14, and CD41a, as well as lymphoid-associated antigens, including CD2, CD5, CD7, CD19, CD10, and CD20, and lineage nonspecific antigens HLA-DR, CD34, and CD56 were used to characterise the phenotypes of the leukaemia cells as previously described (Tien et al., 1995; Chou et al., 2006).

Mutation analysis

Mutation analyses of 13 relevant molecular marker genes, NPM1 (Falin et al., 2005), CEBPA (Lin et al., 2005), FLT3/ITD (Shih et al., 2002), FLT3/TKD (Shih et al., 2004), N-RAS, K-RAS (Chen et al., 2006), JAK2 (Chen et al., 2007), AML1/RUNX1 (Tang et al., 2009), MLL/PTD (Shiah et al., 2002), PTPN11 (Hou et al., 2008), ASXL1 (Chou et al., 2010), and WT1 (Hou et al., 2010) were performed as previously described.

Statistical analysis

The association between chromosomal abnormalities/gene mutations and the Wnt inhibitor hypermethylation was analysed using the Monte Carlo simulation-based Fisher’s exact tests. That is, the statistical significance was calculated using a Monte Carlo simulation corrected for multiple hypothesis testing (each with 10 000 simulations and with a prior type-I error z = 0.01). Mann–Whitney U-tests were used to compare continuous variables and medians of distributions. To evaluate the impact of Wnt hypermethylation on clinical outcome, only the 219 patients who received standard chemotherapy as mentioned above were included in the analysis. Overall survival (OS) was measured from the date of first diagnosis to the date of last follow-up or death from any cause, whereas relapse-free status indicated that the patient achieved CR and did not relapse by the end of this study. Kaplan–Meier estimation was used to plot survival curves, and log-rank tests were used to test the difference between groups. The Monte Carlo simulation-based Fisher’s exact tests were conducted using StatXact-8 (Cytel Inc., Cambridge, MA, USA), and other statistical analyses were performed with the SPSS 16 software (SPSS Inc., Chicago, IL, USA) and Statsdirect (Altrincham, Cheshire, UK).

Cytogenetics

Bone marrow cells were harvested directly or after 1–3 days of unstimulated culture as described previously (Tien et al., 1995). Metaphase chromosomes were banded by trypsin-Giemsa.
is shown in Table 1. Patients with FAB M0 subtype of AML had the highest incidence (100%) of hypermethylation of Wnt inhibitors, whereas those with M4/M5 subtype had the lowest incidence (47.3%, \( P = 0.0006 \)). For better delineation of the clinical impact of hypermethylation of specific Wnt inhibitor, we analysed the association of individual Wnt inhibitor hypermethylation with clinical characteristics, respectively (Table 2). SFRP hypermethylation occurred more frequently in the patients with AML M0 (87.5% of M0 patients vs 38.7% of others, \( P = 0.0079 \)), but was less in M4/M5 subtype (27.5% of M4/M5 vs 49.1% of others, \( P = 0.0009 \)). DKK1 methylation was also more common in AML M0 subtype (75% vs 28.7%, \( P = 0.0104 \)). On the contrary, Wif-1 methylation was preferentially found in AML M1 and M3 (42.1% of M1 vs 21.7% of others, \( P = 0.0035 \) and 63.2% of M3 vs 23.2% of others, \( P = 0.0005 \), respectively).

**Table 1** Comparison of clinical and laboratory characteristics between AML patients with and without promoter hypermethylation of Wnt inhibitors

| Characteristics | Methylated\( ^a \) | Non-methylated | \( P \)-value |
|-----------------|-----------------|----------------|-------------|
| Patient no.     | 166             | 103            | 0.7989      |
| Age (years)\( ^b \) | 47.5 (15–87)    | 48 (15–87)     | 0.023       |
| Gender          | Male            | Female         |             |
|                 | 103             | 63             | 0.002       |
|                 | 49              | 54             |             |
| FAB classification\( ^b \) | M0   | 8 (4.8)        | 0 (0)        | 0.0254      |
|                 | M1   | 39 (23.5)      | 18 (17.5)    | 0.2836      |
|                 | M2   | 54 (32.5)      | 31 (30.1)    | 0.6885      |
|                 | M3   | 15 (9.0)       | 4 (3.9)      | 0.1429      |
|                 | M4   | 37 (22.2)      | 41 (39.8)    | 0.0024      |
|                 | M5   | 6 (3.6)        | 7 (6.8)      | 0.2544      |
|                 | M6   | 4 (2.4)        | 2 (1.9)      | \( >0.9999 \) |
|                 | M7   | 0              | 0            |             |
|                 | Undclassified   | 3 (100)        | 0 (0)       |             |
| WBC \( (\times 10^9 \text{ per l})^k \) | 15950 (310–352300) | 54470 (300–627800) | \( P<0.0001 \) |
|                 | Blast \( (\times 10^9 \text{ per l})^k \) | 7132 (0–348777) | \( P<0.0001 \) |
|                 | LDH \( (\text{U l}^{-1})^l \) | 859 (250–7734) | \( P<0.0038 \) |
|                 | Hb \( (\text{g d}^{-1})^l \) | 79 (2.9–13.9) | \( P<0.0001 \) |
|                 | Plt \( (\times 10^7 \text{ per l})^l \) | 40 (5–802) | 46 (6–268) | \( P<0.0038 \) |

Abbreviations: AML = acute myeloid leukaemia; FAB = French-American-British; Hb = haemoglobin; LDH = lactate dehydrogenase; Plt = platelet; WBC = white blood cell. \( ^a \)Hypermethylation of at least one Wnt inhibitor. \( ^b \)Median (range). \( ^\text{No of patients (\%)} \).

Male patients had a higher incidence of hypermethylation of Wnt inhibitors than females (62.0% vs 47.6%, \( P = 0.023 \)). Patients with hypermethylation of at least one Wnt inhibitor had lower WBC counts, blast counts and serum lactate dehydrogenase (LDH) levels at diagnosis. SFRP-5 hypermethylation alone was associated with higher platelet counts (\( P = 0.0144 \)).

Correlation between promoter hypermethylation of Wnt inhibitors and immunophenotypes of leukaemic cells

Promoter hypermethylation of any Wnt inhibitor as a whole was positively associated with the expression of HLA-DR \( (P = 0.0385) \), CD19 \( (P = 0.0026) \), and CD34 \( (P = 0.005) \), but was inversely associated with the expression of CD14 \( (P = 0.0237) \) on the leukaemic cells (Supplementary Table 3). There was no difference in the expression of other antigens between the patients with and without Wnt inhibitor methylation.

Acute myeloid leukaemia patients with hypermethylation of any SFRP gene as a whole had higher frequency of CD19 \( (P = 0.0004) \), CD7 \( (P = 0.0144) \), and CD34 expression \( (P = 0.012) \), but had lower frequency of CD14 expression \( (P = 0.0395) \) on the leukaemia cells. For individual SFRP, hypermethylation of SFRP1 had similar pattern of association with antigen expression to that of SFRP as a whole (Supplementary Table 5). SFRP2 hypermethylation showed no association with the expression of any antigen studied, whereas SFRP5 hypermethylation had close association with CD11b and CD7 expression, but had inverse correlation with CD33 expression. On the other hand, DKK1 methylation was positively associated with HLA-DR \( (P = 0.0273) \), CD34 \( (P = 0.0002) \), and CD56 expression \( (P = 0.0238) \), and Wif-1 methylation was positively associated with CD7 \( (P = 0.0004) \), but was inversely associated with CD14 \( (P = 0.0377) \) and CD33 expression \( (P = 0.0383) \).

Association of aberrant methylation of Wnt inhibitors with cytogenetic abnormalities

Chromosome data were available in 260 patients at diagnosis, including 157 with and 103 without hypermethylation of Wnt inhibitors (Table 3 and Supplementary Table 3). Hypermethylation of any Wnt inhibitor as a whole occurred more frequently in patients with favourable karyotype (79.4%) than in those with intermediate-risk (52.4%) or unfavourable cytogenetics (67.7%, \( P = 0.0005 \), and more frequently in patients with abnormal cytogenetics than in those with normal karyotype (69% vs 50.8%, \( P = 0.0034 \)). It was also positively associated with t(8;21) \( (P = 0.0014) \). Hypermethylation of any SFRP as a whole was closely associated with favourable cytogenetics \( (P = 0.0132) \) and t(8;21) \( (P<0.0001) \), but negatively correlated with intermediate

**Table 2** Summary of correlation between hypermethylation of Wnt inhibitors and clinical and laboratory features

| Variables\( ^a \) | Wnt\( ^b \) | SFRP\( ^c \) | SFRP1 | SFRP2 | SFRP5 | DKK1 | Wifl |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gender\( ^d \) | \(+0.023\) | \(+0.0254\) | \(+0.0079\) | \(+0.0008\) | \(+0.0104\) | \(+0.0035\) | \(+0.0005\) |
| M0 | \(+0.023\) | \(+0.0009\) | \(+0.0083\) | \(+0.0298\) | \(+0.0005\) | \(+0.0019\) | \(+0.0128\) |
| M1 | \(+0.014\) | \(+0.0001\) | \(+0.0029\) | \(+0.0072\) | \(+0.0053\) | \(+0.0045\) | \(+0.0196\) |
| M2 | \(+0.012\) | \(+0.0001\) | \(+0.0002\) | \(+0.0022\) | \(+0.0033\) | \(+0.0045\) | \(+0.0196\) |
| M3 | \(+0.0003\) | \(+0.0004\) | \(+0.0043\) | \(+0.0072\) | \(+0.0053\) | \(+0.0045\) | \(+0.0196\) |

Abbreviations: blank = no significant association; LDH = lactate dehydrogenase; Plt = platelet; + = positive association (\( P \)-value); – = negative association (\( P \)-value). \( ^a \)No significant correlation of hypermethylation of Wnt inhibitors with age and haemoglobin level, which were not shown in this table. \( ^b \)Hypermethylation of any Wnt inhibitors including SFRP, Wf-1, and DKK1. \( ^c \)Hypermethylation of any SFRP gene including SFRP1, SFRP2, SFRP3, and SFRP5. \( ^d \)Gender (correlation with male); white blood cells, blast, platelet, and LDH (correlation with high level).
cytogenetics ($P = 0.0002$) and normal karyotype ($P = 0.0072$). For individual SFRP, hypermethylation of SFRP1 had the same pattern of association with cytogenetic changes as that of SFRP as a whole (Table 3 and Supplementary Table 4). On the other side, hypermethylation of SFRP2 was positively associated with unfavourable ($P = 0.0069$) and complex cytogenetic ($P = 0.0006$), but was inversely correlated with t(15;17) ($P = 0.0293$). DKK1 hypermethylation was detected more frequently in patients with favourable cytogenetics ($P = 0.0179$) and t(8;21) ($P <0.0001$), but was less common in patients with intermediate cytogenetic ($P = 0.0002$) and normal karyotype ($P = 0.0025$). Wif-1 hypermethylation was more frequently detected in the patients with t(15;17) than in other cytogenetic changes (63.2% of M3 vs 23.2% of others, $P = 0.0005$), but was seldom found in the patients with t(8;21) (0% vs 29.7%, $P < 0.0001$). There was no association of hypermethylation of Wnt inhibitors with other chromosomal abnormalities, including +8, +11, +13, +21, −5/del(5q), and −7/del(7q).

### Table 3

| Wnt methylated | Wnt unmethylated | SFRP methylated | SFRP unmethylated | SFRP1 methylated | SFRP1 unmethylated | SFRP2 methylated | SFRP2 unmethylated |
|----------------|------------------|------------------|-------------------|------------------|-------------------|------------------|-------------------|
| N = 158        | N = 102          | N = 159          | N = 184           | N = 92           | N = 178           | N = 47           | N = 213           |
| Favourable     |                  |                  |                   |                  |                   |                  |                   |
| Unfavourable   |                  |                  |                   |                  |                   |                  |                   |
| t(8;21)        |                  |                  |                   |                  |                   |                  |                   |
| t(15;17)       |                  |                  |                   |                  |                   |                  |                   |
| Inv(16)        |                  |                  |                   |                  |                   |                  |                   |
| t(11q23)       |                  |                  |                   |                  |                   |                  |                   |
| Normal         |                  |                  |                   |                  |                   |                  |                   |
| Complex        |                  |                  |                   |                  |                   |                  |                   |

No significant correlation between hypermethylation of Wnt inhibitors and +8, +11, +13, −21, −7q−, and −5q−, which were not shown in this table. The P-value was calculated using a Monte Carlo simulation corrected for multiple hypothesis testing (each with 10 000 simulations and with a prior type-I error $x = 0.001$). Three hundred and sixty patients, including 158 Wnt-methylated and 102 Wnt unmethylated patients, had chromosome data at diagnosis. Hypermethylation of SFRP4 was not included in the table because only 1.5% of patients had this change. Hypermethylation of any Wnt inhibitor including SFRP, Wif-1, and DKK1, hypermethylation of any SFRP inhibitor including SFRP1, SFRP2, SFRP4, and SFRP5. Favourable, t(15;17), t(8;21), inv (16); unfavourable, −7, del(7q)−, −5, del(5q), 3q abnormality, complex abnormalities; Intermediate, normal karyotype and other abnormalities.

Association of promoter hypermethylation of Wnt inhibitors with molecular gene mutations

Among the 166 patients with hypermethylation of any Wnt inhibitor, 145 (87.3%) patients showed concurrent molecular mutation of at least one gene at diagnosis (Table 4); 98 patients had one gene mutation, 35 had two, 10 had three and 2 patients had four gene mutations. In total, 100 patients (68.5%) had concurrently at least one Class II mutation, including MLL/PTD, CEBPA, and AML1/RUNX1 mutations, t(8;21), t(15;17), inv (16), and (11q23).

Patients with at least one Wnt inhibitor hypermethylation had a trend of higher incidence of CEBPA mutation than those without hypermethylation (16.9% vs 8.7%, $P = 0.0693$), but had significantly lower incidence of FLT3/ITD and NPM1 mutations (17.5% vs 34%, $P = 0.003$ and 12.7% vs 36.9%, $P < 0.0001$, respectively).

Among the 108 patients with SFRP methylations as a whole, 92 (85.2%) showed concurrent molecular gene mutations at diagnosis; 60 had one gene mutation, 23 had two, 7 had three, and 2 patients had four mutations. Sixty-nine (75.0%) of them had at least one Class II mutation concurrently. Patients with SFRP hypermethylation had a significantly lower incidence of NPM1 mutation than those without the gene hypermethylation (11.1% vs 29.2%, $P = 0.0005$, Table 4 and Supplementary Table 3). There was no difference in the incidence of other molecular gene mutations between patients with and without SFRP methylations. For individual SFRP, hypermethylation of SFRP1 was negatively associated with NPM1 mutation, whereas that of SFRP2 was closely associated with AML1/RUNX1 mutation, but negatively correlated with NPM1 mutation, and SFRP5 hypermethylation was positively associated with MLL/PTD (Table 4 and Supplementary Table 4).

Among the 70 patients with Wif-1 methylations, 60 (85.7%) patients showed concomitant molecular gene mutations at diagnosis; 46 had one gene mutation, 10 had two and 4 patients had three. Of these, 38 (63.3%) had concurrently at least one Class II mutation. Patients with Wif-1 hypermethylation had a significantly higher incidence of CEBPA mutation (28.6% vs 8.5%, $P < 0.0001$, Table 4 and Supplementary Table 4).
Supplementary Table 4), but had lower incidences of FLT3/ITD, MLL/PTD, NPM1 mutations, and ASXL1 mutations (14.3% vs 27.1%, P = 0.0338; 0% vs 5.7%, P = 0.0402; 12.9% vs 25.1%, P = 0.043; and 2.9% vs 15.1%, P = 0.0127, respectively) than those without the gene hypermethylation.

Among the 31 patients with DKK1 hypermethylation, 70 (86.4%) showed concurrent gene mutations at diagnosis (Table 4 and Supplementary Table 4); 41 had one gene mutation, 23 had two, and 6 had three. Out of these, 52 (73.2%) had at least one Class II mutation concurrently. Patients with DKK1 hypermethylation had a significantly higher incidence of ASXL1 mutation (18.5% vs 6.9%, P = 0.0078), but lower incidences of FLT3/ITD and NPM1 mutations than those without the gene hypermethylation (14.8% vs 27.7% P = 0.0284 and 9.9% vs 27.1%, P = 0.0013, respectively).

Impact of promoter hypermethylation of Wnt inhibitors on response to therapy and clinical outcome

Of the 219 AML patients undergoing conventional induction chemotherapy, 184 (84.0%) patients achieved a CR. With a median follow-up time of 32 months, the identified poorer prognostic factors for OS included older age (P < 0.001), unfavourable cytogenetics (P = 0.001), FLT3/ITD (P = 0.03), and AML/RUNX1 mutations (P = 0.039). However, hypermethylation of at least one Wnt inhibitor or methylation of any FRP did not influence the CR rate, relapse rate, OS, and relapse-free survival (RFS). Further evaluation of the effect of hypermethylation of individual Wnt inhibitors, including SFRP1, SFRP2, SFRP5, DKK1, and WIF-1 showed the same findings. Subgroup analyses in patients with favourable, intermediate, or unfavourable-risk cytogenetics, and in patients with normal karyotype or those with specific gene mutations could not demonstrate prognostic impact of Wnt inhibitors hypermethylation. The number of genes hypermethylated also did not affect the treatment response.

**DISCUSSION**

In this study, we showed that aberrant promoter methylation of Wnt inhibitors was closely associated with specific cytogenetic abnormalities and molecular gene alterations in the patients afflicted with de novo AML. Most of the AML patients harbouring Wnt inhibitors hypermethylation had concurrent Class II mutations at diagnosis. Furthermore, aberrant Wnt inhibitor methylation was closely associated with male, FAB M0 subtype, lower WBC and blast counts and lower LDH levels, but inversely associated with M4/M5 subtype.

Few studies have focused on the correlation of hypermethylation of Wnt inhibitors with specific cytogenetic abnormalities. To the best of our knowledge, there has been no report concerning the association between hypermethylation of Wnt inhibitors and molecular gene mutations till now. This study recruited a large cohort of de novo adult AML patients for analyses of the association between abnormal promoter methylation of Wnt inhibitors and genetic alterations. We found that hypermethylation of Wnt inhibitors occurred predominantly in the patients with abnormal cytogenetics, especially in the favourable-risk group.

In addition to the close association of SFRP1 and DKK1 hypermethylation with t(8;21) and WIF-1 hypermethylation with t(15;17) (Tables 3 and 4), we found for the first time the close association of hypermethylation of Wnt inhibitors and genetic alterations. We found that hypermethylation of at least one Wnt gene including SFRP2, SFRP4, and SFRP5, Any mutation included the mutation of any Class I, II, or others, as such, NPM1, WIF1, and ASXL1 mutations.
the leukaemogenesis of AML. Muller-Tidow et al (2004) demonstrated AML-associated translocation products, such as RUNX1-RUNX1T1 and PML-RARα activated the plakoglobin production, resulting in the accumulation of endogenous β-catenin in the nucleus and further activation of relevant target genes. Results from this and other studies suggest that downregulation of Wnt inhibitors through promoter hypermethylation might be another mechanism leading to the activation of Wnt signalling pathway in AML with some chromosome translocations and gene mutations, such as t(8;21), t(15;17), MLL/PTD, AML1/RUNX1, CEBPA, and ASXL1 mutations. The reason that these genetic alterations are closely associated with hypermethylation of specific Wnt inhibitors remains elusive.

Although we did not validate our findings by correlating the methylation status with the gene expression, downregulation of the Wnt inhibitors due to abnormal promoter methylation has been demonstrated previously (Roman-Gomez et al, 2007). Furthermore, methylation of Wnt inhibitors was shown to be associated with upregulation of the downstream signalling of the Wnt pathway (Roman-Gomez et al, 2007; Valencia et al, 2009). However, it remains unknown whether the hypermethylation of the Wnt inhibitors examined in this study indeed directly influence the Wnt canonical signalling pathway or the non-canonical pathway activities in AML because Bovolenta et al (2008) showed that SFRPs inhibit both Wnt canonical and non-canonical pathways and different SFRPs may have opposite effect on the same process. Re-expression of methylated Wnt inhibitors and inactivation of the Wnt pathway in the cell lines with aberrant methylation of these Wnt inhibitors were also shown after treatment with the demethylating agent decitabine (Valencia et al, 2009). These findings suggest that Wnt pathway is regulated, at least partially, by methylation of the Wnt inhibitors.

The percentage of patients with aberrant methylation of at least one Wnt inhibitor in this study (61.7%) was similar to that reported by Valencia et al (2009). However, the frequencies of aberrant methylation of SFRP1, SFRP2, SFRP4, and SFRP5 (31.6%, 19.3%, 1.5%, and 12.6%, respectively; total 40.1%) in this study were lower than those (41%, 31%, 4%, and 22%, respectively) reported by Valencia et al (2009) who analysed 184 non-M3 AML patients, but were similar to those reported by Jost et al (2008). These probably reflect the difference in patient selection and ethnic diversity.

To investigate the prognostic relevance of aberrant methylation of Wnt inhibitors in AML patients, we focused on the patients receiving standard chemotherapy. However, we did not find the difference of clinical outcome, including CR rate, OS, and RFS, between AML patients with and without Wnt inhibitor hypermethylation. The same were also true in subgroups of patients with different risk cytogenetics or gene mutations. Jost et al (2008) also did not find any prognostic impact of aberrant methylation in SFRP promoters in 100 AML patients. Contrary to our findings, Chim et al (2006) pointed out that Wif-1 methylation was an independent poor prognostic factor for DFS and Valencia et al (2009) showed AML patients with two or more methylated Wnt inhibitor genes had poorer RFS, but not OS, in the subgroup of patients 60 years or younger with intermediate-risk cytogenetics by multivariate analysis. Large-scale studies with more AML patients are needed to clarify this point.

In summary, our findings address that CpG island hypermethylation in the promoters of Wnt pathway inhibitors including SFRP family, WIF-1, and DKK1 is a common event in AML. More intriguingly, there is distinct association between aberrant methylation of some Wnt inhibitors and specific genetic alterations, such as close association of DKK1 hypermethylation with t(8;21) and ASXL1 mutation, WIF-1 hypermethylation with t(15;17) and CEBPA mutation, SFRP1 hypermethylation with t(8;21), SFRP2 hypermethylation with AML1/RUNX1 mutation, and SFRP5 methylation with MLL/PTD. Epigenetic alterations, such as hypermethylation of Wnt inhibitors, may interact with specific cytogenetic abnormalities or molecular gene mutations, especially Class II mutations, in the leukaemogenesis of AML.

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Author contributions

H-AH was responsible for literature collection, study design, data management and interpretation, and manuscript writing; Y-YK was responsible for literature collection, data interpretation, and manuscript writing; C-YL was responsible for statistical analysis and interpretation of the statistical findings; M-CL was responsible for laboratory research and interpretation; J-LT, C-YC, W-CC and MY, contributed toward patient samples and clinical data; C-FH, F-YL and M-CL, performed the laboratory and chromosomal studies and H-FT planned, designed, managed and interpreted the statistical findings; M-CL was responsible for literature collection, study design, data management and interpretation, and manuscript writing; Y-YK was responsible for literature collection, data interpretation, and manuscript writing; C-YL was responsible for statistical analysis and interpretation of the statistical findings; M-CL was responsible for laboratory research and interpretation; J-LT, C-YC, W-CC and MY, contributed toward patient samples and clinical data; C-FH, F-YL and M-CL, performed the laboratory research and chromosomal studies and H-FT planned, designed, managed and interpreted the statistical findings, and coordinated the study over the entire period.

Conflict of interest

The authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J (2008) Beyond AML-associated translocation products, such as RUNX1-RUNX1T1 and PML-RARα, the Wnt inhibitors examined in this study indeed directly influence the Wnt canonical signalling pathway or the non-canonical pathway activities in AML because Bovolenta et al (2008) showed that SFRPs inhibit both Wnt canonical and non-canonical pathways and different SFRPs may have opposite effect on the same process. Re-expression of methylated Wnt inhibitors and inactivation of the Wnt pathway in the cell lines with aberrant methylation of these Wnt inhibitors were also shown after treatment with the demethylating agent decitabine (Valencia et al, 2009). These findings suggest that Wnt pathway is regulated, at least partially, by methylation of the Wnt inhibitors.

The percentage of patients with aberrant methylation of at least one Wnt inhibitor in this study (61.7%) was similar to that reported by Valencia et al (2009). However, the frequencies of aberrant methylation of SFRP1, SFRP2, SFRP4, and SFRP5 (31.6%, 19.3%, 1.5%, and 12.6%, respectively; total 40.1%) in this study were lower than those (41%, 31%, 4%, and 22%, respectively) reported by Valencia et al (2009) who analysed 184 non-M3 AML patients, but were similar to those reported by Jost et al (2008). These probably reflect the difference in patient selection and ethnic diversity.

To investigate the prognostic relevance of aberrant methylation of Wnt inhibitors in AML patients, we focused on the patients receiving standard chemotherapy. However, we did not find the difference of clinical outcome, including CR rate, OS, and RFS, between AML patients with and without Wnt inhibitor hypermethylation. The same were also true in subgroups of patients with different risk cytogenetics or gene mutations. Jost et al (2008) also did not find any prognostic impact of aberrant methylation in SFRP promoters in 100 AML patients. Contrary to our findings, Chim et al (2006) pointed out that Wif-1 methylation was an independent poor prognostic factor for DFS and Valencia et al (2009) showed AML patients with two or more methylated Wnt inhibitor genes had poorer RFS, but not OS, in the subgroup of patients 60 years or younger with intermediate-risk cytogenetics by multivariate analysis. Large-scale studies with more AML patients are needed to clarify this point.

In summary, our findings address that CpG island hypermethylation in the promoters of Wnt pathway inhibitors including SFRP family, WIF-1, and DKK1 is a common event in AML. More intriguingly, there is distinct association between aberrant methylation of some Wnt inhibitors and specific genetic alterations, such as close association of DKK1 hypermethylation with t(8;21) and ASXL1 mutation, WIF-1 hypermethylation with t(15;17) and CEBPA mutation, SFRP1 hypermethylation with t(8;21), SFRP2 hypermethylation with AML1/RUNX1 mutation, and SFRP5 methylation with MLL/PTD. Epigenetic alterations, such as hypermethylation of Wnt inhibitors, may interact with specific cytogenetic abnormalities or molecular gene mutations, especially Class II mutations, in the leukaemogenesis of AML.

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