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Genomic 5-hydroxymethylcytosine levels correlate with *TET2* mutations and a distinct global gene expression pattern in secondary acute myeloid leukemia

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The TET proteins are 2-oxoglutarate- and Fe(II)-dependent oxygenase catalyzing the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC).[1] The *TET1* (ten–eleven translocation 1) gene was originally identified as an *MLL* fusion partner in rare cases of acute myeloid leukemia (AML) with a t(10;11)(q22;q23).[2,3] The definite function of 5hmC still remains elusive, but hydroxylation of 5mC has been suggested to be involved in the process of DNA demethylation. This suggests a possible role of 5hmC in epigenetic gene regulation. Recently, hemizygous deletions and mutations of *TET2* were found in a wide range of myeloid malignancies, including myelodysplastic syndrome (MDS), myeloproliferative disorders such as chronic myelomonocytic leukemia (CMML) and in secondary AML (sAML).[4–6] Interestingly, very recently, myeloid neoplasias harboring heterozygous *TET2* mutations were shown to have decreased levels of 5hmC.[7]

To explore the relationship among *TET2* mutations, global gene expression profiles (GEPs) and 5hmC levels, we measured 5hmC levels in the genomic DNA in a series of 30 sAML patients using a novel assay method employing β-glucosyltransferase from bacteriophage T4.[8] In addition to the *TET2* mutational status, we screened for *IDH1/2* mutations (see Supplementary Material).

All patients had developed AML after a preceding MDS, refractory anemia with excess blast or CMML phase. The average age at diagnosis was 70.8 years. Eight patients had a normal karyotype (nk), eight patients had a complex aberrant karyotype (ak) with more than three chromosomal aberrations and the remainder of the patients had one or two chromosomal aberrations, which are typical of MDS, that is, del(5)(q) (two patients), +8 (five patients), −7 or del(7)(q) (six patients; see Supplementary Table 1). We sequenced the complete coding region of *TET2* in all 30 patients. In all, 7 of the 30 patients (23.3%) had *TET2* mutations. One patient (no. 16) had single-nucleotide deletions in both alleles of *TET2* at amino-acid positions 218 and 519, which caused truncation of the protein after 250 and 533 amino acids, respectively. Two patients (nos. 15 and 26) had nonsense mutations at positions 1216 and 1274, and four patients (nos. 7, 14, 20 and 30) had missense mutations (see Table 1). All the *TET2* mutations (except for patient no. 16) were heterozygous. We did not detect any deletions in patients with *TET2* mutations using a commercially available fluorescence in situ hybridization probe for the *TET2* locus. There was no significant association between *TET2* mutational status and any particular chromosomal abnormality. Although there was a trend toward a higher frequency of *TET2* mutations in patients with a nk in comparison with patients with an ak (50% (4 out of 8 nk patients) versus 13.6% (3 out of 22 ak patients), χ^2-test: $P=0.17$).

The analysis of the 5hmC levels of the patients' DNA using the β-glucosyltransferase assay revealed a 5hmC content of the DNA, ranging from 0.006 to 0.054%. This range of 5hmC levels, about 9- to 14-fold difference between the lowest and highest measurements, agrees well with the measurements reported by Ko *et al.*,[7] although very different patient groups were assayed. In contrast to the

Table 1 Overview of the clinical diagnosis, *TET2* and *IHD2* mutational status, as well as 5hmC levels of the 30 sAML patients analyzed

No.	*Diagnosis*	*TET2 mutations*	*IDH2 mutations*	*5hmC (%)*	*Standard deviation of 5hmC levels*
1	sAML after MDS	Wt	Wt	0.02550	0.00444
2	sAML M2 after MDS RA	Wt	Wt	0.02920	0.00185
3	sAML M4 after MDS	Wt	Wt	0.01804	0.00315
4	sAML M4 after MDS	Wt	Wt	0.01762	0.00321
5	sAML M2 after MDS	Wt	Wt	0.05458	0.00174
6	sAML M2 after MDS	Wt	Wt	0.03486	0.00186
7	sAML M4 after MDS	p.Asn1753_Tyr1766dup c.5256_5297dup42	p.Arg140Gln c.419G>A	0.01208	0.00412
8	sAML M2 after MDS	Wt	Wt	0.01633	0.00384
9	sAML M4 after 5q syndrome	Wt	Wt	0.03204	0.00189
10	sAML M2 after MDS	Wt	Wt	0.01636	0.00312
11	sAML M2 after MDS	Wt	Wt	0.02774	0.00128
12	sAML M2 after MDS	Wt	Wt	0.04758	0.00087
13	sAML M4 after MDS	Wt	Wt	0.03065	0.00442
14	sAML M0 after CMML	p.Glu1144Lys c.3430G>A	Wt	0.01062	0.00162
15	sAML M1 after CMML	p.Arg1216* c.3646C>T	Wt	0.01010	0.00239
16	sAML M2 after CMML	p.Val218Trpfs*32 c.651delC p.Phe519Leufs*14 c.1557delT	Wt	0.00698	0.00224
17	sAML M2 after MDS	Wt	p.Arg140Gln c.419G>A	0.00630	0.00056
18	sAML M2 after MPS	Wt	p.Arg140Gln c.419G>A	0.02075	0.00385
19	MDS RAEB-2 transformation to AML M2	Wt	Wt	0.01505	0.00325
20	sAML M2 after MDS	p.Thr1270Pro c.3808A>C	Wt	0.01655	0.00255
21	MDS RAEB-2, borderline AML M6	Wt	Wt	0.04156	0.00334
22	sAML M4 after MDS	Wt	Wt	0.03973	0.00386
23	sAML M2 after MDS	Wt	Wt	0.03396	0.00538
24	sAML after 5q syndrome	Wt	Wt	0.02885	0.00230
25	sAML M0 after MDS	Wt	Wt	0.02051	0.00132
26	sAML M0 after OMF	p.Gln1274* c.3820C>T	Wt	0.02618	0.00432
27	MDS RAEB-2, borderline CMML-2 or AML M4	Wt	Wt	0.00648	0.00075
28	sAML after MDS	Wt	Wt	0.00973	0.00202
29	MDS RAEB-2 transformation to AML	Wt	Wt	0.00758	0.00108
30	MDS RAEB-2, borderline AML M2	p.Ile1873Thr c.5618T>C	Wt	0.00699	0.00081

Abbreviations: AML, acute myeloid leukemia; cDNA, complementary DNA; CMML, chronic myelomonocytic leukemia; 5hmC, 5-hydroxymethylcytosine; MDS, myelodysplastic syndrome; MPS, myeloproliferative disorder; OMF, osteomyelofibrosis; RA, refractory anemia; RAEB, RA with excess blast; sAML, secondary AML; Wt, wild type.
Reference sequences: TET2 protein: NP_001120680.1; TET2 cDNA: NM_001127208.1; IDH2 protein: NP_002159.2; and IDH2 cDNA: NM_002168.2.

results presented by Ko *et al.*,[7] we did not observe a clear bimodal distribution of 5hmC values. This could be due to the smaller sample number in our series (30 versus 88) and to different patient characteristics in the two studies (mainly sAML in our study versus a broader range of myeloid malignancies in the study of Ko *et al.*[7]).

When we compared the presence of *TET2* mutation with the 5hmC levels, we found a significant clustering of patients with *TET2* mutations in the lower half of 5hmC levels (Figure 1 a). All but one patient with a *TET2* mutation belonged to the group of the 15 patients with the lowest 5hmC levels (χ^2-test: $P=0.03$). This corresponded to 5hmC levels of $<0.02\%$. Only one patient with a *TET2* mutation, no. 26) had 5hmC levels of $>0.020\%$. These results agree well with the observation of Ko *et al.*[7] Interestingly, there were several patients with very low 5hmC levels that did not have a *TET2* mutation. As it was reported recently that *IDH1/2* mutations can impair TET2 function, which might also correlate with low 5hmC levels,[9,10] we determined the mutational status of the *IDH1* and *IDH2* genes in our patients. Only three patients (nos. 7, 17 and 18) had mutations at amino acid R140 in *IDH2* (Table 1, Figure 1 a). No mutations in *IDH1* were detected. Interestingly, one patient (no. 7) had both mutations in *IDH2* and in *TET2*. In a much larger series of patients reported by Figueroa *et al.*,[10] no patient with both a *TET2* and an *IDH1/2* mutation was discovered. Patient no. 17 who had an *IHD2* mutation had the lowest 5hmC levels in our series. Patient no. 18 had intermediate 5hmC levels. However, there are still eight patients in the lower half of the 5hmC level range who have neither a *TET2* nor an *IDH1/2* mutation. There was no correlation between *TET2* expression levels and 5hmC levels in our patients (data not shown).

To determine the impact of *TET2* mutations and 5hmC levels on cellular function, we obtained GEPs from 28 patients (all except patient nos. 9 and 22) and performed two comparisons for differential gene expression: (1) patients with *TET2* mutations (7 patients) versus patients without *TET2* mutations (21 patients) and (2) the 7 patients with the lowest versus the 7 patients with the highest 5hmC levels. The top differentially expressed genes in the high versus low 5hmC level

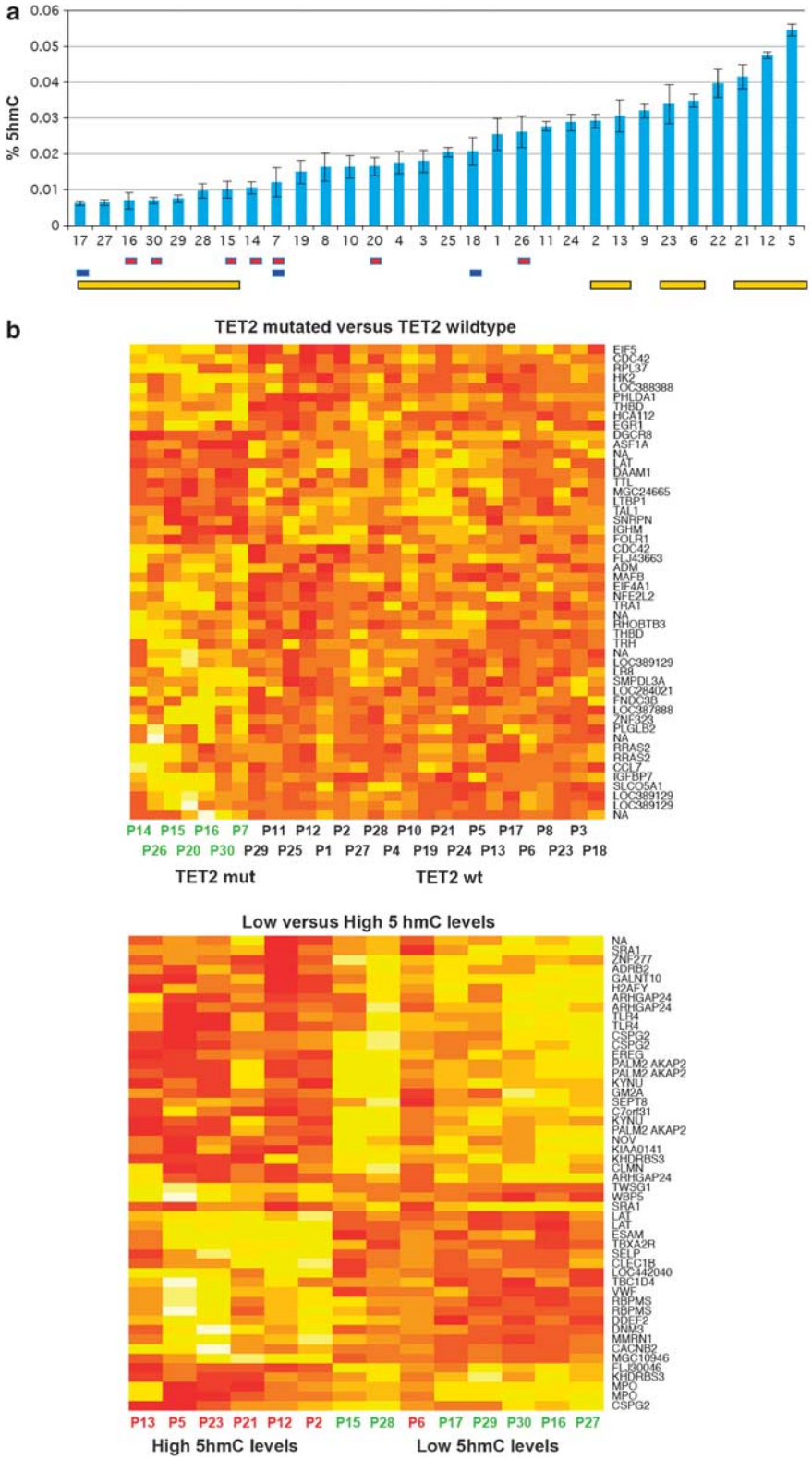

Figure 1 (**a**) Bar graph of 5hmC (%) levels of the 30 patients of this study. Patients with a TET2 mutation or an IDH2 mutation are indicated with a red or a blue rectangle, respectively. The yellow bars indicate the samples that were used for the differential gene expression analysis in the comparison of low versus high 5hmC levels. Note that the patients 9 and 22 did not have gene expression data of sufficient quality to be included in this analysis. (**b**) Heatmaps of the 50 most significantly differentially expressed probe sets comparing samples with TET2 mutations ($n=7$) versus TET2 wild type ($N=21$; top) and samples with low ($n=7$) and high ($n=7$) 5hmC levels (bottom).

comparison had a lower *P*-value and had a higher degree of deregulation than the differentially expressed genes from the comparison *TET2* mutated versus wild type (Figure 1 b; Supplementary Figure 1 and Supplementary Tables 2 and 3). These results indicate that 5hmC levels are most likely a more relevant measurement to define biologically distinct secondary leukemia subtypes than the *TET2* (or *IDH1/2*) mutational status. The fact that in some patient samples with low 5hmC levels neither *TET2* nor *IDH1/2* mutations could be identified suggests that additional genes might be directly or indirectly involved in the regulation of 5hmC levels. To further elucidate the regulation of 5hmC levels and their role in leukemogenesis, larger groups of sAML as well as *de novo* AML patients need to be studied.

Conflict of interest

The authors declare no conflict of interest.

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Author Contributions

NK and FS designed and performed the mutation screening with the help of AD and BK, and wrote the manuscript. SB, AS and HL designed and performed the 5hmC measurements and wrote the manuscript. HL supervised the project. PMK and SS performed cytogenetics and fluorescence in situ hybridization analysis. TH and MM analyzed the GEPs. KS designed experiments and wrote the manuscript. SKB designed experiments, analyzed the data, supervised the project and wrote the manuscript.

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Immunophenotype-defined sub-populations are common at diagnosis in childhood B-cell precursor acute lymphoblastic leukemia

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Neoplasms often display significant heterogeneity in morphology, gene expression (including cell surface markers), genetic aberrations, cell proliferation kinetics and response to therapy.[1] Heterogeneity in antigen marker expression is well known in acute myeloid leukemia (AML);[2] however, has rarely been studied in acute lymphoblastic leukemia (ALL). In ALL, shifts in immunophenotypic and genetic profiles can occur between