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Somatic mutations of the histone methyltransferase gene *EZH2* in myelodysplastic syndromes

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In myelodysplastic syndromes (MDS), deletions of chromosome 7 or 7q are common and correlate with a poor prognosis. The relevant genes on chromosome 7 are unknown. We report here that *EZH2*, located at 7q36.1, is frequently targeted in MDS. Analysis of *EZH2* deletions, missense and frameshift mutations strongly suggests that *EZH2* is a tumor suppressor. As EZH2 functions as a histone methyltransferase, abnormal histone modification may contribute to epigenetic deregulation in MDS.

MDS comprise a heterogeneous group of acquired clonal hematopoietic disorders characterized by dysplasia of the myeloid, erythroid and/or megakaryocytic lineages. MDS is one of the most frequent hematopoietic malignancies, particularly in the elderly[1]. In the majority of affected individuals, a cure for the disease cannot be achieved, and most die from severe cytopenias. Identification of the underlying genetic aberrations in MDS may promote proper classification and prognostication of disease and, eventually, the development of targeted therapy.

Conventional cytogenetic techniques detect large chromosomal aberrations in 40–60% of individuals with MDS, of which deletions of chromosome 5 or 5q (15%) and 7 or 7q (10%) are the most frequent[2]. Several genes have been implicated on chromosome 5, but the relevant genes on chromosome 7 have remained elusive.

Previously, we applied SNP arrays on a cohort of 102 subjects to identify genomic aberrations in MDS. The complete overview of SNP array lesions has been published[3]. Using DNA sequencing, we and others identified perturbation of *TET2*, located at chromosome 4q24, in 26% of the individuals with MDS[3,4]. Here, the same cohort was used to identify affected genes on chromosome 7. SNP array analysis revealed chromosome 7 deletions in 14 out of 102 subjects and loss of heterozygosity due to uniparental disomy (UPD) in two subjects (**Fig. 1a**). In subject 87, we found a monoallelic microdeletion of 130 kb at 7q36.1. This region was shared by 13 out of 14 subjects with deletions, as well as by the two subjects with UPD, and encompassed two genes, *CUL1* and *EZH2* (**Fig. 1a** and **Supplementary Fig. 1**). Genomic sequence analysis of subject 87 covering the coding region and splice sites of the remaining copy of *CUL1* and *EZH2* showed no aberrations in *CUL1*. However, a frameshift mutation in exon 7 (703delGinsAA, nomenclature as described in ref. 5) introducing a premature stop codon was found in *EZH2* (**Table 1**, **Supplementary Figs. 2** and **3**). The presence of this mutation implied that no intact copy of *EZH2* was left in the malignant bone marrow cells of this individual. Because large segmental regions of UPD may harbor acquired homozygous mutations, we sequenced *EZH2* in the bone marrow cells of the two subjects who showed UPD at 7q (**Fig. 1a**; subjects 97 and 103).

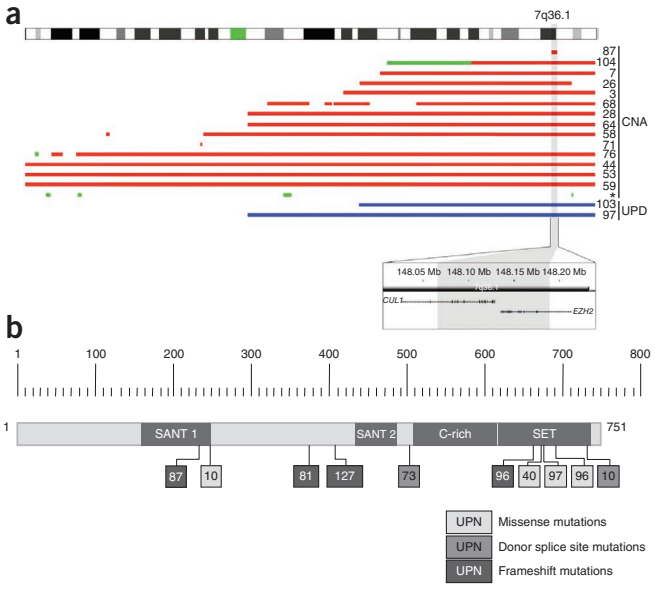

Figure 1 *EZH2* is recurrently affected in individuals with MDS. (**a**) Genomic aberrations of chromosome 7 detected in 102 individuals with MDS by high-resolution SNP arrays. Numbers on the right are unique patient numbers. Two subjects carried large areas of UPD (blue bars), 14 carried deletions (red bars) and 6 showed duplications (green bars, asterisk represents four different subjects). Subject 87 showed a 130-kb microdeletion at 7q36.1, which contains *CUL1* and *EZH2*. CNA, copy number alterations; UPD, uniparental disomy. (**b**) Protein localization of ten different *EZH2* point mutations found in 8 out of 126 subjects with MDS. The ruler indicates amino acids. The catalytic SET domain, a C-rich domain and the two SANT domains are indicated. Numbers in the boxes indicate unique patent numbers (UPN)

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Table 1 Characteristics of subjects with MDS showing 7q36.1 aberrations or *EZH2* mutations

Classification					SNP array		Point mutations in *EZH2*			
UPN	FAB	WHO	IPSS	Karyotype	UPD at 7q36.1	Deletion location 7q36.1	DNA variation	Protein variation	Zygosity	Source
3	RAEB-t	RAEB-2	High	Complex with chr. 7 lesions	No	7q21.13–q36.3				
7	RAEB-t	RAEB-2	Int-2	Complex with chr. 7 lesions	No	7q31.1–q36.3				
10	RAEB-t	RAEB-2	Int-2	Normal	No	No	G745A	E249K	Heterozygous	Acquired
							G2195+1A	Y733LfsX6	Heterozygous	Acquired
26	RA/RAEB	RA/RAEB-1	Int-2	del(7)(q22), del(20)(q11)	No	7q21.3–q36.2				
28	RAEB	RAEB-1	Int-2	+1, der (1;7)(q10;p10)	No	7q11.21–q36.3				
40	RAEB	RAEB-2	High	t(3;16)(q26;q2?3)	No	No	T2020G	L674V	Heterozygous	Acquired
43	MDS-U	MDS-U	Int-2	del(5)(q13q33), -7	No	No				
44	RAEB	RAEB-2	Int-2	Normal	No	7p22.3 –q36.3				
53	RAEB	RAEB-1	Int-2	Complex with chr. 7 lesions	No	7p22.3–q36.3				
58	RAEB	RAEB-2	High	Complex with chr. 7 lesions	No	7p12.3–q36.3				
59	RAEB	RAEB-1	Int-2	–7	No	7p22.3–q36.3				
64	RAEB	RAEB-2	High	Complex with chr. 7 lesions	No	7q11.21–q36.3				
68	RAEB-t	RAEB-2	High	Complex with chr. 7 lesions	No	7q22.1 –q36.3				
73	RAEB	RAEB-1	Int-1	+8	No	No	G1505+1T	Unknown	Heterozygous	Acquired
76	RAEB-t	AML	High	Complex with chr. 7 lesions	No	7p21.2–q36.3				
81	RA	RCMD	Int-1	Normal	No	No	1119-1120insC	T374HfsX3	Heterozygous	N/A
87	RA	RA	Low	Normal	No	7q36.1	703delGinsAA	G235KfsX11	Hemizygous	N/A
96	RA	RCMD	Low	Normal	No	No	1983delA	V662CfsX13	Heterozygous	N/A
							C2068T	R690C	Heterozygous	N/A[a]
97	RA	RCMD	Int-1	Normal	7q11.21–q36.3	No	C2025A	N675K	Homozygous	N/A[a]
103	RAEB	RAEB-1	Int-1	Normal	7q21.2–q36.3	No				
104	RAEB-t	AML	High	Complex	No	7q31.33–q36.3				
106	RA	RA	Int-2	–7	N/A	N/A				
108	RAEB	RAEB-1	Int-1	Complex with chr. 7 lesions	N/A	N/A				
112	RARS	RCMD-RS	Int-1	Complex with chr. 7 lesions	N/A	N/A				
113	RAEB	RAEB-1	Int-2	–7	N/A	N/A				
114	RA	RCMD	Int-2	der(7)t(1;7)(p10;q10)	N/A	N/A				
119	RAEB	RAEB-1	Int-2	del(5)(q15q33), der(7)del(7)(p11)add(7)(q3?3)	N/A	N/A				
121	RAEB	RAEB-1	Int-2	Complex with chr. 7 lesions	N/A	N/A				
126	RA	RCMD	Int-2	Complex with chr. 7 lesions	N/A	N/A				
127	RA	RA	Low	Normal	N/A	N/A	1212-1216delGAAGA	K405RfsX2	Heterozygous	N/A

Aberrations of *EZH2* at chromosome 7q36.1 were detected in 29 bone marrow samples from 126 individuals with MDS. Genomic sequencing of the coding region and the splice donor and acceptor sites revealed missense mutations, donor-splice-site mutations, deletions and insertions, predicting amino acid substitutions and truncations. Sequences were compared to the reference genomic sequence NT_007914.15 and the deduced protein sequences were compared to reference NP_004447.2. In subjects 44 and 104, the 7q deletion that was identified on the SNP array was missed by karyotype analysis. In subject 43, the 7q deletion that was revealed by karyotype analysis was not detected by a SNP array analysis. To assess whether mutations were acquired, T cells from the same individuals were analyzed or allelic discrimination assays were applied to show that the mutations were absent in a cohort of 250 unaffected donors (**Supplementary Methods**, **Supplementary Table 2** and **Supplementary Fig. 5**). Detected SNPs are summarized in **Supplementary Table 3**. UPN, unique patient number; FAB, French-American-British classification; WHO, World Health Organization classification; IPSS, International Prognostic Scoring System; N/A, not analyzed; chr., chromosome.

[a]Mutation was absent in a cohort of 250 unaffected donors.

EZH2 mutations were not detected in subject 103, but a homozygous missense mutation was present in subject 97 (**Table 1**).

To assess the prevalence of *EZH2* mutations in individuals with MDS, we sequenced the gene in all 102 subjects from the initial cohort and in 24 additional individuals with MDS (**Supplementary Table 1**). *EZH2* missense, donor-splice-site and frameshift mutations predicting truncated proteins were observed in 8 out of 126 subjects (6%; **Table 1** and **Fig. 1b**). In three individuals, we could show that mutations were biallelic. In subject 87, one allele was deleted and the other contained a point mutation. In subject 97, a point mutation was found that was homozygous due to UPD, and in subject 96, two different point mutations were found that resided on two different alleles (**Supplementary Fig. 4**). To investigate whether these mutations were somatically acquired, we analyzed DNA of non-neoplastic T cells from the same individuals. Polyclonal T-cell cultures were generated from viably frozen cells from subjects 10, 40 and 73 (**Supplementary Methods**). None of the four mutations detected in the malignant cells were found in these T cells, confirming that these were acquired mutations (**Table 1**). For two additional subjects who carried missense mutations (subjects 96 and 97), we designed allelic discrimination assays (using PCR with allele-specific probes and high-resolution melting analysis, HRM) and tested a cohort of 250 unaffected individuals (controls) (**Supplementary Methods** and **Supplementary Table 2**). None of these mutations were found in any of the unaffected subjects (**Table 1** and **Supplementary Fig. 5**). In three individuals, we examined whether the mutant mRNA was expressed (**Supplementary Fig. 6** and **Supplementary Methods**). In subject 73 (who harbored a heterozygous splice-donor-site mutation in intron 12), only wild-type mRNA was detected. No aberrant splice variants were detected using primers from exon 11 to exon 13 or when exon 11 to exon 20 primers were used (**Supplementary Methods**). In subjects 10 and 127, mutant mRNA was detected, but

pyrosequencing and GeneScan analysis showed that the mutant sequences were expressed at lower levels than wild-type sequences, suggesting a decreased stability of the mutant transcript. Although we could readily detect EZH2 protein in cell lines and in primary T cells, no EZH2 protein expression could be measured in the total bone marrow of individuals with MDS irrespective of the presence or absence of *EZH2* mutations. Therefore, more detailed expression studies in bone marrow subfractions from individuals with MDS are warranted. In addition to the eight subjects with *EZH2* point mutations, the *EZH2* locus at 7q36.1 was entirely deleted at one allele in 22 subjects by 7 or 7q (micro)deletions. Collectively, deletions and point mutations of *EZH2* were present in 23% of the affected subjects. In five out of eight subjects with an *EZH2* mutation, a *TET2* was present (ref. 3 and **Supplementary Table 1**). Overall, in 40% of the subjects showing 7q36.1 aberrations or *EZH2* mutations, *TET2* was affected as well. This is notable, as both EZH2 and the TET family of proteins have been implicated in epigenetic regulation of gene transcription[6–10].

The cohort harboring *EZH2* aberrations was too small to detect a clear association with a specific French-American-British, International Prognostic Scoring System or World Health Organization classification of MDS (**Table 1**). Subjects carrying 7 or 7q deletions showed a significantly worse survival compared to individuals without any *EZH2* abnormalities ($P < 0.001$; **Supplementary Fig. 7**). The adverse effect of chromosome 7 and 7q deletions on the prognosis of individuals with MDS is well known[1]. We show here that *EZH2* point mutations result in a similar adverse tendency in overall survival ($P = 0.076$ when measured from the date of diagnosis and $P = 0.026$ when measured from the time of bone marrow sampling), but a larger cohort from a prospective clinical trial should be analyzed to assess this properly.

EZH2 encodes the histone methyltransferase that constitutes the catalytic unit of the polycomb repressive complex 2 (PRC2)[7–10]. This complex initiates dimethylation and trimethylation of lysine 27 of histone H3 (H3K27), an epigenetic modification associated with gene silencing. All the mutations detected here disrupt highly conserved amino acids (**Supplementary Fig. 8**). Notably, the SET domain, essential for the methyltransferase activity of EZH2 (refs. 8–10), was either altered or truncated in at least one allele in all affected subjects (**Fig. 1b** and **Supplementary Fig. 8**). We conclude that *EZH2* is targeted by various types of deletions and mutations in MDS, probably leading to loss of function of the gene. Recently, a recurring monoallelic *EZH2* point mutation in the SET domain (Tyr641; Tyr646 in NM_004456.3) leading to loss of function has been described in lymphomas[11]. Together with our data, this suggests that EZH2 may act as a tumor suppressor. This is in line with recent investigations showing that *Drosophila* E(z) as well as other PRC1 and PRC2 components may exert tumor suppressor activity by silencing the mitogenic JAK-STAT[12] and Notch[13] signaling pathways. In contrast, in various malignancies, overexpression of *EZH2* has been reported[14], suggesting that EZH2 might also act as an oncogene. This indicates that deregulation of *EZH2* in cancer may not be generalized into one simple mechanism. We postulate that changes in epigenetic modifications caused by defective EZH2 may result in tumor promotion by different mechanisms depending on the cellular context and the oncogenic pathways that are activated.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

G.N., S.M.C.L., R.P.K., T.d.W., B.A.v.d.R. and J.H.J. designed the experiments. G.N., S.M.C.L., R.K., M.M., E.R.L.T.M.T., A.v.d.H., T.N.S., P.V. and T.d.W. provided subject material and clinical data. S.M.C.L. and R.P.K. performed and analyzed the SNP arrays. G.N., E.R.L.T.M.T., M.M., A.v.d.H. and T.N.S. performed sequence analysis and allelic discrimination assays. G.N. and J.H.J. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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