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Research paper

Characterization of acute myeloid leukemia with del(9q) – Impact of the genes in the minimally deleted region

Isabel S. Naarmann-de Vries[a,*], Yvonne Sackmann[a,b], Felicitas Klein[a,b], Antje Ostareck-Lederer[a], Dirk H. Ostareck[a], Edgar Jost[b], Gerhard Ehninger[c], Tim H. Brümmendorf[b], Gernot Marx[a], Christoph Röllig[c], Christian Thiede[c], Martina Crysandt[b,**]

[a] Department of Intensive Care Medicine, University Hospital RWTH Aachen University, Aachen, Germany
[b] Department of Hematology, Oncology, Hemostaseology and Stem Cell Transplantation, University Hospital RWTH Aachen University, Aachen, Germany
[c] Medical Department I, University Hospital of the Technical University Dresden, Dresden, Germany

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ABSTRACT

Acute myeloid leukemia is an aggressive disease that arises from clonal expansion of malignant hematopoietic precursor cells of the bone marrow. Deletions on the long arm of chromosome 9 (del(9q)) are observed in 2% of acute myeloid leukemia patients. Our deletion analysis in a cohort of 31 del(9q) acute myeloid leukemia patients further supports the importance of a minimally deleted region composed of seven genes potentially involved in leukemogenesis: *GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK*, *RMI1*, *SLC28A3* and *NTRK2*. Importantly, among them *HNRNPK*, encoding heterogeneous nuclear ribonucleoprotein K is proposed to function in leukemogenesis. We show that expression of *HNRNPK* and the other genes of the minimally deleted region is significantly reduced in patients with del(9q) compared with normal karyotype acute myeloid leukemia. Also, two mRNAs interacting with heterogeneous nuclear ribonucleoprotein K, namely *CDKN1A* and *CEBPA* are significantly downregulated. While the deletion size is not correlated with outcome, associated genetic aberrations are important. Patients with an additional t(8;21) show a good prognosis. *RUNX1-RUNX1T1*, which emerges from the t(8;21) leads to transcriptional down-regulation of *CEBPA*. Acute myeloid leukemia patients with mutations in *CEBPA* have a good prognosis as well. Interestingly, in del(9q) patients with *CEBPA* mutation mRNA levels of *HNRNPK* and the other genes located in the minimally deleted region is restored to normal karyotype level. Our data indicate that a link between *CEBPA* and the genes of the minimally deleted region, among them *HNRNPK* contributes to leukemogenesis in acute myeloid leukemia with del(9q).

1. Introduction

Acute myeloid leukemia (AML)[1] arises from clonal expansion of malignant hematopoietic precursor cells of the bone marrow. The broad variety of clinical features is the result of different alterations in multiple cellular pathways. This leads to a wide range of subgroups and different treatment options. Recent studies revealed distinct molecular subgroups of AML harboring single or combined somatic mutations [1]. The deletion of a portion of the long arm of chromosome 9, del(9q), is a recurring abnormality in malignant myeloid diseases reported in approximately 2% of AML cases [2]. In about 24% of these cases, del (9q) is observed as sole karyotypic abnormality, while in the remaining 76%, it is observed in association with t(8;21) or other abnormalities [2–4]. Among all del(9q) AML cases, 36%–50% exhibit an additional t (8;21), whereas 7%–14% of AML cases with t(8;21) show del(9q) as additional aberration [2,5,6]. The *RUNX1-RUNX1T1* fusion, which results from the t(8;21) is not sufficient to cause leukemia [7,8], but requires cooperating genetic aberrations. Recurrent deletions involving segments of chromosome arms 5q, 7q, and 20q are observed in AML as well [2]. However, the molecular consequences of these specific

* Corresponding author at: Department of Hematology, Oncology, Hemostaseology and Stem Cell Transplantation, University Hospital, RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen Germany.
** Corresponding author at: Department of Intensive Care Medicine, University Hospital, RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen Germany.
E-mail addresses: inaarmann@ukaachen.de (I.S. Naarmann-de Vries), mcrysandt@ukaachen.de (M. Crysandt).

[1] Acute myeloid leukemia (AML), commonly deleted region (CDR), minimal deleted region (MDR), heterogeneous ribonucleoprotein K (hnRNP K), normal karyotype (NK), white blood cell count (WBC), platelet count (PLT), hemoglobin (Hb), lactate dehydrogenase (LDH), complete remission after first induction cycle (CR1), overall survival time in months (OSTM), relapse free survival time in months (RFSTM), bone marrow blasts (BMB), peripheral blood blasts (PBB).

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chromosomal deletions and functions of critical genes in AML pathogenesis remain unclear. For AML del(9q), a commonly deleted region (CDR) was defined and studied to identify cooperating factors [9]. *TLE1* and *TLE4*, which are localized adjacent to the CDR contribute to leukemogenesis due to haploinsufficiency [10], but their deletion is not required for the development of AML. They were described to be expressed at lower levels as well in cases of AML without del(9q) [10]. Further analysis specified a minimal deleted region (MDR) within the CDR to 9q21.32–9q21.33 including seven annotated genes (*GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK*, *RMI1*, *SLC28A3*, *NTRK2*) [11].

For most genes located in the MDR, a typical tumor suppressor or oncogene function could not be assigned. *RMI1* is part of *BLM*-containing complexes required for genome stability [12] and a common polymorphism in *RMI1* is associated with a higher risk for AML [13]. Homozygous deletion of *RMI1* is embryonic lethal and *RMI1/TP53* double heterozygous knockout mice show accelerated tumor formation [14]. Furthermore, a recent study demonstrated that reduction of heterogeneous ribonucleoprotein K (hnRNP K), which is encoded by *HNRNPK* can contribute to leukemogenesis in AML [15]. In contrast to that overexpression of hnRNP K protein has been described in different solid tumors [16,17]. Thus, the *HNRNPK* gene can neither be classified as oncogene nor as tumor suppressor. Instead its role seems to depend on the cellular context [17]. HnRNP K is a multifunctional protein that interacts with other proteins, DNA and RNA [18] and modulates the activity of genes and gene products on different levels. For example, hnRNP K not only regulates transcription of the *SRC* gene [19], but also translation of the *SRC* mRNA [20] and c-Src kinase activity [21,22].

In this study, we screened a cohort of 40 del(9q) patients and included 31 patients with at least 40% del(9q) metaphases to delineate specific biologic effects of this chromosomal aberration. We determined the deleted region and confirmed the previously identified MDR. RT-qPCR analysis revealed a significantly reduced expression of MDR genes. The comprehensive analysis of clinical parameters and associated genetic alterations uncovered a link between the MDR located genes and the transcription factor *CEBPA*, which is also recurrently mutated in AML. Interestingly, the mRNA levels of MDR encoded genes were restored in del(9q) patients with associated *CEBPA* mutation, indicating a relationship of *CEBPA* and the del(9q) MDR. A promising candidate to fulfill this function is *HNRNPK*, whose gene product was shown to interact with the *CEBPA* promoter and mRNA [15]. This analysis provides first insights to delineate the contribution of the del(9q) MDR genes to pathogenesis of AML.

2. Material and methods

2.1. Patient samples

Cryopreserved bone marrow or peripheral blood samples at the timepoint of diagnosis from AML patients with normal karyotype (NK) (n = 24) or del(9q) (n = 31) were obtained from the RWTH Aachen University Hospital biobank (RWTH cBMB) or the SAL Study Alliance Leukemia biorepository, Dresden, respectively. The presence of del(9q) was detected with standard chromosome banding techniques and patients with at least 40% metaphases showing del(9q) were included for further analysis. The percentage of del(9q) metaphases is given in Supplementary Table 8. All patients gave informed consent for treatment and genetic analysis according to the declaration of Helsinki. Positive statement of the ethics committee was obtained.

2.2. Isolation of genomic DNA and deletion analysis

A detailed protocol of the deletion analysis can be found in the Supplementary information.

Table 1
Clinical characteristics of the analyzed patient cohorts. The clinical parameters of 24 N K and 31 del(9q) AML patient samples are summarized. Abbreviations: WBC, white blood cell count; PLT, platelets; Hb, hemoglobin; LDH, lactate dehydrogenase; CR1, complete remission after first induction cycle; OSTM, overall survival time in months; RFSTM, relapse free survival time in months; BMB, bone marrow blasts; PBB, peripheral blood blasts. Median and range are given for age, WBC, PLT, Hb, LDH, BMB and PBB. For sex, CR1 and associated mutations absolute numbers and percent of the respective patient cohort are depicted (* = $p < 0.05$, n.a. = not applicable).

	normal karyotype, n = 24	del(9q), n = 31	p-value
Age (range)	67 (34–83)	59 (19–78)	0.0157 (*)
Sex (%)	9 (37.5)	14 (45.2)	0.5945
female	15 (62.5)	17 (54.8)	
male			
WBC [Gpt/l] (range)	34.8 (1.2–228.6)	18.3 (2.0–224.0)	0.2596
PLT [Gpt/l] (range)	65 (5–291)	40 (7–442)	0.3920
Hb [mmol/l] (range)	6.1 (2.8–9.2)	5.4 (3.0–8.4)	0.0357 (*)
LDH [U/l] (range)	540 (160–5390)	686 (192–4446)	0.3177
CR1 (%)	10 (41.7)	21 (67.7)	0.0623
yes	14 (58.3)	10 (32.3)	
no			
OSTM (range)	0.3–30.2	0.1–156.3	0.5886
RFSTM (range)	0.9–34.4	4.3–155.7	0.0197 (*)
BMB [%] (range)	75 (20–97)	62 (27–97)	0.3901
PBB [%] (range)	37 (0–97)	47 (1–99)	0.4466
FLT3-ITD (%)	8 (33.3)	7 (22.6)	0.6547
NPM1 mut (%)	8 (33.3)	8 (25.8)	0.4037
CEBPA mut (%)	2 (8.3)	2 (6.5)	n.a.

2.3. Isolation of RNA and RT-qPCR analysis

RNA from cryopreserved patient samples was isolated using TRIZOL (Thermo Fisher Scientific). 400 ng RNA was reverse transcribed (RT) using Maxima First Strand cDNA synthesis kit with dsDNase (Thermo Fisher Scientific) and Random Primers. Quantitative PCR (qPCR) was performed in triplicates with Power SYBR Green Master Mix (Thermo Fisher Scientific) on a StepOnePlus PCR System (Thermo Fisher Scientific). MRNA levels were determined by the ΔΔCt method [23] and normalized to *ACTB* mRNA and the mean of the NK samples. Residual genomic DNA contamination after dsDNase digestion was excluded by qPCR analysis with primers specific for a genomic region of *DDX58* (Supplementary Table 1). Primer sequences for RT-qPCR analysis are shown in Supplementary Table 2. Correct product amplification was verified by melt curve analysis (not shown).

2.4. Statistical analyses

All statistical analyses were conducted with GraphPadPrism (GraphPad Software, La Jolla, California, USA). Assuming non-Gaussian distribution, statistical significance of two groups was assessed using the Mann-Whitney test. Data sets with more than two groups were compared using Kruskal-Wallis-test followed by Dunn's multiple comparison test. Survival curves were compared using Log-rank (Mantel-Cox) test. For correlation analysis Spearman correlation coefficient was calculated. Significance level were defined: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3. Results

3.1. Patient characteristics

In the present study, diagnostic samples from untreated AML patients with normal karyotype (NK) (n = 24) and AML patients carrying a deletion on the long arm of chromosome 9 (del(9q)) (n = 31) were analyzed. Cytogenetic characteristics are summarized in Supplementary Table 3. Median age of NK patients was 67 years, whereas del(9q)

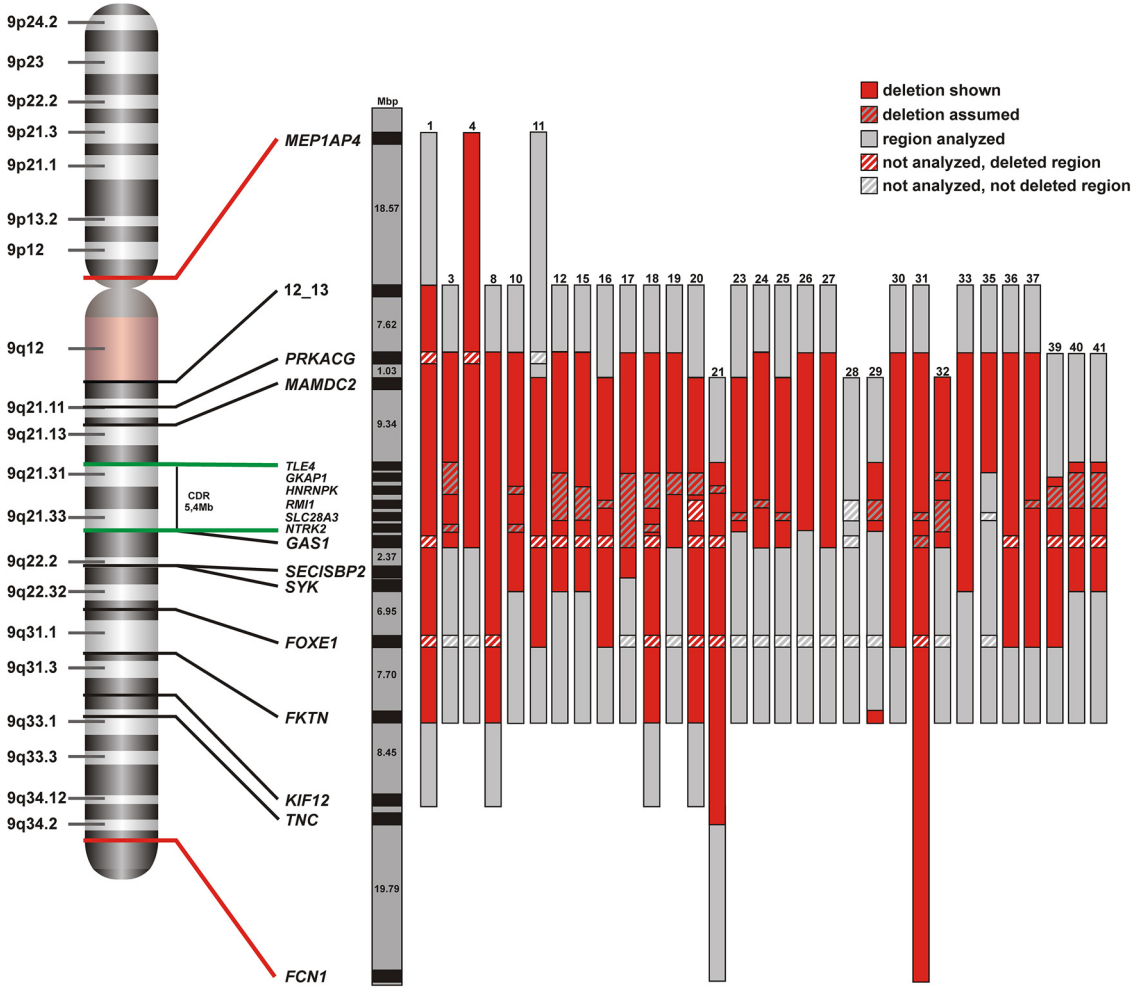

Fig. 1. Characterization of the deleted region on chromosome 9. Schematic representation of the PCR-based deletion analysis with specific primers for depicted genes. The CDR is indicated by green lines. The analyzed region is marked by red lines and shown enlarged in grey with analyzed genes black. The results of the deletion analysis for every patient are shown on the right. The analyzed region is marked light grey, deleted regions red. Hatched boxes indicate that either the respective gene was not analyzed or that the deletion was not detected but can be expected due to deleted flanking regions (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

patients had a median age of 59 years (Table 1). The relapse free survival time (RFSTM) was significantly higher in del(9q) patients than in NK patients. Both patient cohorts were well balanced concerning other clinical parameters (Table 1).

The outcome of del(9q) AML patients is dependent on additional chromosomal rearrangements [24]. They were therefore divided into three subgroups: del(9q) only, del(9q) with t(8;21) and del(9q) associated with other cytogenetic abnormalities. Del(9q) patients with associated t(8;21) showed a 100 percent complete remission after the first induction cycle (CR1), which is significantly better than all other groups analyzed (Supplementary Table 4). Del(9q),t(8;21) is mutually exclusive to mutations in *NPM1* and *CEBPA*, whereas *NPM1* mutations are strongly associated with del(9q) only (Supplementary Table 4).

3.2. Characterization of deletion

All samples were routinely characterized by standard chromosome banding techniques (Supplementary Table 3). We used PCR-based characterization for the precise determination of gene deletions on this specific chromosomal arm. The results of the deletion analysis are summarized in Fig. 1. Agarose gel images and quantification results are shown in Supplementary Figure 5 and Supplementary Table 8, respectively. As *TLE4* is located directly adjacent to the CDR, deletion was assessed using two primer pairs binding at a central position and at the 3′end of the gene. Del(9q) could not be confirmed for Q28. For the remaining samples, the deletion size was in the range of 5.5–65.9 Mbp (Supplementary Table 8). The CDR [9] and MDR [11] determined in previous studies were affected in all samples with confirmed deletion (Fig. 1), except sample Q35. Clinical parameters and associated genetic aberrations or mutations showed no correlation with deletion size. Furthermore, deletion size was not associated with response to therapy and outcome (Supplementary Table 5).

The deleted region in AML del(9q) was previously analyzed by different methods [9,11,25]. We compared the results of our deletion analysis with these data. Patient samples analyzed by standard chromosome banding techniques show 9q22.1–9q22.33 as most commonly deleted region and identified terminal deletions in 15–19% of cases (Fig. 2, left panel) [25]. These results are challenged by gene-specific deletion analysis by SNP-array or PCR based methods (Fig. 2, right panel). A terminal deletion was detected here only in one case. Furthermore, these analyses revealed 9q21.32 as most frequently deleted region (Fig. 2, right panel). By PCR or SNP-array 9q21.32 was shown to be deleted in all patients with a confirmed deletion except Q35 (Fig. 2, right panel) [9,11]. Standard chromosome banding techniques revealed a deletion of this region only in 55–68 percent of patient samples (Fig. 2, left panel) [25]. Six genes of the MDR, namely *GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK*, *RMI1* and *SLC28A3* are located at position 9q21.32.

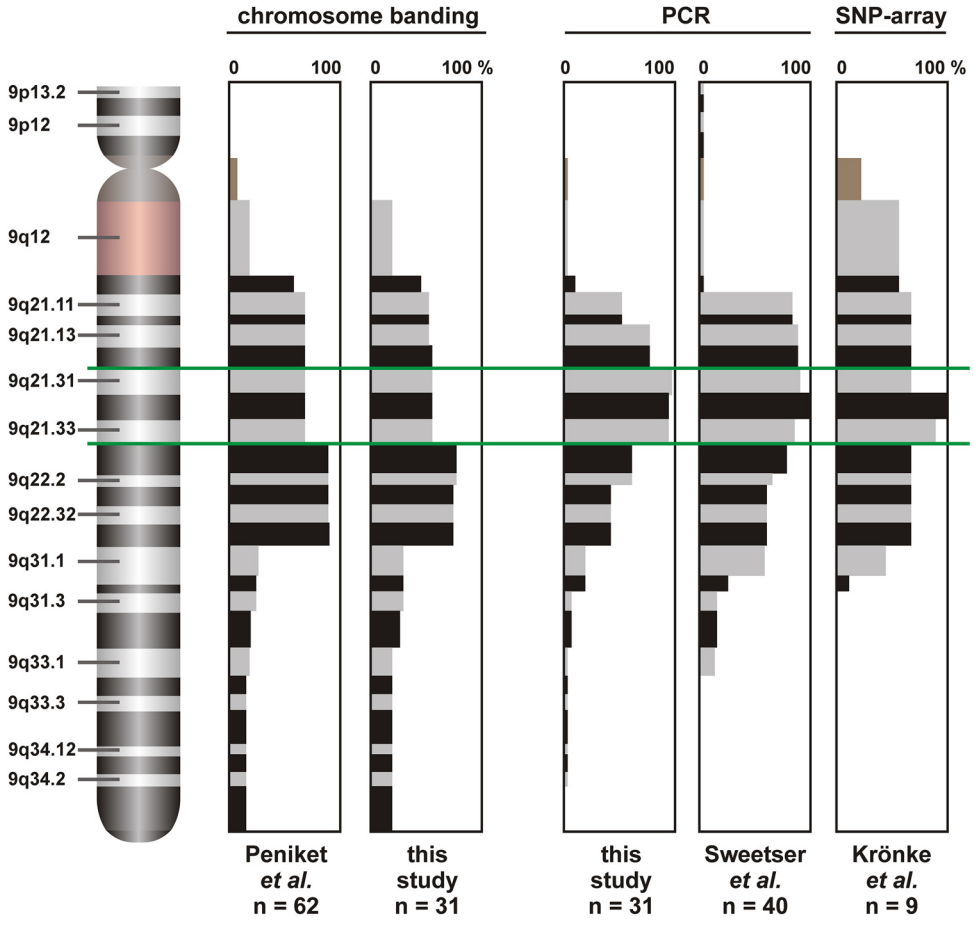

Fig. 2. Comprehensive analysis of the deleted region in del(9q). The fraction of patients with deleted chromosomal bands (in percent) was calculated for this study and previous published studies [9,11,25] dependent on the method used for deletion analysis. In the left panel the analysis of patient samples with standard chromosome banding techniques is shown (this study and Peniket et al. [25]). In the right panel samples are summarized analyzed by PCR or SNP-array (this study, Sweetser et al. [9], Kronke et al. [11]). The CDR is indicated by flanking green lines (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

These data point to the importance of the MDR in pathogenesis of AML with del(9q) and indicate that other genes located on 9q seem to be less relevant in this context.

3.3. Expression and impact of MDR genes

The deletion analysis confirmed the MDR in the cohort of 31 del(9q) patients. Thus, we further focused on the genes located in the MDR and analyzed their expression by RT-qPCR. Levels of *GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK* and *RMI1* mRNAs were significantly reduced in del (9q) patients compared to NK patients (Fig. 3A–E). *SLC28A3* and *NTRK2* mRNA level could not be determined due to their low expression in both patient groups (data not shown). On the other hand, expression of *B2M* and *RPLP0*, which are not located on chromosome 9 was not reduced in del(9q) patients (Fig. 3F–G). Importantly, expression of the MDR genes correlated strongly with each other, especially in del(9q) patients (Supplementary Table 6), which suggests that these genes are deleted as one entity. Expression of *SYK*, which is located on 9q, but outside the MDR and deleted in 42% of del(9q) patients analyzed, is significantly reduced in patients with *SYK* deletion, but not in patients bearing both *SYK* alleles (Fig. 3H). *SYK* showed no correlation with expression of MDR genes (Supplementary Table 6). Expression of *TLE4*, which is located adjacent to the MDR is not significantly reduced in del(9q) patients (Fig. 3I, Supplementary Fig. 1) compared to NK patients. This is in accordance with previous results that *TLE4* is decreased in AML patients also in absence of a genetic deletion [10]. *TLE4* expression correlates with MDR gene expression in NK, but not del(9q) patients (Supplementary Table 6).

Expression levels of the MDR genes were analyzed for correlation with clinical parameters. The MDR genes showed a higher expression in patients with a high peripheral blood blast count. Furthermore, they were significantly inversely correlated with age (Supplementary Table 7). Thus, younger patients have higher *HNRNPK* and *RMI1* mRNA levels. Other clinical parameters and response to therapy showed no consistent correlation with expression of MDR genes (Supplementary Table 7).

HnRNP K is a well described RNA and DNA binding protein [18,26,27] and was proposed to be involved in pathogenesis of AML del (9q) [15]. Therefore, we analyzed expression of the known hnRNP K target genes *CDKN1A* and *CEBPA* [15]. *CDKN1A* (p21) and *CEBPA* (C/EBPα) mRNA levels were significantly reduced in del(9q) compared to NK (Fig. 3J–K), as described for an hnRNP K haploinsufficient mouse model [15]. Expression of *CEBPA* and most MDR genes, among them *HNRNPK* correlates well in NK patients, but not in del(9q) patients, indicating a disturbed complex regulation on transcriptional and post-transcriptional levels (Supplementary Table 6).

3.4. Influence of additional genetic abnormalities on MDR gene expression

It is well known that the outcome of del(9q) patients is strongly influenced by additional genetic abnormalities. For example, t(8;21) confers a significantly improved outcome (Supplementary Table 4) [28,29]. We therefore analyzed expression of the genes located in the MDR in different genetic subgroups. The levels of mRNAs encoded by MDR genes were not correlated with deletion size. Also, no difference in mRNA levels between the subgroups del(9q) only, with t(8;21) and with other genetic abnormalities was observed (Table 2, Supplementary Fig. 1). *RUNX1-RUNX1T1*, the gene product resulting from the t(8;21) translocation, mediates *CEBPA* down-regulation on the transcriptional level [30]. *CEBPA* is mutated in 6.5% of analyzed del(9q) and 8.3% NK patients (Table 1) and *CEBPA* mutations are mutually exclusive with t(8;21) [31–33]. Accordingly, patients were divided in *CEBPA* wt

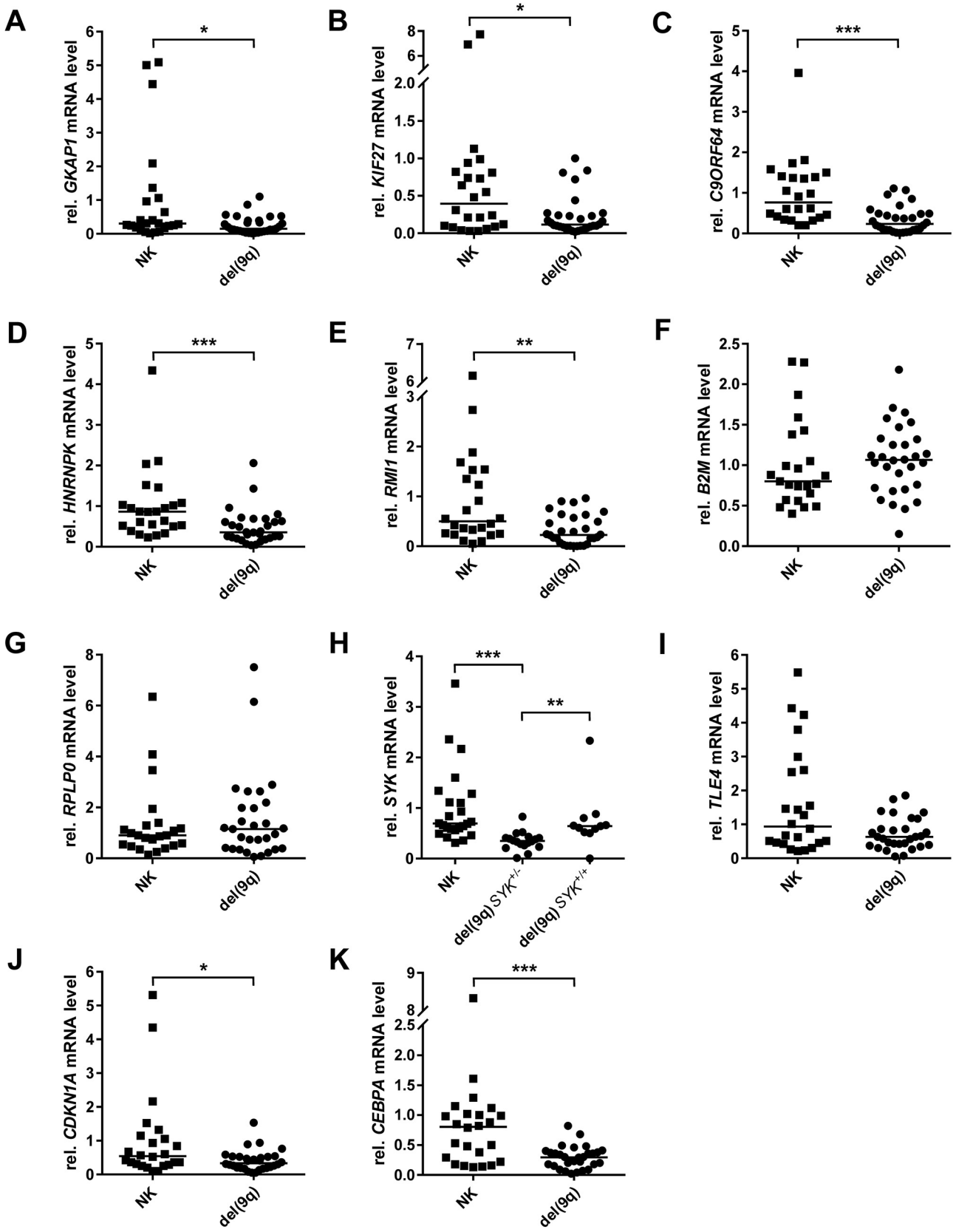

Fig. 3. Determination of mRNA levels in NK and del(9q) AML. MRNA levels were determined with specific primers by RT-qPCR analysis for genes located in the MDR as indicated: *GKAP1* (A), *KIF27* (B), *C9ORF64* (C), *HNRNPK* (D), *RMI1* (*E*) as well as for *B2M* (F), *RPLP0* (G), *SYK* (H), *TLE4* (I), *CDKN1A* (*J*) and *CEBPA* (K). Data were normalized to *ACTB* mRNA as housekeeping gene and to the mean of the NK using the ΔΔCt method. Mann-Whitney test for statistical significance (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Table 2
Association of MDR encoded mRNA levels with additional genetic abnormalities. Relative expression of MDR genes (*GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK*, *RMI1*) as well as of *SYK*, *TLE4*, *CDKN1A* and *CEBPA* determined by RT-qPCR was analyzed for correlation with deletion size as well as for differences in different genetic subgroups. Complex karyotype was defined as three or more genetic aberrations (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.a. = not applicable).

	GKAP1		*KIF27*		*C9ORF64*		*HNRNPK*		*RMI1*		*SYK*		*TLE4*		*CDKN1A*		*CEBPA*	
Spearman	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)	NK	del(9q)
deletion size																*		
r		0.185		−0.115		−0.308		−0.062		0.050		−0.246		−0.123		−0.385		0.088
p-value		0.3376		0.5533		0.1042		0.7505		0.7987		0.2063		0.5335		0.0393		0.6483
median																		
subgroups																		*
only		0.15		0.16		0.27		0.30		0.23		0.43		0.76		0.49		0.36
t(8;21)		0.10		0.10		0.20		0.35		0.17		0.38		0.57		0.36		0.15
other		0.36		0.13		0.43		0.55		0.26		0.41		0.42		0.31		0.33
p-value		0.0696		0.9722		0.4558		0.5227		0.9699		0.9180		0.1367		0.7733		0.0118
FLT3-ITD																		
yes	0.29	0.10	0.60	0.11	1.00	0.15	0.99	0.31	0.82	0.18	0.70	0.46	1.09	0.41	0.36	0.41	0.65	0.24
no	0.31	0.17	0.31	0.13	0.91	0.34	0.86	0.53	0.42	0.27	0.73	0.42	1.01	0.64	0.60	0.30	0.85	0.30
p-value	0.7165	0.7536	0.9882	0.8379	0.7646	0.2525	0.8874	0.1889	0.1688	0.7944	0.7284	0.6921	0.5580	0.2298	0.6232	0.8166	0.4281	0.4187
NPM1												*						**
yes	0.36	0.07	0.64	0.09	1.38	0.33	0.95	0.32	1.13	0.19	0.83	0.56	0.48	0.69	0.36	0.51	0.99	0.40
no	0.28	0.18	0.23	0.13	0.60	0.25	0.74	0.43	0.41	0.30	0.65	0.37	1.35	0.58	0.59	0.30	0.49	0.21
p-value	0.7303	0.1769	0.4797	0.3343	0.2315	0.4762	0.5995	0.6446	0.2381	0.1986	0.3503	0.0315	0.0833	0.4921	0.1761	0.0813	0.0808	0.0033
CEBPA																		
yes	0.64	0.40	0.65	0.24	0.72	1.02	0.70	0.75	0.30	0.63	0.67	0.48	1.37	0.67	0.58	0.11	0.84	0.53
no	0.28	0.13	0.28	0.11	0.77	0.20	0.87	0.34	0.56	0.20	0.70	0.41	0.77	0.63	0.55	0.38	0.81	0.27
p-value	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
complex																		
yes		0.36		0.13		0.19		0.52		0.24		0.41		0.44		0.30		0.26
no		0.12		0.11		0.29		0.36		0.23		0.42		0.70		0.38		0.32
p-value		0.1260		0.9176		0.9908		0.4965		0.5724		0.4307		0.2893		0.6208		0.1810

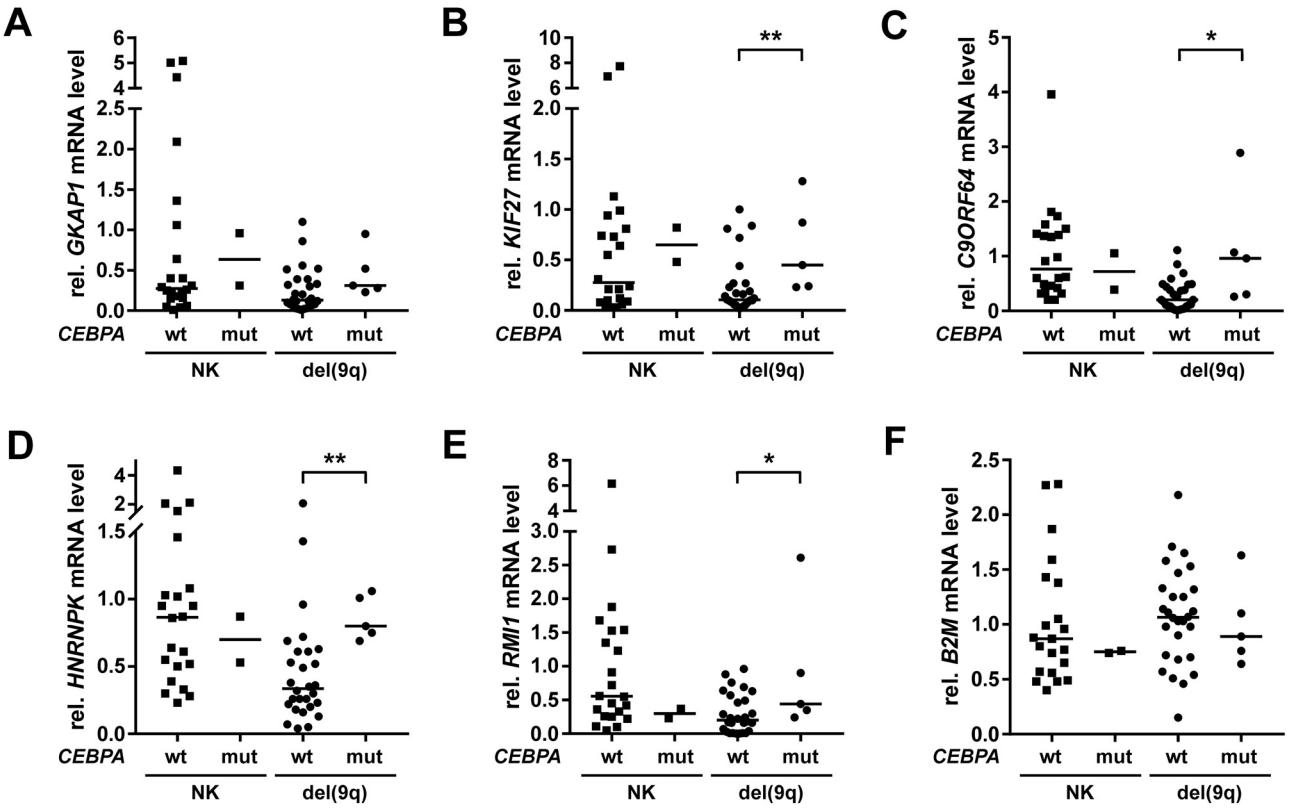

Fig. 4. Expression of MDR genes in *CEBPA* wt and mut patients. MRNA levels determined by RT-qPCR analysis in NK and del(9q) AML patient samples shown in Fig. 3 as well as in three additional del(9q), *CEBPA* mut samples were plotted according to the *CEBPA* mutation status for *GKAP1* (A), *KIF27* (B), *C9ORF64* (C), *HNRNPK* (D) and *RMI1* (E) as well as for the control gene *B2M* (F). Kruskal-Wallis test followed by Dunn's multiple comparison test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

(n = 22 for NK, n = 29 for del(9q)) and *CEBPA* mut patients (n = 2 for both groups). Interestingly, del(9q) samples with mutations in *CEBPA* exhibited an elevated expression of the mRNAs encoded by MDR genes (Table 2, Supplementary Fig. 2 A–E). This effect is not observed in NK samples and for *B2M* mRNA (Supplementary Fig. 2F). From the SAL biorepository three additional patient samples harboring del(9q), *CEBPA* mut could be included in the expression analysis (Fig. 4). In the total group of five del(9q) *CEBPA* mut patients, the *CEBPA* mutation was biallelic in three samples and monoallelic in two samples. This analysis confirmed the elevated expression of MDR genes in del(9q), *CEBPA* mut samples (Fig. 4A–E). The effect was most prominent for *HNRNPK*, whose expression is restored in all samples analyzed (Fig. 4D). *FLT3*-ITD and *NPM1* mutations had no impact on expression of the MDR genes (Table 3, Supplementary Figs. 3 and 4). These data indicate that expression of the del(9q) MDR genes is specifically upregulated by mutant C/EBPα, which might contribute to improved prognosis.

4. Discussion

AML is the most frequent acute leukemia in adults and characterized by heterogeneous genetics. In about 2% of AML cases a deletion on the long arm of chromosome 9 (del(9q)) is detected [2].

Here we confirm the presence of an MDR (Fig. 1) that is affected in all del(9q) patients analyzed on the gene level (PCR, SNP-array), except Q35 (Fig. 2, [9,11]), albeit the deletion size strongly differs between patients. The comparison of standard chromosome banding techniques with PCR and SNP-array analysis revealed that precise molecular methods are required to accurately determine the deleted region (Fig. 2). The comprehensive analysis of clinical parameters showed no correlation with deletion size (Supplementary Table 5). Our study points to an important function of the MDR, which harbors seven genes: *GKAP1*, *KIF27*, *C9ORF64*, *HNRNPK*, *RMI1*, *SLC28A3* and *NTRK2*. Accordingly, the deletion size has no effect on the outcome of these patients (Supplementary Table 5). Expression of the MDR genes is significantly reduced in del(9q) patients (Fig. 3A–E), corresponding to deletion of one allele. The high correlation confirms the simultaneous deletion of these genes. *TLE4* expression was not significantly reduced in del(9q) compared to NK samples (Fig. 3I), although it was also frequently deleted (Fig. 1, Supplementary Table 8). A reduced expression of *TLE4* in AML was shown also in absence of genetic deletion [10]. Thus, expression may be reduced in NK samples as well.

The outcome of AML del(9q) patients is influenced strongly by other genetic aberrations. It is well documented that del(9q) associated with t(8;21), resulting in expression of the fusion gene *RUNX1-RUNX1T1* confers a good prognosis [24,28]. Expression of *RUNX1-RUNX1T1* results in transcriptional down-regulation of the transcription factor *CEBPA* [30]. Furthermore, C/EBPα protein function is disturbed by different other mechanisms in AML. Among others, the fusion gene products of t(3;21) and inv(16) activate expression of Calreticulin [34,35], which in turn represses translation of the *CEBPA* mRNA [36]. Hypermethylation of the *CEBPA* promoter leads to reduced *CEBPA* expression as well [37] and is associated with favorable prognosis [38]. Mutations in *CEBPA* are found in 5–14% of AML cases [31,39]. T(8;21) and *CEBPA* mutations are mutually exclusive [31–33], which also applies to our patient cohort (Supplementary Table 3). Among the group of 31 del(9q) patients analyzed, 12 (38.7%) harbor a t(8;21) and 2 (6.5%) bear a *CEBPA* mutation. Mutation of *CEBPA* confers an improved overall survival, similar to t(8;21) [39]. In summary, *CEBPA* loss of function is an important aspect for a good outcome of AML patients.

Among the MDR genes, *HNRNPK* is best studied. HnRNP K is a multifunctional protein, which binds DNA and RNA and serves as

interaction platform for other proteins [18,26,27]. It is well known as regulator of mRNA translation in erythroid maturation and inflammation [20,40–42]. However, hnRNP K also binds DNA and either functions as gene-specific inhibitor or activator of transcription [19,43–46]. It has been shown in mice that hnRNP K interacts with the *CEBPA* and *CDKN1A* mRNAs [15], which harbor hnRNP K binding motifs [47] that might confer regulation of translation. Interestingly, hnRNP K haploinsufficency in mice leads to reduced *CEBPA* mRNA and C/EBPα-p42 protein, but not C/EBPα-p30 protein levels [15], pointing to an effect on start codon selection. In addition, it was shown that hnRNP K binds the *CEBPA* and the *CEBPB* gene [15] and regulates *CEBPB* transcription [44]. Here we show that the deletion of the MDR including *HNRNPK* in AML del(9q) patient samples results in reduced expression of its target genes *CDKN1A* and *CEBPA* as well (Fig. 3J–K).

Strikingly, *HNRNPK* mRNA as well as the other mRNAs encoded by MDR genes are enhanced in a group of five del(9q), *CEBPA* mut patients (Fig. 4). This may indicate a regulatory interaction between *CEBPA* and the MDR genes. However, a larger patient cohort with *CEBPA* mutations currently not available is required to investigate this trend further, which would also allow to analyze the outcome of these patients. In contrast to *CEBPA* mutation, t(8;21) does not restore expression of the MDR genes. Supplementary Figure 6 schematically highlights key characteristics of the different genetic backgrounds in AML.

Deletion of the MDR genes seems to be important for the development of AML in del(9q) patients. Our data in conjunction with previously published data employing a haploinsufficient mouse model [15] point to *HNRNPK* as an important gene in this chromosomal aberration. Its gene product hnRNP K in turn seems to influence expression of C/EBPα [15]. Down-regulation of C/EBPα activity or of its expression by different mechanisms (promoter methylation, repression of transcription, repression of mRNA translation, inhibitory post-translational modifications) are key elements in the development of AML [48–50]. Future work should focus on the mechanism by which hnRNP K regulates C/EBPα expression as well as on a potential contribution of the other MDR genes, especially *GKAP1*, *KIF27*, *C9ORF64* and *RMI1*. Studies in cellular models of AML are required to functionally dissect the underlying mechanisms using defined knock out cell lines of single MDR genes.

Authors contributions

MC, ISNdV, AOL, DHO and EJ designed the study and analyzed data. ISNdV, YS and FK performed the experiments and statistical analyses. MC, GE, CR and CT selected patient samples and collected patient data. MC and ISNdV wrote this manuscript. THB and GM discussed the data and critically revised the manuscript. All authors critically read the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2018.11.007.

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