Secondary mutations in t(4;11) leukemia patients

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MLL rearrangements are a genetic hallmark of acute leukemia patients, which exhibit a particular poor outcome. To date, more than 70 *MLL* rearrangements have been described at the molecular level.[1] For the most frequently diagnosed *MLL* rearrangements, for example, *AF4-MLL*, *MLL-AF9*, *MLL-AF10* or *MLL-ENL*, it has been shown that these fusion proteins are sufficient for acute leukemia onset in murine model systems.[2] However, these models had a latency time of 4–12 months for the disease phenotype to become overt. This argues in favor of pre-leukemic clones that carry the fusion genes, but need, in addition, complementing mutations to develop a malignant disease.

In case of *MLL* rearrangements—specifically *MLL-AF4*—it has been shown that copy number aberrations could not be detected through whole genome high-resolution technologies,[3] favoring the possibility that single nucleotide activating mutations, such as mutated *Fms-like tyrosine kinase 3* (*FLT3*) or *RAS*, are more important than previously reported.[4–6] Therefore, we investigated the incidence of *RAS* and *FLT3* mutations in a large cohort of *MLL*-rearranged leukemia patients ($n = 144$). This cohort of *MLL*-associated leukemias was split into three subgroups. The t(4;11) group, which was of major interest includes 21 t(4;11) patients displaying complex genomic rearrangements (>2 fusions alleles; 11 pediatric, 10 adult), whereas 79 t(4;11) patients had a balanced translocation (31 pediatric, 48 adult). The control group of 44 patients comprised cases with *MLL-AF9* ($n = 22$; 19 pediatric, 3 adult), *MLL-ENL* ($n = 14$; 13 pediatric, 1 adult), *MLL-AF10* ($n = 4$; 4 pediatric), *MLL-ELL* ($n = 2$; 2 pediatric) and, finally, 1 case with *MLL-AF6* (adult) and one with *MLL PTD* (adult), respectively. All patient material has been made available by the I-BFM and German multicenter acute lymphoblastic leukemia (ALL) study groups.

Genomic DNA of these patients was investigated by amplifying the corresponding genomic regions of the *N-RAS* and *K-RAS* genes (exons 2 and 3), and of the *FLT3* receptor gene (exons 13–15 and exon 20) and, subsequently, by sequencing the resulting amplimers. FLT3 is predominantly expressed in hematopoietic stem/progenitor cells. In addition, FLT3 is expressed at considerable levels in most clinical samples from acute myeloid leukemia (AML) and B-cell precursor ALL patients. Internal tandem duplication of the juxtamembrane domain of the *FLT3* gene is frequently observed in AML. AML patients bearing a particular *FLT3* mutation (D835; *FLT3-TKD*) tend to have poor prognosis, suggesting that *FLT3*-TKD mutations have an important role in AML.[7] Otherwise, it has been published that *FLT3-TKD* mutations were found in approximately 15% of patients with *de novo* mixed-lineage leukemia (MLL) rearrangements.[5] However, these patients were screened by Southern blot experiments, without gaining information about the involved *MLL* fusion partner.

In the cohort of 144 *MLL*-rearranged leukemia samples we analyzed, none of the investigated patients—regardless of whether diagnosed with balanced, complex t(4;11) rearrangements or other *MLL* fusions—displayed a genomic *FLT3* mutation. This is in line with data reported in the literature that mutant *FLT3* is mostly diagnosed in hyperdiploid leukemia cases.[8]

The reason for the absence of mutant *FLT3* receptor genes in *MLL*-rearranged leukemia might be that a mutant *FLT3* receptor gene confers a selective disadvantage for *MLL*-rearranged cells. Published data demonstrates that *MLL*-rearranged leukemia cells seem to overexpress wild-type FLT3 receptor and that stimulation of *MLL*-rearranged leukemia cells with FLT3-ligand (FL) causes quiescence, leading to chemoresistance.[9] As a result, *MLL*-rearranged leukemia cells get protected against chemotherapy when residing in the bone marrow and binding to FL-expressing stroma cells. This also explains why a high FLT3 expression in leukemia cells directly predicts a high relapse rate and poor outcome.[10] Thus, MLL-rearranged leukemia seems to be addicted to wild-type FLT3 receptor, and not to mutant FLT3. This becomes obvious when recent findings are considered: FLT3–internal tandem duplication mutations cause oncogenic signaling from the endoplasmatic reticulum, which—different from wild-type FLT3 receptors—causes a direct phosphorylation of STAT5, and subsequently, an upregulation of SOCS proteins. SOCS proteins block cytokine signaling from the cell surface, by inhibiting the family of Janus kinases (JAK1-3, TYK2), which results in a "shielding effect" against external cytokine signals.[11] However, *MLL*-rearranged cells depend on such external signals deriving from the expressed FL on bone marrow stroma cells. Thus, inhibition of cell surface signaling through a mutant FLT3 receptor represents a counter-productive event that seems to be selectively omitted in *MLL*-rearranged leukemia, which could be a reason for the absence of FLT3 mutations in our study.

By contrast, our *RAS* gene analyses revealed mutations in *MLL*-rearranged patients. The majority of point mutations was identified in pediatric patients ($n = 31$) bearing a balanced t(4;11) translocation ($n = 8$; $1 \times$ *N-RAS*, $7 \times$ *K-RAS*), whereas adult patients with balanced t(4;11) translocations ($n = 48$) had significantly less mutated *RAS* genes ($n = 4$; $2 \times$ *N-RAS*, $2 \times$ *K-RAS;* $P = 0.035$). Four additional *RAS* mutations were identified in pediatric patients with *MLL-AF9* ($n = 1$; $1 \times$ *K-RAS*), *MLL-ENL* ($n = 2$; $1 \times$ *N-RAS*, $1 \times$ *K-RAS*) and *MLL-AF10* ($n = 1$; $1 \times$ *K-RAS*). As we only had six adult cases in this subgroup of *MLL*-rearranged leukemia patients, statistical evaluation was not eligible. Regarding the three different subgroups of the investigated cohort (Figure 1), no statistical significance could be observed. All identified *N-RAS* point mutations—except one G12C exchange—resulted in missense G12D mutations. In one pediatric t(4;11) patient, an *N-RAS* G12D/G13D double mutation was identified. By contrast, the spectrum of *K-RAS* mutations was more diverse, including G10A, G12A, G12D, G12V and G13D missense mutations. Of interest, the group of patients with complex *MLL* rearrangement ($n = 21$; $11 \times$ pediatric, $10 \times$ adult) did not exhibit any *RAS* mutation. All these data were summarized in Figure 2.

To understand the unusual bias of mutant *RAS* genes in the group of infant/pediatric t(4;11) patients, we tried to analyze the matched relapse samples. For most t(4;11) patients, this material was not available, because the patients are either still in remission ($n = 5$) or no information for a relapse could be provided ($n = 8$). Thus, we obtained only three matched relapse samples (one adult and two pediatric cases with balanced t(4;11) translocation).

To our surprise, two patients lost their mutated *RAS* allele upon relapse, whereas one pediatric case still retained its mutation (Figure 2). This clearly argues in favor for the presence of different subclones in the diagnostic tumor sample, which are (1) only supportive during disease onset, but presumably not necessary for disease maintenance, or (2) that mutated *RAS* genes are recognized by the host immune system, and thus effectively cleared by T-cell clones, or (3) that *RAS* mutations identified in diagnostic samples are just coincidence.

In conclusion, we found *N-* and *K-RAS* mutations in 26% of pediatric patients that exhibit a balanced t(4;11) translocation. We also demonstrate that *RAS* mutations can be readily diagnosed in

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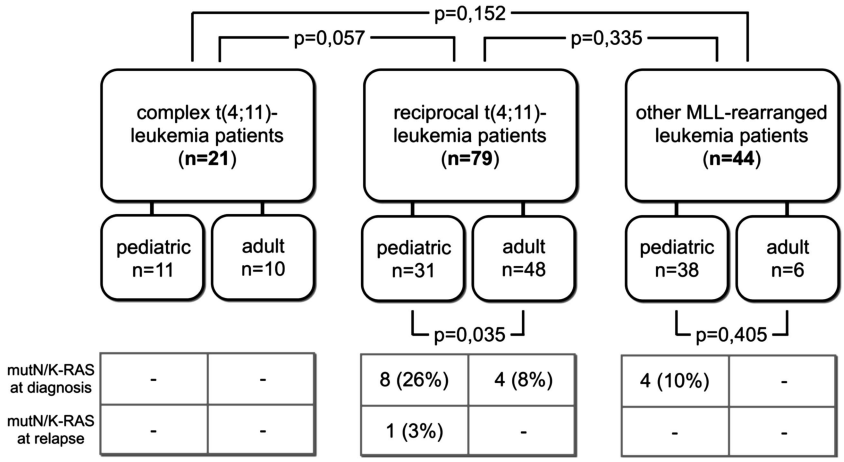

Figure 1. The cohort of 144 patients is split into 21 t(4;11) leukemia patients that displayed a complex rearrangement of the *MLL* gene (with 3–4 fusion genes), 79 leukemia patients that displayed a balanced t(4;11) translocation and 44 leukemia patients that displayed several distinct *MLL* rearrangements. The number of pediatric and adult patients is displayed for each subgroup. We identified a total of 12 pediatric patients with *K-* or *N-RAS* mutations. A similar situation was found in four adult t(4;11) patients that displayed again *K-* or *N-RAS* mutations. Of three investigated relapse samples, only one patient retained its *RAS* mutation.

UPN	TL	age @ diagnosis	mutation	sequence @ diagnosis	sequence @ relapse
P08_1316	t(4;11)	2.5 months	NRAS - G12D/G13D		no information
P09_1409	t(4;11)	2.9 months	KRAS - G12V		no information
P05_394	t(4;11)	5 months	KRAS - G12D		no information
P05_399	t(4;11)	5 months	KRAS - G12V		
P08_1180	t(4;11)	154 months	KRAS - G13D		MRD negative
P09_1414	t(4;11)	3 months	KRAS -G13D		
P09_1421	t(4;11)	1 month	KRAS - G12A		no information
P10_1628	t(4;11)	10 months	KRAS - G13D		no information
P10-1783	t(11;19)	4.6 months	KRAS - G12D		no information
P10-1758	t(9;11)	4 years	NRAS - G12D		MRD negative
P10-1755	t(10;11)	0.6 months	KRAS - G12D		no information
P10-1754	t(11;19)	14 months	NRAS - G12C		no information
P03_196	t(4;11)	adult	KRAS - G10A		MRD negative
P06_821	t(4;11)	adult	NRAS- G12D		
P03_208	t(4;11)	adult	KRAS - G13D		MRD negative
P03_209	t(4;11)	adult	NRAS - G12D		MRD negative

Figure 2. Summary of all data obtained in patient analyses. UPN, genetic rearrangement, age at diagnosis, identified mutation and sequence chromatogram is displayed. For most patients with RAS mutations, no remission samples were available, because these patients are still in remission or no information was available for these patients.

adult t(4;11) leukemia patients, however, in a much lower frequency (8%). In view of this, it is interesting that a recent study demonstrated how ectopic expression of MLL-AF4, MLL-AF5 or MLL-LAF4 fusion proteins led to activated ELK-1 protein, a downstream target of the RAS/RAF signaling pathway.[12] Thus, these fusion proteins are *per se* able to activate the RAS/RAF signaling cascade by themselves and, therefore, *RAS* mutations are actually not necessary. By contrast, in a humanized *MLL-AF10* model, the additional expression of mutant *K-RAS* led to an AML M5 leukemia disease phenotype, whereas *MLL-AF10* expression alone did not.[13] This indicates that a certain threshold of RAS signaling may be necessary to establish a leukemic disease in the murine system.

Considering the overall frequency of RAS mutations in our analyses, the above mentioned mouse data are hardly comparable to the human system. The fact that we found a significant higher mutation rate in pediatric t(4;11) patients (26% pediatric versus 8% adult) could be interpreted in a way that RAS mutations are a potential second hit for early leukemia onset. However, we disagree with this notion because a 26% frequency of additional

mutations is not sufficient to postulate a two-hit model for ALL childhood leukemia. Therefore, other interpretations are necessary. By now, we can only speculate about a biological function, but there is a significant difference between mutant and physiological RAS signaling. Downregulation of phosphoinositide-3 kinase signaling and activation the ATM/ATR-induced DNA damage response system may cause a delay of tumor development.[14] In addition, an additional RAS mutation might be recognized by the mother's immune system, and thus preventing tumor outgrowth already *in utero*.

To this end, further work will be necessary to find a satisfactory explanation for these observations, and to understand at the molecular mechanisms triggered by mutated RAS signaling. Understanding the importance of oncogenic signaling for the biology of *MLL*-rearranged leukemia may bear the potential to identify novel drug targets that can be therapeutically addressed in future therapy regimens. This might be true for signaling events deriving from highly expressed FLT3, as inhibition of this receptor by PKC412 and CEP-701 resulted in a selective killing of childhood leukemia cells.[15]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Low GFI1 expression in white blood cells of CP–CML patients at diagnosis is strongly associated with subsequent blastic transformation

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Chronic myeloid leukemia (CML) is characterized by the *BCR–ABL1* fusion gene, resulting in uncontrolled proliferation of myeloid progenitor cells. Growth factor independence 1 (GFI1) is a transcription factor with a crucial role in haematopoiesis, including preserving haematopoietic stem cell (HSC) quiescence and enhancing granulocytic differentiation, but is not required for inducing myeloid differentiation in p210BCR/ABL-transformed cells.[1] Recently, Soliera *et al.*[2] demonstrated that ectopic GFI1 expression inhibited proliferation and colony formation both in p210BCR/ABL-expressing cell lines and in primary CD34+ CML cells through the repression of STAT5B and/or Mcl-1. This study, along with their previous work[1] demonstrated the biological importance of the GFI1/STAT5B/Mcl-1 regulatory pathway on proliferation and survival of CML cells. However, the association between GFI1 expression and the achievement of response to imatinib therapy, be it major molecular response (MMR; $<0.1\%$ *BCR–ABL1* (IS)) or early molecular response (*BCR–ABL1* $\leqslant 10\%$ following 3 months of imatinib therapy) in *de-novo* chronic phase–CML (CP–CML) patients has not been examined. We hypothesized, based on the findings of Soliera *et al.* that increased GFI1 expression would be associated with a favorable outcome in CP–CML treated with imatinib.

The expression of GFI1 was examined using TaqMan Low Density array (RQ-PCR, Applied Biosystems, Carlsbad, CA, USA) in white blood cells of 40 *de-novo* CP–CML patients enrolled in clinical trials conducted in our Centre, who received imatinib as frontline therapy. Data analysis was performed using the statistical program *R* version 2.15.1, with the Bioconductor package high-throughput analysis and visualization of quantitative real-time PCR (HTqPCR)[3] and GraphPad Prism 5. GFI1 expression (Hs00382207_m1, Applied Biosystems) was normalized to *GUSB* expression (Hs99999908_m1, Applied Biosystems) using the $\Delta\Delta$Ct method.[4] As GFI1 is primarily expressed in granulocytes,[5] we rationalized that the use of white cells would enable accurate representation of total GFI1 expression from each patient.

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