A comparative study of molecular mutations in 381 patients with myelodysplastic syndrome and in 4130 patients with acute myeloid leukemia

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ABSTRACT

Background and Objectives

The precise relationship between myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) is unclear and the role of molecular mutations in leukemic transformation in MDS is controversial. The aim of this study was to clarify the relationship between AML and MDS by comparing the frequency of molecular mutations in the two conditions.

Design and Methods

We compared the frequency of *FLT3*-length mutations (*FLT3*-LM), *FLT3*-TKD, *MLL*-partial tandem duplications (*MLL*-PTD), *NRAS*, and *KIT*D816 in 381 patients with MDS refractory anemia with excess blasts [RAEB] n=49; with ringed sideroblasts [RARS] n=310; chronic monomyelocytic leukemia [CMML] n=22) and in 4130 patients with AML (*de novo*: n=3139; secondary AML [s-AML] following MDS: n=397; therapy-related [t-AML]: n=233; relapsed: n=361).

Results

All mutations were more frequent in s-AML than in MDS and all but the *FLT3*-TKD were more frequent in RAEB than in RA/RARS. The higher incidences in s-AML were significant for *FLT3*-TKD (p=0.032), *MLL*-PTD (p=0.034), and *FLT3*-LM (RA/RARS: 0/45; RAEB: 8/293; 2.7%; s-AML: 45/389; 11.6%; p<0.0001). The incidence of NRAS-mutations increased from 17/272 (6.3%) in MDS to 41/343 in s-AML (12.0%) and that of *KIT*D816-mutations from 2/290 (0.7%) to 5/341 (1.5%) (p=n.s.). *FLT3*-LM-acquisition occurred in 3/22 cases (13.6%) during MDS transformation; *NRAS*-acquisition occurred in 1/24 (4.2%). *FLT3*-LM and *MLL*-PTD were more frequent in AML relapse than in *de novo* AML or s-AML (p<0.0001).

Interpretation and Conclusions

The increase of molecular mutations from low- to high-risk MDS, to s-AML, and to relapsed AML emphasizes the value of these mutations as markers of progressing disease. Finally, we found a low rate of 5q- in the molecularly mutated cases in MDS which might explain the stability of this subtype.

Key words: myelodysplastic syndromes (MDS), acute myeloid leukemias (AML), molecular mutations, progression, leukemogenesis.

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From clinical, cytomorphologic, cytogenetic, and molecular aspects the myelodysplastic syndromes (MDS) are heterogeneous diseases. If left untreated, the survival of patients with MDS varies between a few months and 20 years. The World Health Organization (WHO) classification (based on the French-American British [FAB] classification of 1982) subdivides MDS into eight subtypes according to the percentages of bone marrow blasts, ringed sideroblasts, and the number of dysplastic cell lineages.[1-4] Karyotype represents a strong prognostic parameter:[5] 5q-, -Y, and 20q- as sole abnormalities are associated with a favorable prognosis. Complex aberrant karyotype (defined by ≥3 chromosomal abnormalities) and chromosome 7 abnormalities predict a poor prognosis, whereas all other chromosome abnormalities are associated with an intermediate prognosis. The International Prognostic Scoring System (IPSS) categorizes MDS patients into four risk groups based on blast percentage, karyotype, and the number of cell lines showing cytopenia.[3,5] However, cytogenetic abnormalities are observed in only 30-50% of *de novo* MDS cases.[6,7] Thus, molecular mutations may serve as potential markers to extend the spectrum of diagnostic and prognostic parameters in MDS. As MDS and AML are conceived as end points of a stepwise process of leukemogenesis in some patients, research in MDS should concentrate as well on the analysis of molecular mutations which occur frequently in AML.[8,9]

Mutations of the *FLT3*-gene, a member of the class-III-receptor tyrosine kinase family, play a central role in AML.[10-14] The *FLT3* length mutations (LM) (internal tandem duplications or insertions) (20%-27%) are, together with *NPM*1,[15] the most frequent mutations in AML. While *FLT3*-LM are prognostically unfavorable,[16-18] *NPM*1 mutations are associated with a favorable outcome.[15,19] Furthermore, small mutations in the tyrosine kinase domain (TKD) of *FLT3* (*FLT3*-TKD) have been found in 5%-8% of all AML cases.[11,12,20-22] Their prognostic impact in AML is not yet clarified.[16,22] In MDS the *FLT3*-mutations are less frequent. LM were found in 2%-3% (Horiike *et al*. n=58; Shih *et al*. n=198),[23,24] and TKD mutations in 3%, as shown by Yamamoto *et al*. (to our knowledge the only analysis of this marker in MDS; n=29).[12]

Mutations of the *KIT*-proto-oncogene are a further example of class-III-receptor tyrosine kinase mutations in AML. *KITD816*-mutations occur with a frequency of 2% in unselected AML, are localized in the intracellular protein tyrosine kinase domain,[25,26] and have an unfavorable prognostic impact in the subgroup of AML with t(8;21)/*AML1-ETO*.[27-29] In MDS, the single study focused on this mutation reported a frequency of 3/39 (6%) when combining all cytomorphologic subtypes in MDS.[30] Mutations of the *NRAS*-proto-oncogene are identified in 10-15% of cases of AML. These mutations increase the activity of the RAS-pathway and lead to cell proliferation and reduction of apoptosis.[31-35] Their influence on prognosis in AML seems dependent on cytogenetics and additional molecular mutations.[36] They show a favorable trend in CBF-leukemias and normal karyotype AML lacking *FLT3*-LM and partial tandem duplications of the *MLL* gene (*MLL*-PTD). In MDS frequencies of *NRAS* mutations were reported to be between 7% and 48% in previous studies including series of up to 220 patients.[37-42] *MLL*-PTD occur in 10% of AML with normal karyotype and are associated with a poor prognosis.[43-46]

Here we performed a study on the incidence of *FLT3*-LM, *FLT3*-TKD, *MLL*-PTD, *NRAS*- and *KITD816*-mutations in different cytomorphologic subtypes of MDS (n=381) and compared these data to those for AML (n=4130) in order to gain a better understanding of the leukemic transformation of MDS. In addition, we analyzed the correlation of these mutations with cytogenetics in MDS.

Design and Methods

Bone marrow samples – in many cases accompanied by peripheral blood samples – from 381 consecutive patients with MDS at diagnosis and from 4130 patients with AML (*de novo* AML at diagnosis: n=3139; secondary AML [s-AML] at diagnosis: n=397; therapy-related [t-AML] at diagnosis: n=233; and relapsed AML: n=361) were included in the study. The patients' clinical data and biological characteristics are shown in Table 1. The cytomorphological classification was made according to the FAB-classification, as the cohort was analyzed in part before WHO-criteria were defined in these patients.[2,47] The patients with refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS) were combined to form the RA/RARS cohort. The cohort with refractory anemia with excess blasts (RAEB) included patients with RAEB-1 (≤10% of blasts) and with RAEB-2 (<20% of blasts). The third MDS subgroup was represented by the dysplastic subtype of chronic myelomonocytic leukemia (CMML). Only patients with MDS at diagnosis were included. AML patients were subdivided according to the history of disease: *de novo* AML, secondary AML (s-AML) following MDS, and therapy-related AML (t-AML) in association with previous chemotherapy or radiotherapy of a malignant disease. Screening for *FLT3*-LM, *FLT3*-TKD, *MLL*-PTD, *NRAS*- and *KIT*D816-mutations was performed as described before. All methods for mutation analysis have been reported in detail. Briefly, screening for *FLT3*-LM was performed by gel electrophresis[17] and fragment analysis[20] in parallel; *MLL*-PTD were analyzed by quantitative real-time polymerase chain reaction (PCR),[48] and

analysis for *FLT3*-TKD, *NRAS*- and *KIT*D816-mutations was performed using melting curve based Light Cycler analysis and subsequent sequencing of the positive samples.[29] In addition, chromosome banding analyses and fluorescence *in situ* hybridization were performed as previously described.[49]

The cytogenetic subgroups were categorized as follows: normal karyotype, reciprocal translocations, complex aberrant karyotype (≥3 chromosomal anomalies), deletion of 5q (5q-), chromosome 7 abnormalities, numerical gain of 8 (+8), deletion of 20q (20q-), loss of Y (-Y), inv(3)/t(3;3)(q21;q26), and other aberrations.

Results

Distribution of molecular mutations in low grade MDS, RAEB, and s-AML

First, the distribution of the mutations in the total cohorts of MDS and AML was analyzed. The complete results of the molecular analyses within the different subgroups are shown in Table 2. In MDS *NRAS*-mutations were detected in 17/272 patients (6.3%) and, from among the analyzed mutations, was the one with the highest frequency in MDS, followed by the *MLL*-PTD (10/368; 2.7%) and by the *FLT3*-LM (8/367; 2.2%). In contrast, in the total AML cohort *FLT3*-LM was the most frequent mutation of all those analyzed (783/3718; 21.1%), followed by the *NRAS* mutation (290/2856; 10.2%), and *FLT3*-TKD (144/3052; 4.7%).

Considering the different cytormophologic subtypes of MDS, in RA/RARS a *FLT3*-TKD-mutation was observed in 1 of 28 cases whereas *FLT3*-LM, *NRAS*, and *KITD816* were not observed at all. In RAEB *NRAS* mutations represented the most frequent molecular marker (15/223; 6.7%), followed by the *FLT3*-LM

Table 1. Clinical data and cytomorphologic subtypes of 381 patients with MDS at diagnosis and of 4130 patients with AML at diagnosis or relapse.

MDS	
Sex	240 males, 141 females
Age	18.1-97.8 years (median 68.1)
RA/RARS	49
RAEB	310
CMML	22
MDS total	381
AML	
Sex	2260 males, 1870 females
Age	16.2-96.8 years (median 61.2)
de novo AML	3139
s-AML	397
t-AML	233
Relapsed AML	361
AML total	4130
Total MDS + AML	**4511**

Table 2. Mutated cases in the different cytomorphologic subtypes of MDS and in the cohorts with AML.

Mutation	*RA/RARS*	*RAEB*	*CMML*	*Total MDS*	*de novo AML*	*s-AML*	*t-AML*	*Relapsed AML*	*Total AML*
***FLT3*-LM**									
N. mutated	0	8	0	8	629	45	26	83	783
Total number	45	293	29	367	2813	389	216	300	3718
%	0.0	2.7	0.0	**2.2**	22.4	11.6	12.0	27.7	**21.1**
	(<0.0001)	(<0.0001)	(0.034)		(<0.0001)	(<0.0001)	(0.004)	(<0.0001)	
***FLT3*-TKD**									
N. mutated	1	0	0	1	130	6	3	5	144
Total number	28	209	19	256	2357	322	167	206	3052
%	3.6	0.0	0.0	**0.4**	5.5	1.9	1.8	2.4	**4.7**
	(1.000)	(<0.0001)	(1.000)		(0.030)	(0.032)	(0.064)	(0.096)	
MLL-PTD									
N. mutated	1	8	1	10	176	28	7	25	85
Total number	46	292	30	368	2735	378	213	303	1197
%	2.2	2.7	3.3	2.7	6.0	7.4	3.3	8.3	**7.1**
	(0.517)	(0.032)	(0.627)		(0.017)	(0.034)	(0.166)	(0.036)	
NRAS									
N. mutated	0	15	2	17	209	41	21	19	290
Total number	29	223	20	272	2128	343	174	211	2856
%	0.0	6.7	10.0	**6.3**	9.8	12.0	12.1	9.0	**10.2**
	(0.128)	(0.108)	(1.000)		(1.000)	(0.177)	(0.294)	(0.810)	
KITD816									
N. mutated	0	2	0	2	39	5	2	3	49
Total number	32	237	21	290	2136	341	172	213	2862
%	0.0	0.8	0.0	**0.7**	1.8	1.5	1.2	1.4	**1.7**
	(1.000)	(0.431)	(1.000)		(0.227)	(1.000)	(1.000)	(1.000)	

The p-values (in brackets) indicate whether the frequencies of the molecular mutations differ significantly from those in the other MDS or AML stages.

(8/293; 2.7%) and *MLL*-PTD (8/292; 2.7%).

Subsequently the distribution of the respective markers in the different hematologic subgroups was analyzed. The *FLT3*-TKD were heterogeneously distributed, most probably because of the very low frequency of this mutation overall. Statistically significant differences in distribution were found for *FLT3*-LM ($p<0.0001$) and *MLL*-PTD ($p=0.004$), whereas the distribution of the *NRAS*- and *KITD*816-mutations did not vary significantly. The incidence of the markers in early MDS categories (RA/RARS) was compared with that in the advanced stages (RAEB) and in s-AML. The incidence of *FLT3*-LM, *MLL*-PTD, *NRAS*-, and *KIT*D816-mutations increased from RA/RARS to RAEB and to s-AML. The differences were statistically significant for *FLT3*-LM ($p<0.0001$), *FLT3*-TKD mutations ($p<0.0001$), and *MLL*-PTD ($p=0.032$). The sharpest increase was observed for *FLT3*-LM which were found in no case with RA/RARS (0/45; 0.0%), in 8/293 (2.7%) cases of RAEB, and in 45/389 (11.6%) cases of s-AML ($p<0.0001$) (Figure 1).

Molecular mutations in CMML

In our small series of CMML with dysplastic subtype *NRAS*-mutations were found in 2/20 (10%) rates, which was similar to the frequency in AML. The other mutations analyzed were rarely (*FLT3*-TKD) or never (*FLT3*-LM and *KITD816*) observed in CMML (Table 2).

Incidence of molecular mutations with respect to history of AML

FLT3-LM were significantly more frequent in *de novo* AML and in AML at relapse than in s-AML or t-AML ($p<0.0001$). *FLT3*-TKD were significantly more frequent in *de novo* AML ($p=0.030$) and in s-AML ($p=0.032$) than in t-AML or in relapsed AML. The frequencies of *MLL*-PTD, *NRAS*-, and *KITD816*-mutations did not differ significantly between the different AML cohorts. These results confirmed those of two previous studies (Table 2).[17,36]

Concomitant molecular mutations in MDS

The concomitant presence of different molecular mutations was observed in only 2/381 MDS patients (0.5%): one case with RAEB showed *FLT3*-LM and *MLL*-PTD, whereas another RAEB patient had *FLT3*-LM, *MLL*-PTD, and *NRAS*-mutations.

Acquisition of molecular mutations during progression of MDS

Finally, we analyzed whether leukemic transformation of MDS was accompanied by acquisition of the molecular markers. For this analysis, 25 paired MDS/AML cases were available. We found that the mutation status was stable in all cases screened for *FLT3*-TKD (n=24), *MLL*-PTD (n=22), and *KITD816* (n=24). However, acquisition of *FLT3*-LM was observed in 3/22 cases (13.6%) during leukemic transformation and acquisition of the *NRAS* mutation was observed in 1/24 cases (4.2%) during leukemic transformation.

Distribution of chromosomal abnormalities in molecularly mutated MDS cases

We analyzed the frequency of chromosomal abnormalities in the molecularly mutated MDS cases. The cases with *FLT3*-LM, *NRAS* mutation, and *MLL*-PTD showed a high incidence of normal karyotype (*FLT3*-LM: 4/8 [50%]; *NRAS* mutation: 11/14 [79%]; *MLL*-PTD: 7/9 [78%]). We analyzed whether deletions of 5q or monosomy 7 showed any association with *FLT3*-LM or *NRAS* mutations: del(5q) was rare in *FLT3*-LM positive MDS (1/8; 12%) and was not found in *NRAS*- or *MLL*-PTD mutated cases. Chromosome 7 abnormalities were not detected in any of the *FLT3*-LM-, *NRAS*-, or *MLL*-PTD-positive MDS cases (Table 4).

Discussion

Given the new therapeutic options for MDS, such as allogeneic stem cell transplantation with reduced intensity conditioning for elderly patients,[50-52] intensive chemotherapy regimens for high-risk MDS,[53] and new compounds including azacitidine[54] and lenalidomide,[55] risk assessment and prognostic stratification in MDS have become increasingly important. As cytogenetics included in the IPSS provide the basis for prognostic predictions only in some patients,[6,7] additional parameters are needed for a more detailed characterization of the biology and prognosis of this heterogeneous disorder. In AML it has been established that 80-85% of all cases with normal karyotype can be further characterized by molecular markers, which are found alone or in combination with others (*NPM1*: 50%, *MLL*-PTD: 10%, *CEBPA*: 15%, *FLT3*-LM: 35%, *FLT3*-TKD: 6%, *NRAS*: 10%). In contrast, in MDS screening for molecular mutations is not currently included in routine practice, as the frequency and prognostic impact of these mutations are less well determined. However, there are many questions also with respect to the role of molecular mutations in the leukemic transformation process of MDS. Thus, in this study, we focused not only on the incidence of different molecular mutations in MDS, but also compared the distribution of these markers within the different stages of MDS and in AML.

In consideration of the central role of *FLT3*-mutations in AML (≥30% of all AML patients show either internal tandem duplications or mutations of the tyrosine kinase domain), *FLT3*-mutations have been hypothesized to be important in MDS transformation.[11,12,20-22] Indeed, our study gives additional support to an association of *FLT3*-LM with progression. These

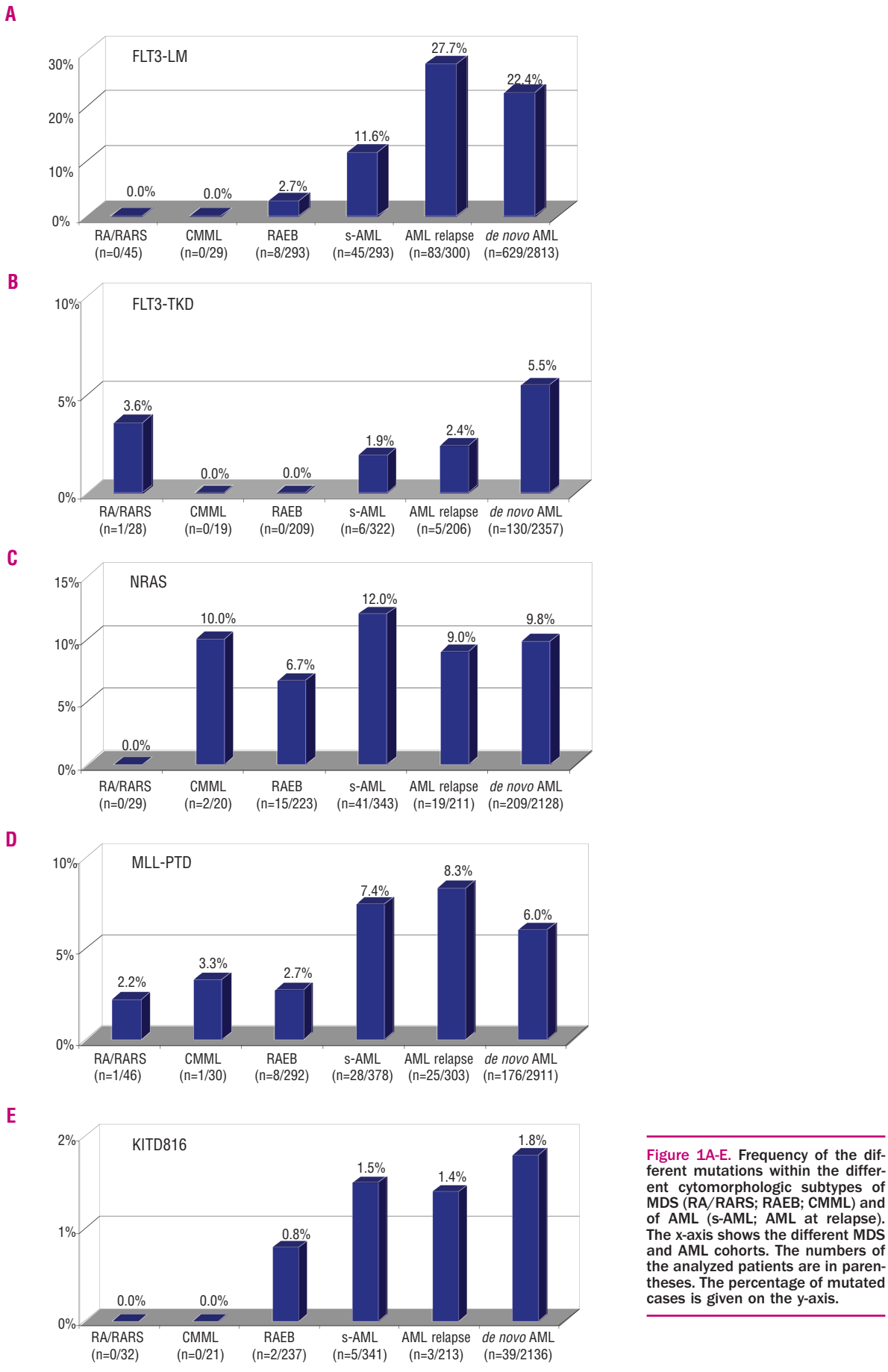

Figure 1A-E. Frequency of the different mutations within the different cytomorphologic subtypes of MDS (RA/RARS; RAEB; CMML) and of AML (s-AML; AML at relapse). The x-axis shows the different MDS and AML cohorts. The numbers of the analyzed patients are in parentheses. The percentage of mutated cases is given on the y-axis.

Table 3. Frequency of molecular mutations in previous studies.

FLT3-LM	*This study*	*Shih et al., 2004*[24]	*Horiike et al., 1997*[23]	*Total*
RA	0% (0/45)	0% (0/27)	0% (0/13)	0.0% (0/98)
RARS			0%	0%
	(0/21)	(0/4)		
RAEB	2.7% (8/293)	2% (2/99)	0% (0/20)	2.4% (10/412)
RAEB-t	–	–	8% (1/12)	8% (1/12)
CMML	0% (0/29)	6% (3/51)	11% (1/9)	4.5% (4/89)
MDS total	**2.2% (8/367)**	**2.5% (5/198)**	**3% (2/58)**	**0.2% (15/623)**

FLT3-TKD	*This study*	*Yamamoto et al.,2001*[12]	*Total*
RA	3.6% (1/28)	–	3.6% (1/28)
RARS		–	
RAEB	0% (0/209)	0% (0/6)	0% (0/215)
RAEB-t	–	8% (1/13)	8% (1/13)
CMML	0% (0/19)	0% (0/10)	0% (0/29)
MDS total	**0.4% (1/256)**	**3% (1/29)**	**0.7% (2/285)**

NRAS	*This study*	*Nakagawa et al., 1992*[41]	*Paquette et al., 1993*[57]	*Mitani et al., 1997*[58]	*Padua et al., 1998*[42]	*Total*
RA	0% (0/29)	0% (0/10)	8% (6/72)	0% (0/9)	35% (6/17)	10.0% (20/201)
RARS		0% (0/1)	9% (4/46)	0% (0/1)	25% (4/16)	
RAEB	6.7% (15/223)	67% (2/3)	8% (5/63)	0% (0/8)	50% (5/10)	8.8% (27/307)
RAEB-t	–	100% (1/1)	13% (3/23)	9% (2/23)	not specified	12.8% (6/47)
CMML	10.0% (2/20)	20% (1/5)	12% (2/16)	–	66% (21/32)	35.6% (26/73)
MDS total	**6.3% (17/272)**	**20% (4/20)**	**9% (20/220)**	**2% (2/44)**	**48% (36/75)**	**12.5% (79/631)**

KITD816	*This study*	*Lorenzo et al., 2006*[30]	*Total*
RA	0.0% (0/32)	0% (0/10)	0.0% (0/42)
RARS		–	
RAEB	0.8% (2/237)	0% (0/10)	0.8% (2/247)
RAEB-t	–	13% (2/15)	13% (2/15)
CMML	0.0% (0/21)	0% (0/15)	0.0% (0/36)
MDS total	**0.7% (2/290)**	**4% (2/49)**	**1.2% (4/339)**

MLL-PTD	*This study*
RA	1/46 (2.2%)
RARS	
RAEB	8/292 (2.7%)
RAEB-t	–
CMML	1/30 (3.3%)
MDS total	**2.7% (10/368)**

Table 4. Distribution of chromosomal aberrations in the molecularly mutated MDS cases.

	FLT3-LM	*FLT3-TKD*	*cKITD816*	*NRAS*	*MLL-PTD*
Normal karyotype	50% (4/8)	100% (1/1)	0% (0/2)	79% (11/14)	78% (7/9)
Reciprocal translocations	0% (0/8)	0% (0/1)	0% (0/2)	0% (0/14)	0% (0/9)
Complex aberrant	12% (1/8)	0% (0/1)	0% (0/2)	7% (1/14)	0% (0/9)
inv(3)/t(3;3)	0% (0/8)	0% (0/1)	50% (1/2)	0% (0/14)	0% (0/9)
del(5q)	12% (1/8)	0% (0/1)	0% (0/2)	0% (0/14)	0% (0/9)
Chromosome 7 anomalies	0% (0/8)	0% (0/1)	0% (0/2)	7% (1/14)	0% (0/9)
+8	25% (2/8)	0% (0/1)	0% (0/2)	0% (0/14)	22% (0/9)
del(20q)	0% (0/8)	0% (0/1)	0% (0/2)	0% (0/14)	0% (0/9)
-Y	0% (0/8)	0% (0/1)	0% (0/2)	0% (0/14)	0% (0/9)
Others	0% (0/8)	0% (0/1)	0% (0/2)	7% (1/14)	0% (0/9)

mutations were not found in low-risk MDS, but their incidence increased over the proceeding stages (RAEB; RAEB-t) to s-AML, following MDS. In ≥10% of all cases the progression of MDS to AML is accompanied by the acquisition of *FLT3*-LM (this study; Shih *et al.*).[23,56] The occurrence of *FLT3*-LM at diagnosis of MDS is associated with leukemic transformation and shorter survival.[56] Furthermore, the incidence of *FLT3*-LM was significantly higher in relapsed AML than in *de novo* and s-AML at first diagnosis, underlining the importance of the *FLT3*-LM also in AML progression (Table 3). Thus, rather than being considered as initial events in the development of MDS, *FLT3*-LM should be considered as secondary events involved in MDS progression. The inclusion of the respective mutation status into MDS risk assessment at diagnosis and during follow-up might improve the identification of patients who may benefit from therapy intensification and might be considered also in routine diagnostics.

The role of *FLT3*-TKD mutations in MDS progression is less clear. *FLT3*-TKD are also significantly more frequent in s-AML than in MDS as shown by this study and by Yamamoto *et al.*,[12] and slightly more frequent in AML relapse than in s-AML at diagnosis (*this study*). This points to a role for *FLT3*-TKD in the transformation of MDS and possibly also in relapse of AML.[12] However, definite conclusions cannot yet be drawn as the numbers of cases and studies are too low – so far only two cases of *FLT3*-TKD mutations in

MDS have been reported: one patient with RA in this study and one patient with RAEB in transformation in the study by Yamamoto *et al*.[12]

Due to the rather high incidence of *NRAS*-mutations in AML, interest was focused on the role of this marker in MDS.[31-36] In this study, as in most previous analyses, *NRAS*-mutations were among the most frequent mutations in MDS (≥6.5% of all cases),[38,41,42,57] and more frequent than *FLT3*-LM (≤3%).[23,24] Although the reported incidences of *NRAS* mutations in MDS range widely (probably due to different proportions of MDS subtypes in the various analyses), this study and all mentioned previous analyses found higher frequencies of *NRAS* mutations in the advanced stages of MDS than in the initial stages.[41,42,57,58] This demonstrates an association between *NRAS* mutations and MDS transformation. *NRAS* mutations were further shown to be associated with karyotype evolution, e.g. with the acquisition of monosomy 7, during MDS transformation,[59,60] and with inferior survival in MDS.[57] Based on these results the inclusion of *NRAS*-screening at diagnosis and during follow-up in MDS might be discussed. With respect to the *MLL*-PTD, our data showed a significantly higher incidence in AML than in MDS, whereas the frequency of *MLL*-PTD did not vary significantly within the diverse cytomorphologic MDS subtypes. To our knowledge there are no further studies on this molecular marker in MDS, so the role of *MLL*-PTD in MDS and in leukemogenesis needs further clarification.

*KIT*D816 mutations play a minor role in AML. In MDS, these mutations seem to be restricted to the advanced stages of MDS, as found both in this study and a study by Lorenzo *et al*.,[30] suggesting involvement in the transformation towards AML. We found a slightly higher frequency of *KIT*D816 in AML than in MDS, but the numbers are too small to comment on this fact. We found no influence of AML progression to relapse on the incidence of *KIT*D816 mutations.

Another aim of our study was an analysis of the cytogenetic characteristics in the molecularly mutated MDS cases. The high rates of normal karyotype in *NRAS*-mutated cases (79% of all *NRAS*-mutated MDS patients in this study, 57% in the study by De Souza *et al*.)[61] support the hypothesis that *NRAS* mutations might represent the initial event in a proportion of MDS cases while additional aberrations induce leukemic transformation.[61] Some authors have suggested a co-operation of chromosome 7 abnormalities with *RAS* and *FLT3*-LM mutations in leukemogenesis. Side *et al*. found monosomy 7 in two patients who progressed from t-MDS to AML with a positive *NRAS* or *FLT3*-LM mutation status.[62] In a report by Stephenson *et al*., *RAS* mutations occurred in three out of seven patients with RAEB in transformation and monosomy 7.[60] A single case of *NRAS*-positive RAEB with -7 was reported by de Souza *et al*.[61] In contrast to these reports, we found no assocation between *NRAS* mutations or *FLT3*-LM and chromosome 7 abnormalities in MDS in our study. Therefore, a co-operation of -7 with these molecular markers can be discussed in single cases, but general conclusions should not be drawn at this time due to the low number of reported cases.

We found a 5q- syndrome in 12% of cases with *FLT3*-LM but in no case of MDS with NRAS or *MLL*-PTD mutations. This observation corresponds to that of Fidler *et al*. who found no case of *FLT3*-LM, *NRAS*, or *p53* mutations in four patients with the 5q- syndrome and thus suggested that the stability of this MDS syndrome might be a consequence of the absence of other molecular mutations.[63]

In conclusion, the progression from the initial stages of MDS to secondary AML can be accompanied by the acquisition of molecular mutations which are known to play an important role in AML, such as the *FLT3*-LM or *NRAS*-mutations. This allows the interpretation of these mutations as markers of progression in MDS and supports the two-hit theory, according which at least two different types of mutations are needed for the development of AML: the class I mutations (which are frequently represented by mutations of receptor tyrosine kinases) mediate myeloproliferation, while the class II mutations lead to an arrest in differentiation in hematopoiesis.[64-66] It can, therefore, be hypothesized that a single molecular event leads to the early stages of MDS but additional mutations are needed to cause leukemic transformation.

Finally, further evaluation of molecular markers in MDS, especially *FLT3*-LM, *NRAS*, *FLT3*-TKD, *MLL*-PTD, and *KITD816* mutations can be recommended. Such an evaluation should, of course, be completed by analysis of other mutations which are frequent in MDS, such as point mutations in the *AML1/RUNX* gene[67,68] or mutations of *TP53*.[69] These studies may lead to new approaches to the subclassification of MDS and to early detection of progression to AML. Thus, complete understanding of the picture of molecular markers in MDS may also be of therapeutic value.

Aujthors' Contributions

UB: principal investiator. TH, WK, CH: contribution to the design of the study, conducting the work, interpretation of results, and revision of the manuscript. Primary responsibility for the publication, for the tables and figures: UB. Supervision of study: SS.

Conflict of Interest

The authors reported no potential conflicts of interest.

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