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ORIGINAL ARTICLE

Age-dependent frequencies of *NPM1* mutations and *FLT3*-ITD in patients with normal karyotype AML (NK-AML)

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Abstract Prognosis of AML in elderly patients is poor due to adverse patient characteristics and comorbidities. In addition, disease-associated parameters reveal differences between older and younger patients with AML. Survival in

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normal karyotype AML (NK-AML) is influenced by different clinical and molecular markers. The aim of this work was to investigate the frequencies of molecular markers in patients with NK-AML with a focus on *NPM1* mutations and *FLT3*-ITD in different age groups. In the present study, we analyzed the frequencies of mutations of *NPM1* and *FLT3*-ITD in a cohort of 1,321 adult patients and 148 children with AML treated within the AMLCG99, the AML98, and AML04 trials and their distribution in different age groups. Additionally, the frequencies of mutations in *CEBPA* genes, *FLT3*-TKD, and *MLL*-PTD were analyzed in the cohort with NK-AML (n=729). Our data show that the presence of mutations of *NPM1* (from 60% to 40%) and *FLT3*-ITD (from 50% to 20%) significantly decreased with age in adult AML. Consequently, the proportion of *NPM1*−/*FLT3*-ITD− patients increased with age. The decreasing frequency of *NPM1* mutations in elderly patients was paralleled by a reduced complete remission (CR) rate in the elderly of 55% compared to 80% in the younger patients. By contrast, the frequencies of other gene mutations, like *FLT3*-TKD and *MLL*-PTD, and mutations in *CEBPA* were not age-dependent. The decreasing frequency of the favorable *NPM1* mutations with increasing age may partially explain the worse outcome in the elderly patients. Furthermore, the increasing amount of elderly patients without *NPM1* mutations or *FLT3*-ITD suggests that other molecular and clinical risk factors may influence prognosis in this age group.

Keywords Neoplasia · Acute myeloid leukemia · Normal karyotype · *NPM1* · *FLT3-ITD* · Elderly patient

Introduction

Prognosis in elderly patients with AML is poor. Besides adverse patient characteristics, such as age [1] and comorbidities, this is partly due to an age-dependent increase in the number of patients with an unfavorable complex-aberrant karyotype and unbalanced translocations, whereas the incidence of patients with a normal karyotype AML (NK-AML) moderately increases with age. The favorable prognostic groups with the balanced translocations t(8;21)(q22;q22), inv(16)(p13;q22) or t(15;17)(q22;q11-12) remain almost stable in different age groups [2].

In this work, we focused on patients with a normal karyotype. Long-term survival in NK-AML is influenced by different clinical and molecular markers. Whereas the presence of an *NPM1* mutation (*NPM1*+) [3, 4] is associated with a positive prognostic effect on long-term outcome, the presence of an *FLT3*-internal tandem duplication (*FLT3*-ITD+) has a negative impact on survival. Interestingly, a significant interaction between *NPM1* and *FLT3*-ITD has been shown. In adults, the positive prognostic impact on clinical outcome was evident predominantly in patients with NK-AML carrying *NPM1* gene mutations when *FLT3*-ITD was absent. In contrast, the survival in all other groups of combinations between wildtype or mutated *NPM1* and *FLT3*-ITD or wildtype *FLT3* was not different so far. In children with NK-AML, the positive prognostic impact of an *NPM1* mutation on EFS and OS was seen independent of the *FLT3*-ITD mutation status [5, 6]. A clinical parameter with negative impact on all outcome parameters [overall survival (OS), event-free survival (EFS), relapse-free survival (RFS), and the rate of complete remission (CR)] is patient age at diagnosis. Certainly, the worse prognosis in elderly patients is due to adverse patient characteristics and comorbidities. Nevertheless, also disease-associated parameters reveal differences between older and younger patients with AML. Therefore, we investigated the frequencies of molecular markers in patients with NK-AML with a focus on *NPM1* mutations and *FLT3*-ITD in different age groups.

Methods

Patients and samples

Analyses were based on 1,321 patients treated in the AMLCG (German AML Cooperative Group) 1999 trial until January 2006 and 148 children with AML who were treated within different AML trials (AML98, AML04). Patients gave their informed consent prior to the study. The studies have been approved by the ethics committees. Inclusion criteria, treatment protocols, and patient outcome in this study have been published [7, 8].

Molecular analyses

Cytomorphology: Bone marrow cells underwent staining with May–Grünwald–Giemsa, myeloperoxidase, and non-specific esterase [9] and were then classified according to the French–American–British (FAB) criteria [10, 11].

Cytogenetics and fluorescent in situ hybridization analyses: Chromosomal and fluorescent in situ hybridization analyses were performed according to standard protocols [12–16].

Molecular genetics: Mutation analyses of *NPM1*, *FLT3*-ITD, *FLT3*-TKD, and *MLL*-PTD were [12–16] performed according to standard protocols previously described [17]. After screening for the *NPM1* mutation by melting curve analyses, the *NPM1* mutation was confirmed by nucleotide sequencing [3]. *CEBPA* mutational screening was performed using a multiplex PCR-based fragment length

analysis followed by nucleotide sequencing [18]. In the total cohort of 1,469 patients, 1,244 and 1,450 samples were analyzed for *NPM1* mutations and *FLT3*-ITD, respectively. In all 729 NK-AML patients, *FLT3*-ITD and *NPM1* mutation status were known. *FLT3*-TKD, *CEBPA* mutations, and *MLL*-PTD were analyzed in 619, 665, and 673 patient samples, respectively. In children with NK-AML, molecular analyses of *NPM1* and *FLT3*-ITD were performed in all patients, whereas molecular analyses of *CEBPA* mutations and *MLL*-PTD were available for minor proportions of pediatric patients only (≤50%). *FLT3*-TKD were only accessible in three children.

Statistical analyses: Patients were divided into eight age groups [group 1, 0–9 years (n_{all}=64, $n_{NK\text{-}AML}$=16); group 2, 10–19 years (n_{all}=92, $n_{NK\text{-}AML}$=28); group 3, 20–29 years (n_{all}=49, $n_{NK\text{-}AML}$=21); group 4, 30–39 years (n_{all}=105, $n_{NK\text{-}AML}$=56); group 5, 40–49 years (n_{all}=198, $n_{NK\text{-}AML}$=107); group 6, 50–59 years (n_{all}=258, $n_{NK\text{-}AML}$=140), group 7, 60–69 years (n_{all}=449, $n_{NK\text{-}AML}$=238); group 8, ≥70 years (n_{all}=254, $n_{NK\text{-}AML}$=123)]. The frequency and comparison of all molecular markers as well as the four *NPM1* and *FLT3*-ITD combinations were calculated in cross tables (Pearson's chi-square test) in the different age groups. In addition to the frequency of molecular markers, we analyzed the rate of complete remissions in the different age groups. The Mann–Whitney *U* test was applied for the analyses of the differences of median age between *NPM1*+ and *NPM1*− patients.

Results

Patient characteristics

In the entire patient cohort of 1,469 AML patients consisting of 1,321 adults and 148 children of all karyotypes, median age was 59 years (0–85 years) (Supplementary Figure 1). Median age in the children cohort was 10 years (0.06–19 years). Three patients were ≥18 years and treated according to children AML protocols. Median age in the adult cohort was 61 years (10–85 years). Four patients treated according to the adult protocols were younger than 18 years. In adult patients and children, 57.5% and 35.3%, respectively, had AML with a normal karyotype.

Median age in the 729 patients with NK-AML was 59 years (0–85 years), 10 years (0–18 years) in 41 children, and 60 years (17–85 years) in 688 adults. *NPM1* mutations were significantly more frequent in adults (51.0%; 351/688) compared to children (26.8%; 11/41). Distribution of *FLT3*-ITD was 28.3% (195/688) in adults and 41.5% (17/41) in children (p=0.072).

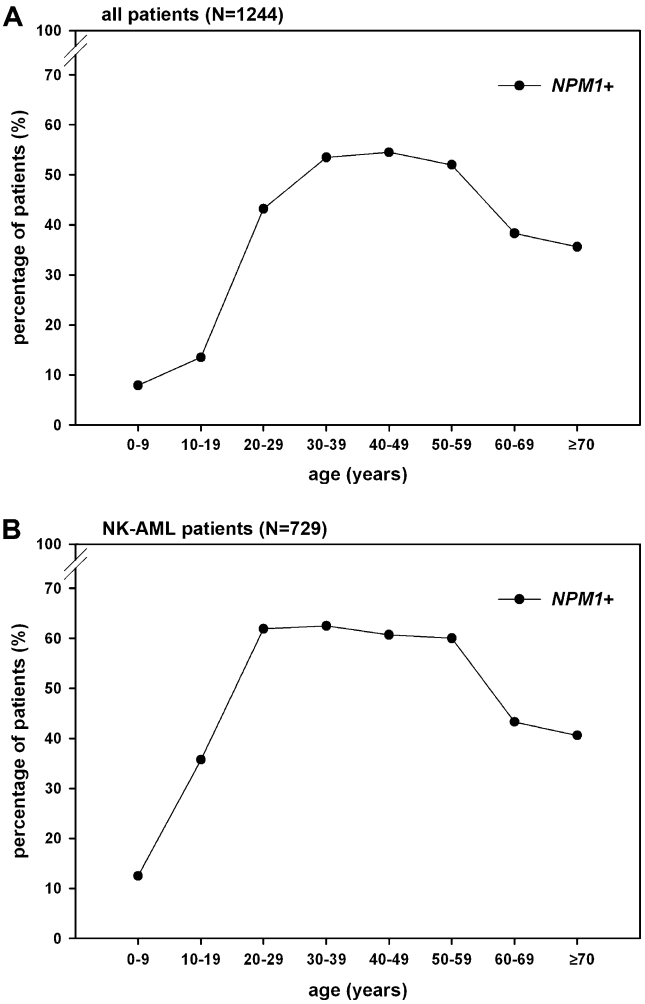

Fig. 1 Frequencies of *NPM1* mutations in different age groups. **A** in patients with different karyotypes (N=1,244); **B** in patients with NK-AML (N=729). **A** Frequency of *NPM1* mutations in 1,244 patients including children AML and complex and NK-AML. Patients were divided into eight age groups: 0–9 years (n=63), 10–19 years (n=89), 20–29 years (n=37), 30–39 years (n=86), 40–49 years (n=167), 50–59 years (n=223), 60–69 years (n=363), ≥70 years (n=216). Frequencies of *NPM1* mutations were calculated in cross tables (Pearson chi square): 7.9% (0–9 years), 13.5% (10–19 years), 43.2% (20–29 years), 53.3% (30–39 years), 54.5% (40–49 years), 52.0% (50–59 years), 38.3% (60–69 years), and 35.6% (≥70 years). **B** Frequencies of *NPM1* mutations in 729 patients with NK-AML. Patients were divided into eight age groups: 0–9 years (n=16), 10–19 years (n=28), 20–29 years (n=21), 30–39 years (n=56), 40–49 years (n=107), 50–59 years (n=140), 60–69 years (n=238), ≥70 years (n=123). Frequencies of *NPM1* mutations were calculated in cross tables (Pearson chi square). *NPM1*+ Presence of an *NPM1* mutation. The analyses were performed in cross tables (Pearson chi square).

Frequency of *NPM1* mutations and *FLT3*-ITD in 1,469 patients with all karyotypes

In 1,469 patients, the *NPM1* and *FLT3* mutation statuses were available in 1,244 and 1,450 cases.

NPM1 mutations occurred in 40.4% (502/1,244), predominantly in adults (44.3% (486/1,096) adult cohort vs. 10.8% (16/148) in children; $p<0.001$).

In the eight age groups, we found the lowest frequencies of mutated *NPM1* in children between 0 and 9 years (7.9%, 5/63) and 10 and 19 years (13.5%, 23/89). Between 30–59 years, more than 50% (53.1%, 253/476) of patients carried an *NPM1* mutation. Interestingly, the frequency of an *NPM1* mutation decreased to 37.3% (216/579) in patients ≥60 years (60–69 years, 38.3%, 139/363; ≥70 years, 35.6%, 77/216; Fig. 1a).

The overall frequency of an *FLT3*-ITD was 24.3% (353/1,450), detected in 24.9% (327/1,314) of adult patients and 19.1% (26/136) of children. The lowest frequency of an *FLT3*-ITD (8.3%, 5/60) was found in 60 children between 0 and 9 years, whereas 84 patients between 10 and 19 years showed a frequency of an *FLT3*-ITD of 27.4% (23/84). The rates of *FLT3*-ITD positivity in patients <20 years (19.3%; 28/145) and ≥60 years (20.7%; 145/701) were comparably low in contrast to patients between 20 and 59 years in which an *FLT3*-ITD could be detected in up to 35% (20–29 years, 34.7%, 17/49; 30–39 years, 34.6%, 36/104; 40–49 years, 29.2%, 57/195; 50–59 years, 27.2%, 70/257; Fig. 2a).

Frequency of *NPM1* mutations and *FLT3*-ITD in 729 patients with NK-AML

In 729 patients with available mutation statuses of both *NPM1* and *FLT3*-ITD, we found a significant decrease in the frequency of mutations in *NPM1* and *FLT3*-ITD with higher age.

The lowest frequency of mutated *NPM1* was seen in children between 0 and 9 years (12.5%) and between 10 and 19 years (35.7%). In the adult cohort, the frequency of mutated *NPM1* was relatively constant in the patient cohorts up to 60 years of age but decreased abruptly from 60.0% in patients aged between 50 and 59 years to 42.4% (153/361) in patients ≥60 years. (Fig. 1b, Table 1). The median age of the *NPM1*+ versus the *NPM1*− patients was significantly different in the Mann–Whitney *U* test [56 years (5–85 years) versus 62 years (0–83 years); $p=0.004$].

Similarly to *NPM1*, an *FLT3*-ITD was a rare event in children between (0 and 9 years). The frequency of an *FLT3*-ITD peaked in children between 10 and 19 years and decreased continuously with increasing age from 57.1% in patients between 10 and 19 years of age to 19.5% in elderly patients ≥70 years (<0.001). The median age in *FLT3*-ITD positive patients was significantly lower compared to patients with wildtype *FLT3* (55 vs. 61 years; $p<0.001$). The frequency of an *MLL*-PTD showed a trend to an increase with age, whereas the frequency of the other molecular markers *FLT3*-TKD and mutated *CEBPA* in the different age groups did not differ significantly (Fig. 2b, Table 1).

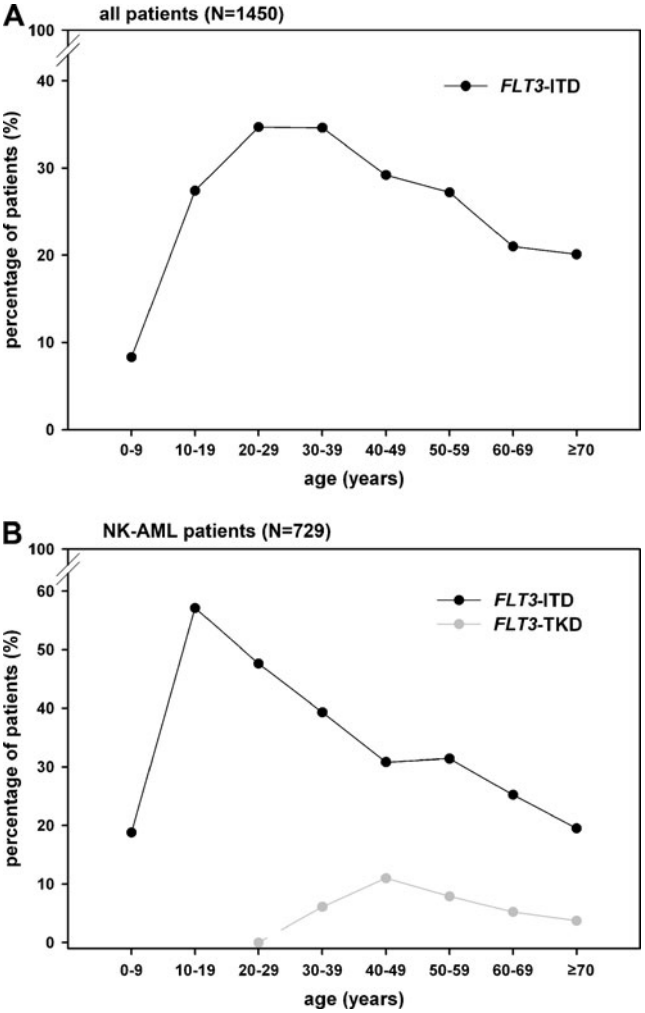

Fig. 2 Frequencies of *FLT3* mutations in different age groups. **A** in patients with different karyotpyes (N=1,450); **B** in patients with NK-AML (N=729). **A** Frequency of *FLT3*-ITD in 1,450 patients including children AML and complex and NK-AML. Patients were divided into eight age groups: 0–9 years (n=60), 10–19 years (n=84), 20–29 years (n=49), 30–39 years (n=104), 40–49 years (n=195), 50–59 years (n=257), 60–69 years (n=447), ≥70 years (n=254). Frequencies of *FLT*-ITD were calculated in cross tables (Pearson chi square): 8.3% (0–9 years), 27.4% (10–19 years), 34.7% (20–29 years), 34.6% (30–39 years), 29.2% (40–49 years), 27.2% (50–59 years), 21.0% (60–69 years), and 20.1% (≥70 years). **B** Frequencies of *FLT3*-ITD and *FLT3*-TKD in 729 patients with NK-AML. Patients were divided into eight age groups: 0–9 years (n=16), 10–19 years (n=28), 20–29 years (n=21), 30–39 years (n=56), 40–49 years (n=107), 50–59 years (n=140), 60–69 years (n=238), ≥70 years (n=123). Frequencies of *FLT3*-ITD and *FLT3*-TKD were calculated in cross tables (Pearson chi square). *FLT3*-TKD was not available for pediatric patients. *FLT3*-ITD+ Presence of an *FLT3*-ITD (ITD internal tandem duplication) mutation. *FLT3*-TKD+ Presence of an *FLT3*-TKD (TKD tyrosine kinase domain) mutation. The analyses were performed in cross tables (Pearson chi square).

Distribution of the *NPM1*/*FLT3*-ITD genotypes in different age decades in 729 patients with NK-AML

An association between *NPM1* mutations and *FLT3*-ITD has previously been described by different authors [5, 19, 20].

Table 1 Frequencies of gene mutations and CR rate in different age groups

	NPM1+ (*N*=729)	*FLT3*-ITD+ (*N*=729)	*MLL*-PTD+ *N*=673)	*FLT3*-TKD+ (*N*=619)	*CEBPA*+ (*N*=665)	CR rate (*N*=729)
0–9 years (*n*=16)						
Number/total number	2/16	3/16	1/10	NA	1/10	13/16
Percent	12.5	18.8	10.0		10.0	81.2
10–19 years (*n*=28)						
Number/total number	10/28	16/28	0/13	NA	2/12	24/28
Percent	35.7	57.1	0.0		16.7	85.7
20–29 years (*n*=21)						
Number/total number	13/21	10/21	1/20	0/20	4/19	17/21
Percent	61.9	47.6	5.0	0.0	21.0	81.0
30–39 years (*n*=56)						
Number/total number	35/56	22/56	1/55	3/49	7/52	42/56
Percent	62.5	39.3	1.8	6.1	13.5	75.0
40–49 years (*n*=107)						
Number/total number	65/107	33/107	6/101	11/100	10/98	84/107
Percent	60.7	30.8	5.9	11.0	10.2	78.5
50–59 years (*n*=140)						
Number/total number	84/140	44/140	5/136	10/127	7/135	88/140
Percent	60.0	31.4	3.7	7.9	5.2	62.9
60–69 years (*n*=238)						
Number/total number	103/238	60/238	21/224	11/211	24/224	152/238
Percent	43.3	25.2	9.4	5.2	10.7	63.9
≥70 years (*n*=123)						
Number/total number	50/123	24/123	15/114	4/109	10/115	68/123
Percent	40.6	19.5	13.2	3.7	8.7	55.3
p value	<0.001	0.001	0.059	0.275	0.350	0.001

Frequency of mutations of *NPM1*, *MLL*, *CEBPA*, and *FLT3* and CR rate in 729 patients with NK-AML. The presence of an *NPM1* mutation and an *FLT3*-ITD significantly decreased with age, whereas the presence of an *MLL*-PTD showed a trend to increase with age in adult patients. In adult patients, the frequencies of *NPM1* mutations and CR rate decreased in a parallel manner with increasing age. All frequencies were calculated in cross tables (Pearson chi-square). The analyses were performed in cross tables (Pearson chi-square).

n number of patients in each age group, *number* number of patients carrying the mutation, *total number* total number of patients in the age group with available mutation status, *CR* complete remission, *NA* not available (the frequency of an *FLT3*-TKD was analyzed only in a minority of patients between 0 and 19 years and is therefore not illustrated).

Patients carrying an *NPM1* mutation without an *FLT3*-ITD achieved an improved long-term outcome in comparison to the non-*NPM1*+/*FLT3*-ITD− cohort. We investigated the distribution of the different *NPM1*/*FLT3*-ITD combinations in different age groups. The majority of children between 0 and 9 years (68.8%) lacked mutations of *NPM1* and/or *FLT3*-ITD. In contrast, only one third of children between 10 and 19 years lacked both mutation markers, one third showed an isolated *FLT3*-ITD and one third displayed the *NPM1*+/*FLT3*-ITD+ genotype. In adults, we found a significant relative increase of *NPM1*−/*FLT3*-ITD− patients from 23.8% in the youngest adult patient cohort (*n*=20–29 years) to 50.4% in patients aged 70–85 years ($p<0.001$). In analogy to the abrupt decrease of the frequency of an *NPM1* mutation in patients older than 60 years, we found a strong increase in the *NPM1*−/*FLT3*-ITD− genotype at the age of 60 years from 31.4% in patients aged 50–59 years to 49.6% in patients aged 60–69 years. With the exception of children, the percentage of patients with a *NPM1*+/*FLT3*-ITD+ genotype decreased continuously with advancing age ($p=0.042$). In the eight age groups (0–9, 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, and ≥70 years), the proportion of the favorable prognostic group of *NPM1*+/*FLT3*-ITD− patients was lowest in children with AML, then showed an increase up to 49 years and a decrease afterwards ($p=0.007$). Nevertheless, comparing the presence of the favorable prognostic genotype *NPM1*+/*FLT3*-ITD− only in adult patients (six age groups: 20–29, 30–39, 40–49, 50–59, 60–69, and ≥70 years), the difference did not reach a statistical significance (chi-square test) (Table 2, Fig. 3).

Table 2 Frequencies of the four *NPM1*/*FLT3*-ITD subgroups in different age groups

	NPM1−/*FLT3*-ITD− (*N*=297)	*NPM1*+/*FLT3*-ITD− (*N*=215)	*NPM1*−/*FLT3*-ITD+ (*N*=70)	*NPM1*+/*FLT3*-ITD+ (*N*=147)
0–9 years (*n*=16)				
Number/total number	11/16	2/16	3/16	0/16
Percent	68.8	12.5	18.8	0.0
10–19 years (*n*=28)				
Number/total number	10/28	2/28	8/28	8/28
Percent	35.7	7.1	28.6	28.6
20–29 years (*n*=21)				
Number/total number	5/21	6/21	3/21	7/21
Percent	23.8	28.6	14.3	33.3
30–39 years (*n*=56)				
Number/total number	15/56	19/56	6/56	16/56
Percent	26.8	33.9	10.7	28.6
40–49 years (*n*=107)				
Number/total number	32/107	42/107	10/107	23/107
Percent	29.9	39.3	9.3	21.5
50–59 years (*n*=140)				
Number/total number	44/140	50/140	12/140	34/140
Percent	31.4	35.7	8.6	24.3
60–69 years (*n*=238)				
Number/total number	118/238	59/238	17/238	44/238
Percent	49.6	24.8	7.1	18.5
≥70 years (*n*=123)				
Number/total number	62/123	35/123	11/123	15/123
Percent	50.4	28.5	8.9	12.2
*P*1	<0.001	0.007	0.028	0.018
*P*2	<0.001	0.080	0.861	0.042

Distribution of the four *NPM1*−/*FLT3*-ITD subgroups in different age groups in 729 patients with NK-AML. In adults, the amount of patients without mutations of *NPM1* and *FLT3* significantly increased with age, whereas the frequency of double-mutated patients significantly decreased with age. The positive prognostic *NPM1*+/*FLT3*-ITD− group was most common in patients between 30 and 59 years. All frequencies were calculated in cross tables (Pearson chi square). The analyses were performed in Cross tables (Pearson chi-square).

*P*1 *p*-values when comparing all eight age subgroups, *P*2 *p*-values when comparing only patients six age groups from 20–29 to ≥70 years excluding children from the analyses.

The decreased frequency of an *NPM1* mutation in older patients is associated with a reduced CR rate

An important parameter to measure early treatment response of AML is the achievement of a complete remission (CR). In the total cohort of 729 patients, 488 (66.9%) achieved a CR. In younger patients, CR rate was significantly higher than in elderly patients (Pearson chi-square, $p=0.001$). CR rates differed from more than 80% in young patients to about 55% in the elderly (0–9 years, 81.2%; 10–19 years, 85.7%; 20–29 years, 81.0%; 30–39 years, 75.0%; 40–49 years, 78.5%; 50–59 years, 62.9%; 60–69 years, 63.9%; 70–85 years, 55.3%). In 41 children <18 years, the frequency of an *NPM1* mutation was significantly lower (24.4%, 10/41) compared to 688 adults (51.2%, 352/688; $p=0.003$).

In contrast to children, in the adult cohorts, the frequencies of mutated *NPM1* and CR rate diminished in an almost parallel manner with increasing age (Table 1).

Interestingly, patients carrying an *NPM1* mutation did not have significant different CR rates in different age groups ($p=0.131$). In contrast, elderly patients with *NPM1* wildtype achieved significantly lower CR rates ($p=0.004$) (data not shown).

Discussion

The aim of this study was to explore the relation between age and the distribution of mutations of several molecular markers in NK-AML. Our data show in a large cohort of 729 patients with NK-AML that the presence of mutations

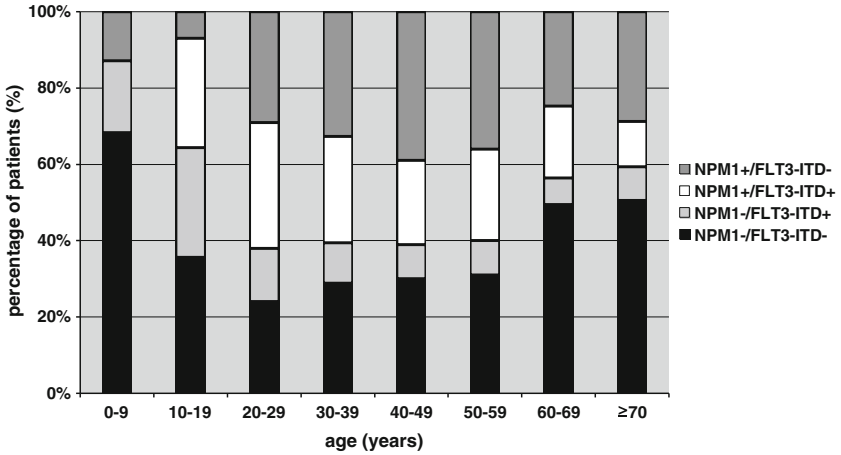

Fig. 3 Distribution of the four *NPM1*/*FLT3*-ITD subgroups in different age groups in 729 patients with NK-AML. Patients were divided into eight age groups: 0–9 years (n=16), 10–19 years (n=28), 20–29 years (n=21), 30–39 years (n=56), 40–49 years (n=107), 50–59 years (n=140), 60–69 years (n=238), ≥70 years (n=123). Frequencies of the *NPM1*/*FLT3*-ITD subgroups were calculated in cross tables (Pearson chi square). *NPM1*+ Presence of an *NPM1* mutation. *FLT3*-ITD+ Presence of an *FLT3*-ITD (ITD internal tandem duplication).

in *NPM1* and *FLT3*-ITD in adult AML significantly decreased with age, whereas the frequency of other gene mutations, like *FLT3*-TKD, *MLL*-PTD, and mutated *CEBPA* were not age-dependent. This is the largest study of frequencies of *NPM1* and *FLT3*-ITD in NK-AML. These data are in a relative contradiction to previous published reports that showed that an *NPM1* mutation was more common in elderly patients [3, 20–22]. Nevertheless, all of these analyses included patients with different karyotypes, the majority of which were not NK-AML. Thus, these data cannot be fully compared to our data restricted to NK-AML unless they are reduced to the actual numbers of patients with NK-AML, which were lower (between 67 and 230 cases) compared to our large study. Including patients regardless of karyotype in our analysis (N=1469), the lowest frequencies of mutated *NPM1* were seen in patients <35 years (22.3%; 50/224). These results are in accordance with Verhaak et al. [20].

The decrease in *FLT3*-ITD positivity with older age is in accordance with analyses from Schnittger et al. performed in 1,003 patients, 428 of which had NK-AML [23]. Other analyses that did not reveal any age-dependency of *FLT3*-ITD were performed in cohorts including other cytogenetic groups, smaller patient numbers of NK-AML, or restriction to patients up to 60 years of age [24–26]. In our cohort, more than one third of patients in the elderly cohort (>70 years) lacked a defined molecular marker compared to one fifth of younger patients (≤40 years) (data not shown). Thus, the pathophysiological role of mutated *NPM1* and *FLT3*-ITD in the development of NK-AML in elderly patients is less evident, and other mutations, differences in gene expression patterns, or changes in epigenetic alterations might be relevant for prognosis and have to be further studied. Newly discovered mutations associated with cytogenetically normal AML include the following genes: *TET2*, *IDH1/2*, *DNMT3A*, and *RUNX1*. Interestingly, median age of patients carrying these mentioned mutations (*TET2*, *IDH1/2*, *DNMT3A*, and *RUNX1*) has been shown to be higher compared to wildtype. A negative prognostic effect of *IDH1/2* mutations (relapse free survival) and *TET2* mutations (overall survival) on survival has been demonstrated only in patients with the favorable *NPM1*+/*FLT3*-ITD− genotype, but not in patients with wildtype *NPM1*/wildtype *FLT3* [27, 28]. *DNMT3A* mutations have been described recently and shown to be associated with a negative effect on overall survival in patients with NK-AML. The effect of *DNMT3A* mutations in different *NPM1/FLT3* subgroups still warrants further investigation [29]. Interestingly, *RUNX1* mutations have been found to display a negative prognostic effect on overall survival and event-free survival in the intermediate cytogenetic risk group without molecular mutations [30, 31]. Thus, the higher occurrence of *RUNX1* mutations in elderly patients with NK-AML who have a lower frequency of *NPM1* and *FLT3*-ITD might in part explain the dismal prognosis in this patient cohort.

In our results, an *FLT3*-TKD mutation occured with equal frequencies in all adult age groups, whereas the frequency of an *FLT3*-ITD showed a strong dependence on age. Additionally, in the literature, an *FLT3*-TKD occurs in all genetic AML subgroups. An overrepresentation of *FLT3*-TKD has been shown for AML with normal karyotype or complex aberrant karyotype. *FLT3*-TKD is significantly associated with an *NPM1* mutation, whereas an *FLT3*-TKD hardly occurs together with an *FLT3*-ITD. A positive prognostic impact on EFS has been described for patients with an *FLT3*-TKD in the combination with the

NPM1 mutation [32]. In our cohort, the frequency of an *NPM1*+/*FLT3*-TKD+ genotype in adult patients ≤60 years was 6.4% (19/298), whereas only 3.1% (10/320) of patients ≥60 years showed this genotype (p=0.056). The effect of a *NPM1*+/*FLT3*-TKD+ genotype is probably similar in all age groups, but the lower frequency of this prognostic favorable subgroup may explain at least in part the worse EFS in the elderly subgroup.

Furthermore, we could show that the decreased frequency of an *NPM1* mutation with increasing age was associated with an almost parallel decrease of achievement of a CR in adult NK-AML patients. The achievement of a CR is associated with a longer overall survival [33]. Thus, we postulate that the inferior prognosis in elderly patients is partly due to a lower frequency of an *NPM1* mutation resulting in a reduced CR rate. The independence of the prognostic impact of *NPM1* mutations and age on overall survival has been proven in a multivariate cox regression model (data not shown).

In earlier investigations, we and others have shown that the presence of an *NPM1* mutation is significantly associated with the achievement of a complete remission as a marker of early response to therapy [34]. We found a higher rate of adequate blast cell reduction in the bone marrow 1 week after the end of the first induction cycle (day 16 blast cell clearance) in *NPM1*-mutation-positive AML compared to AML with wildtype *NPM1* [34], thus postulating a higher in vivo chemosensitivity of *NPM1*-mutated blasts. As the presence of an *NPM1* mutation and CR rate decrease equally with higher age, we propose that the reduced frequency of the *NPM1* mutation leads to a lower CR rate and consequently contributes to a dismal prognosis in elderly patients.

The analysis of the four *NPM1*/*FLT3*-ITD subgroups confirmed the results of the single markers. In adult AML, we found a significant higher number of *NPM1*−/*FLT3*-ITD− patients with older age, whereas the double positive fraction significantly decreased. The *NPM1*+/*FLT3*-ITD− patients showed an increase to up to almost 40% in patients between 40 and 49 years and a decrease in older patients. Comparing adult patients ≤40 years to patients >70 years, there was no statistical difference in the occurrence of the different *NPM1*/*FLT3*-ITD subgroups (data not shown). Thus, the simplest explanation for the inferior survival of the older patient—which would have been the decrease of the favorable prognostic *NPM1*+/*FLT3*-ITD− group—cannot be substantiated by our results. This is clinically relevant since the *NPM1*+/*FLT3*-ITD− genotype has been shown to be associated with a better prognosis in the elderly cohort [1].

Büchner et al. [1] investigated the frequency of *NPM1* mutations and *FLT3*-ITD in a larger patient cohort of de novo AML patients from the AMLCG 92 and 99 trials including all cytogenetic risk groups. In contrast to their analyses, our analyses of *NPM1*/*FLT3* subgroups focused on patients with NK-AML allowing inclusion of secondary AML and therapy-related AML. Furthermore, our adult patients were treated only within the AMLCG 99 trial, except for three patients treated within children AML protocols.

In accordance with the observation of Büchner et al., we found a higher proportion of older patients lacking mutations of *NPM1* and *FLT3*-ITD compared to the younger patient cohort (49.9% versus 29.6% in our analysis; 40.2% versus 26.3% in the Büchner publication).

Whereas Büchner et al. found an almost equal frequency of the favorable *NPM1*+/*FLT3*-ITD− genotype in patients <60 years compared to ≥60 years (36.5% versus 33.2%), in our cohort of NK-AML, the frequency of the favorable *NPM1*+/*FLT3*-ITD− genotype was significantly lower in adult patients <60 years compared to those ≥60 years (35.0% versus 26.0%).

The distribution of the *NPM1*/*FLT3*-ITD subgroups seemed to be comparable in patients <20 years compared to ≥70 years: The majority of children lacked mutations of *NPM1* and *FLT3*, and the frequency of the favorable prognostic *NPM1*+/*FLT3*-ITD− group in children was even lower compared to the elderly patients (9% versus 29%). The fact, that the highest CR rates were achieved by children and the lowest CR rates were achieved by elderly patients suggests that children and adult NK-AML might have to be considered as different entities with different disease biologies.

In conclusion, the decreasing frequency of the *NPM1* mutation in elderly patients with NK-AML contributes to the lower CR rate and might explain part of the drug resistance to chemotherapeutic agents. These observations shed light on the disease biology in older patients with AML and might in part explain the dismal prognosis.

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