## Molecular subtypes of *NPM1* mutations have different clinical profiles, specific patterns of accompanying molecular mutations and varying outcomes in intermediate risk acute myeloid leukemia

*NPM1* mutations are important markers for acute myeloid leukemia (AML) and are already included in the World Health Organization classification of 2008 as indicating a provisional entity of AML.[1] In addition, it is accepted that *NPM1* mutations are prognostically favorable in the absence of *FLT3*-ITD mutations.[2-4] Falini and colleagues showed that there are different types of *NPM1* mutations.[5] The "type A" mutation is characterized by insertion of the four nucleotides thymine, cytosine, thymine and guanine and results in a lengthening of the protein. In addition, other mutations were detected showing diverse insertions that are all located at the terminal end of exon 12 and were named alphabetically in the order of detection. Although the length of insertion of base pairs is the same in most mutations, the resulting sequence of the amino acids differs. For example, in type A the insertion results in the amino acid leucine, whereas in type B it results in methionine. Some research groups found that patients with these types had different outcomes, whereas other groups did not.[6,7] Little is known about accompanying mutations besides *FLT3*-ITD, *FLT3*-TKD and the absence of *MLL*-PTD or double-mutated *CEBPA* mutations.[2,3,8] Only *FLT3*-ITD seems to influence prognosis negatively.[2,3,8] Whether the different types of *NPM1* mutations, and therefore the different sequences of amino acids, are associated with different cytogenetics, concomitant molecular markers, biological or prognostic profiles remains unclear and is the subject of this study.

This study included 660 *NPM1*-mutated newly diagnosed adults with AML with an intermediate-risk karyotype who were investigated for mutations in *CEBPA, CBL, DNMT3A*, *FLT3*-ITD, *FLT3*-TKD, *IDH1*R132, *IDH2*R140, *IDH2*R172, *MLL*-PTD, and *WT1*. Detailed information on the study criteria and patients, cytomorphology, cytogenetics, molecular genetics, and statistics are provided in the *Online Supplementary Material and Methods*.

Regarding the different *NPM1* mutation types, type A, which results in an insertion of TCTG (thymine, cytosine, thymine, guanine) between nucleotides 860 and 863 (c.863_864insTCTG), was found most frequently, being present in 458 (69%) of the cases. Type B (c.863_864insCATG) was found in 72 (11%) cases and type D (c.863_864insCCTG) in 51 (8%). All other types were found in less than 20 cases each. Details on types A,

**Table 1.** Clinical, cytomorphological, cytogenetic, and molecular characteristics of the total cohort, type A, type B, and type D patients.

| | Total cohort (n=660) | | Type A (n=458) | | Type B (n=72) | | Type D (n=51) | | Remaining (n=79) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| **Clinical parameters** | | | | | | | | | | |
| Male | 309 | 47 | 214 | 47 | 42 | 58 | 23 | 45 | 30 | 38 |
| Female | 351 | 53 | 244 | 53 | 30 | 42 | 28 | 55 | 49 | 62 |
| Age (median; mean; range) | 63; 61; 18-88 | | 64; 61; 21-88 | | 60; 59; 29-87 | | 67; 64; 22-85 | | 63;60; 18-85 | |
| WBC x10⁹/L (median; mean; range) | 40.0; 65.9; 0.4-600.0 | | 45.0; 64.0; 0.7-370.0 | | 29.6; 60.8; 0.9-600.0 | | 39.0; 48.3; 1.0-231.5 | | 24.0; 45.1; 0.4-258.0 | |
| Hb g/dL (median; mean; range) | 9.4; 9,5; 2.8-16.0 | | 9.4; 9.5; 2.8-14.8 | | 9.0; 9.2; 5.8-14.6 | | 9.5; 9.4; 5.9-12.7 | | 9.6; 9.7; 5.2-16.0 | |
| PLT x10⁹/L (median; mean; range) | 60; 86; 5-637 | | 61; 86; 9-637 | | 53; 90; 12-410 | | 73; 90; 13-400 | | 55; 81; 5-334 | |
| % BM blasts (median; mean; range) | 71; 77; 20-99 | | 78; 71; 20-99 | | 72; 67; 20-99 | | 79; 72; 23-98 | | 73; 71; 20-99 | |
| **FAB classification** | | | | | | | | | | |
| M0 | 6 | 1 | 3 | 1 | 1 | 1 | 0 | 0 | 2 | 3 |
| M1 | 239 | 36 | 166 | 36 | 24 | 33 | 21 | 41 | 28 | 35 |
| M2 | 166 | 25 | 107 | 23 | 26 | 36 | 14 | 28 | 19 | 24 |
| M4 | 197 | 30 | 148 | 32 | 16 | 22 | 15 | 29 | 18 | 23 |
| M5 | 40 | 6 | 26 | 6 | 4 | 6 | 0 | 0 | 10 | 13 |
| M6 | 11 | 2 | 7 | 2 | 1 | 1 | 1 | 2 | 2 | 3 |
| M7 | 1 | 0.2 | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cytogenetics | | | | | | | | | | |
| Normal karyotype | 570 | 86 | 400 | 87 | 61 | 85 | 46 | 90 | 63 | 80 |
| Aberrant karyotype | 90 | 14 | 58 | 13 | 11 | 15 | 5 | 10 | 16 | 20 |
| Molecular mutations | | | | | | | | | | |
| *CBL*mut | 5 | 1 | 2 | 0.4 | 2 | 3 | 0 | 0 | 1 | 1 |
| *CEBPA*mut | 40 | 6 | 29 | 6 | 4 | 7 | 4 | 8 | 2 | 3 |
| *CEBPA* double-mutated | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *DNMT3A*mut | 337 | 55 | 271 | 59 | 22 | 34 | 19 | 37 | 26 | 33 |
| *FLT3*-ITD | 270 | 41 | 191 | 42 | 33 | 46 | 17 | 33 | 29 | 37 |
| *FLT3*-TKD | 82 | 12 | 54 | 12 | 8 | 11 | 11 | 22 | 9 | 11 |
| *IDH1*R132mut | 88 | 13 | 70 | 15 | 2 | 3 | 6 | 12 | 10 | 13 |
| *IDH2*R140mut | 97 | 15 | 68 | 15 | 7 | 10 | 13 | 26 | 9 | 11 |
| *IDH2*R172mut | 1 | 0.2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| *MLL*-PTD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *WT1*mut | 38 | 6 | 16 | 4 | 12 | 17 | 4 | 8 | 6 | 8 |

*WBC: white blood cells: Hb: hemoglobin; PLT: platelets; BM: bone marrow; FAB: French-American-British.*

B, and D, accounting for 581 (88%) cases, as well as on the remaining 38 types, present in 79 (12%) cases, are available in *Online Supplementary Table S1*. For the analyses, types A, B and D were considered individually; however, all non-type A patients were also considered as "rare types" and all non-type A, B, and D patients were additionally considered as "remaining types".

Concerning biological parameters, patients with the different *NPM1* types showed no differences in age, except for type D cases being older than type B cases (64 *versus* 59 years; *P*=0.036) and a trend to type D cases being older than cases with "remaining types" (64 *versus* 61 years; *P*=0.077). More details on differences between the subtypes with respect to clinical parameters as well as cytomorphological and cytogenetic aspects are given in Table 1 and the *Online Supplementary Results*.

With respect to the accompanying molecular markers, all genes analyzed were found to be mutated within the cohort with the only exception of *MLL*-PTD. *CEBPA* was never found to be double mutated. *DNMT3A* was the most frequently mutated gene (n=337; 55%), followed by *FLT3*-ITD (n=270; 41%) and *IDH2*R140 (n=105; 18%) (Table 1). Comparing type A cases to "rare types" we found both *DNMT3A* and *IDH1*R132 were more frequently mutated in the former (59% *versus* 33%, *P*=0.001; and 15% *versus* 9%, *P*=0.026, respectively). In contrast, *WT1* was less frequently mutated (4% *versus* 11%; *P*=0.001). The same results were found comparing type A *versus* type B cases (*DNMT3A*mut 59% *versus* 31%; *P*<0.001; *IDH1*R132mut: 15% *versus* 3%; *P*=0.002; *WT1*mut: 4% *versus* 17%; *P*<0.001). *DNMT3A* mutations were also more frequent in type A than type D (59% *versus* 37%; *P*=0.004). Comparing type B cases with "remaining types" we found *IDH1*R132 less often in the former (3% *versus* 12%; *P*=0.022) and comparing type D with "remaining types" we found only that *IDH2R*140 mutations were more often in the former than within the "remaining types" (26% *versus* 11%; *P*=0.019). Furthermore, *IDH2*R140 mutations were more frequent in type D than in type B cases (26% *versus* 10%; *P*=0.026). Figure 1 depicts the relationships between the subtypes and markers. Interestingly, in 96 (15%) patients no mutation in addition to that in *NPM1* was detected. No additional mutation was found in 11% of type A patients, 22% of type B cases, 14% of type D and 29% of "remaining types" (type A *versus* "remaining types" *P*<0.001).

With regards to prognostic impact, a sub-cohort of 562 patients were analyzed: these patients had been treated according to one of the German standard protocols (all containing anthracyclines as well as cytarabine and/or high-dose cytarabine) with a curative intent.[10] Type A patients (n=410), as compared to "rare type" patients (n=152), showed a trend towards inferior overall survival (OS) (median 44 *versus* 63 months; *P*=0.052), significantly worse event-free survival (EFS) (14 *versus* 19 months; *P*=0.048), and overall survival censoring the patients on the day of allogeneic stem cell transplantation ($OS^{TXcens}$) (45 *versus* 63 months; *P*=0.044; Figure 2A-C; *Online Supplementary Table S2*). Furthermore, we compared type A (n=410) *versus* type B (n=57) and *versus* type D (n=38). As numbers of patients are limited, some effects might be obscured within these cohorts. Interestingly, type D patients showed a strong trend to better OS than type A patients (median not reached *versus* 44.0 months; *P*=0.051) and a trend to better OS compared to type B patients (37.9 months; *P*=0.090). However, this effect was not evident for EFS and only to a much smaller extent for $OS^{TXcens}$ (*Online Supplementary Figure S1A-C; Online Supplementary Table S2*). In addition, we evaluated whether the accompanying molecular markers result in differences in outcome. As expected, we found that patients with *FLT3*-ITD had a worse prognosis than those without *FLT3*-ITD in the total cohort (OS and $OS^{TXcens}$: *P*<0.001; EFS *P*=0.001). Comparing the effect of *FLT3*-ITD within patients with the different *NPM1* types, we found a significantly worse prognosis only within type A patients (OS *P*=0.001; EFS and $OS^{TXcens}$: *P*<0.001), but not within type B or D patients, a result which might be related to small numbers (*Online Supplementary Figure S1A-C; Online Supplementary Table S2*). The same effects were seen when the cohort was restricted to those with a normal karyotype or younger than 60 years of age (*data not shown*). Similarly, *DNMT3A* mutations conferred a worse effect on prognosis within the total cohort (OS and $OS^{TXcens}$: *P*=0.001; EFS *P*<0.001). However, in addition to conferring a worse outcome to type A patients (OS: *P*=0.006; EFS: *P*<0.001; $OS^{TXcens}$: *P*=0.045), *DNMT3A* mutations also worsened the prognosis within type D patients (OS: *P*=0.016; EFS: *P*=0.012; $OS^{TXcens}$: *P*<0.001). Again, type B patients seemed unaffected by the additional molecular mutations, although this effect might also be due to small numbers (*Online Supplementary Figure 3A-C; Online Supplementary Table S2*). The same effect was found when restricting the patients to those with a normal karyotype or younger patients (*data not shown*). No differences were seen for the other molecular markers. Performing univariate Cox regression analysis, we

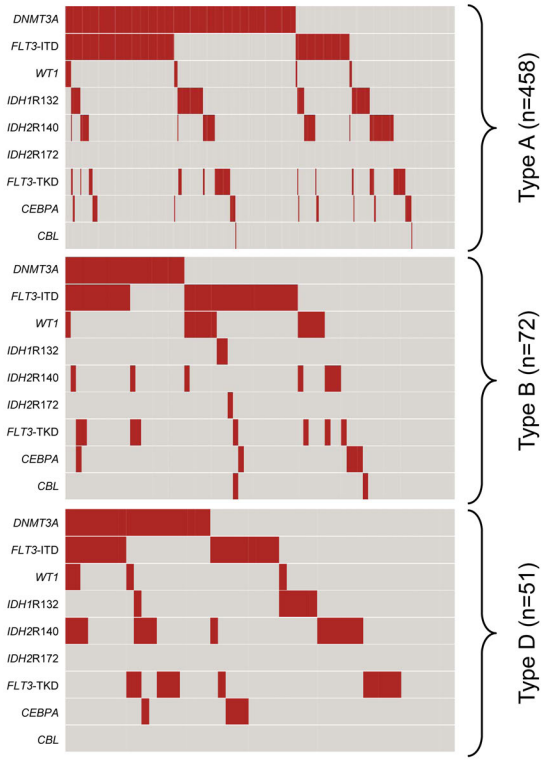


**Figure 1. Mutation pattern of 581 *NPM1* mutated patients with types A, B, and D.** Relationships of patients with *NPM1* mutation types A, B, and D are indicated within the columns (type A n=458; type B n=72, type D n=51) whereas the accompanying molecular markers are delineated within the lines. Red represents patients with mutations whereas patients with no mutation in the respective gene are represented in gray.

found that age, white blood cell count, *NPM1* type, *FLT3*-ITD and *DNMT3A* were prognostic factors for OS and $OS^{TXcens}$ and the same factors with the addition of *IDH2*R140 were prognostic factors for EFS. Importantly, multivariate Cox regression analysis revealed that age, white blood cell count, *FLT3*-ITD and *DNMT3A* were independently associated with OS and $OS^{TXcens}$. Again, in addition to the above-mentioned parameters, *IDH2*R140 was independently associated with EFS. (*Online Supplementary Table S3*).

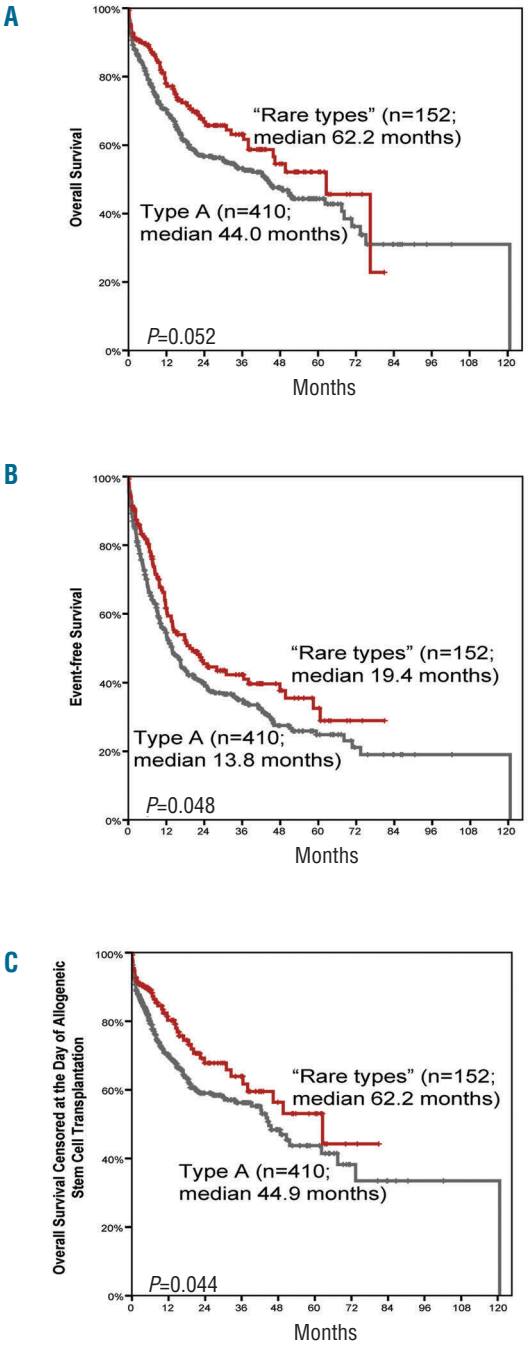


Figure 2. Kaplan Meier analyses of (A) overall survival, (B) event-free survival, and (C) overall survival censored at the day of allogeneic stem cell transplantation comparing type A patients (n=410) with "rare types" comprising all non-type A patients (n=152).

In this study we evaluated whether different types of *NPM1* mutations show different patterns regarding clinical, additional cytogenetic and molecular genetic parameters and prognosis.

Only two studies have so far investigated the impact of *NPM1* types on survival. Koh *et al.* presented data on 18 *FLT3*-ITD-negative patients, describing a tendency for type A patients to have a favorable outcome, whereas Pastore *et al.*[7] were not able to show a prognostic impact of *NPM1* types in 349 patients with normal karyotypes, even though they included cases with *FLT3*-ITD in their analysis. In our study we evaluated not only *FLT3*-ITD but also nine other molecular markers and found a poor prognostic impact of both *FLT3*-ITD and *DNMT3A* mutations in the total cohort of *NPM1*-mutated patients. Separating the cohort according to subtype of *NPM1* mutation, differences for the above mentioned markers were seen, as *FLT3*-ITD showed an adverse effect in type A patients only, and *DNMT3A* mutations in types A and D. The prognosis of patients with type B seemed unaffected by these mutations. As frequencies of the mutations differ within the various *NPM1* mutation types, we hypothesize that differences in outcome could be explained by accompanying molecular markers or by the limited numbers of patients.

In conclusion, we found that among patients with cytogenetically determined intermediate-risk AML, different subtypes of *NPM1* mutation were associated with different profiles with respect to clinical parameters as well as accompanying molecular markers. Furthermore, different outcomes were seen in subgroups of these patients. We therefore recommend analyzing *NPM1*-mutated patients not only for *FLT3*-ITD, which is already recommended, but also for *DNMT3A* mutations as these mutations worsen the outcome of patients with type A and type D *NPM1* mutations.

Further studies need to be conducted to evaluate these relationships in even larger cohorts.

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