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LETTER TO THE EDITOR



# Detection of prognostically relevant mutations and translocations in myeloid sarcoma by next generation sequencing

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## Introduction

Myeloid sarcoma (MS) is a subgroup of acute myeloid leukemia (AML) where myeloid blasts form a tumoral mass in extramedullary tissues [1]. MS may occur at any point during the disease course and almost every site of the body can be affected. Although MS is often diagnosed as an isolated event without concomitant bone marrow involvement, virtually all of these patients will develop overt hematologic disease if left untreated [2,3], which further highlights the systemic nature of this disease. Beside isolated MS, extramedullary manifestations might also occur simultaneously with leukemic bone marrow (BM) infiltration. The incidence rate of this situation in newly diagnosed AML patients is estimated to be around 9%, however, some authors report incidence rates of up to 20–40% [2,4]. MS is usually treated with standard AML induction regimens, even if it occurs as isolated event. However, little is known about optimal consolidation strategies after achievement of complete remission [5,6]. This is particularly true for cases with isolated MS, which is due to the fact that material from MS biopsies is often sparse and usually Formalin-Fixed-Paraffin-Embedded (FFPE), thereby often precluding comprehensive AML risk stratification. In this study analyzing 18 cases of MS, we developed a next-generation sequencing (NGS) based approach, which enables the detection of both mutations and translocations with prognostic relevance from FFPE tissues of MS. Additionally, we analyzed the effects of this MS tissue based NGS profiling on AML risk stratification in cases where MS coincides with systemic AML and where cytogenetic/molecular analyses have already been performed from leukemic BM.

All patient specimens of MS and corresponding BM biopsies were collected at the Division of Hematology, Medical University of Graz (MUG), between June 2003 and December 2016 and stored as FFPE samples. Patient characteristics are presented in Table 1. Briefly, MS coincided with systemic AML in 11 patients, whereas isolated MS was present in seven cases. The study was approved by the MUG-ethical committee (vote number 24-036 ex 11/12) and performed in accordance with the Declaration of Helsinki.

In a first step, we focused on the detection of translocations with relevance for AML risk stratification [5]. This is of relevance, as complete cytogenetic analysis from MS specimens is usually not possible, which is due to the frequent unavailability of fresh material. Additionally, although fluorescence in situ hybridization (FISH) was successfully applied to MS specimens previously [7], this approach is limited by the fact that the amount of MS material is often sparse and that every abnormality tested requires at least one FFPE-section slide. Significant progress in this area came from Mirza et al., who recently analyzed six cases of MS by chromosomal microarray analysis (CMA) [8]. Without the need to target their analyses to selected abnormalities, they successfully detected unbalanced chromosomal aberrations and complex karyotypes. However, a limitation of this approach was the fact that prognostically relevant balanced chromosomal rearrangements could not be detected. To circumvent this limitation, we now performed NGS-based translocation analysis using only 100 ng of RNA extracted from FFPE-MS specimens discovering CBFB-MYH11, DEK/CAN-NUP214, DEK-NUP214, MLL-MLLT3, PML-RARA, RBM15-MKL1, RPN1-MECOM, and RUNX1-RUNX1T1 (for details see also the Supplementary information). By studying 18 MS specimens, we were able to unambiguously detect the presence of CBFB-MYH11 in three of them (Figure 1). In two of these cases, MS presented as an isolated event without any evidence of systemic disease. While the follow up of these patients was too short to allow any conclusions about a prognostic relevance of this lesion in MS, previous reports suggested a potential correlation of

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Supplemental data for this article can be accessed here.

**Table 1.** Clinical characteristics of 18 patients with MS.

| | |
|---|---|
| Age at diagnosis (years) | 54 (21–77) |
| AML subtype | |
| *De novo*, *n* = | 10/18 (55%) |
| Secondary, *n* = | 5/18 (28%) |
| Therapy-related, *n* = | 3/18 (17%) |
| Sex | |
| Male, *n* = | 13/18 (72%) |
| Female, *n* = | 5/18 (28%) |
| WBC at diagnosis ($10^9$/L) | 9.49 (2.77–64.98) |
| LDH (U/L) | 276 (119–3023) |
| MS onset | |
| Isolated, *n* = | 7/18 (39%) |
| Concomitant with systemic AML, *n* = | 11/18 (61%) |
| MS sites | |
| Lymphatic system, *n* = | 7/18 (38%) |
| Cutaneous, *n* = | 3/18 (16%) |
| Gastrointestinal tract, *n* = | 1/18 (6%) |
| Bone, *n* = | 2/18 (11%) |
| Soft tissue, *n* = | 2/18 (11%) |
| Salivary glands, *n* = | 1/18 (6%) |
| Liver, *n* = | 1/18 (6%) |
| Spleen, *n* = | 1/18 (6%) |
| Therapy | |
| High-Dose incl. HSCT, *n* = | 9/18 (50%) |
| High-Dose, *n* = | 4/18 (22%) |
| Low-Dose, *n* = | 4/18 (22%) |
| BSC, *n* = | 1/18 (6%) |

WBC: white blood cell count; LDH: lactate dehydrogenase; HSCT: hematopoietic stem cell transplantation; BSC: best supportive care.

CBFB-MYH11 with a favorable clinical course in isolated MS [9]. It will, however, be necessary to corroborate these data in larger, preferably prospective clinical cohorts. In the third case, MS coincided with systemic disease. Importantly, CBFB-MYH11 could also be confirmed in leukemic BM of this patient by conventional karyotyping and FISH, which further supports the validity of our technique. Another advantage of this technique is that all translocations can be tested in one experimental reaction from the same batch of RNA, which makes it more time and cost effective as FISH and which makes it feasible to increase the number of aberrations analyzed without increasing the amount of MS-specimen needed. One might hypothesize, that a potential combination with CMA might enable clinicians to get a complete chromosomal picture of MS in the future, even if the limitations in available material are taken into account. By this means, it will also be possible to further corroborate a potential relevance of classical favorable cytogenetic markers in AML for risk stratification in isolated MS, as their analysis in larger, preferably prospective clinical cohorts will be enabled.

In a next step, we focused on the mutational landscape and therefore subjected all FFPE-MS samples to NGS analysis covering a set of 39 genes recurrently mutated in myeloid neoplasias as detailed in the Supplementary information and reported previously [10]. Briefly, we analyzed genes involved in the following categories: (a) RAS pathway (NRAS, KRAS, PTPN11, CBL, NF1, and BRAF); (b) activated signaling (FLT3, CSF3R, JAK2, MPL, KIT, SFRP1, and ETNK1); (c) DNA methylation (DNMT3A, TET2, IDH1, and IDH2); (d) Cohesins (STAG2); (e) splicing (SRSF2, DDX41, SF3B1, SF3B2, ZRSR2, and U2AF1); (f) transcription factors (WT1, GATA2, RUNX1, CEBPA, ETV6, PHF6, BCOR, and STAT3); (g) chromatin modification (ASXL1, EZH2); and (h) others (TP53, NPM1, SETBP1, CALR, and SRP72). Additionally, we analyzed for FLT3 internal tandem duplication (FLT3-ITD) including its allelic ratio using PCR fragment length analysis as previously described [5,11]. As recommended in a recent update of the European LeukemiaNet guidelines of AML [5], only high ratios (>0.5) were considered positive. In accordance with two previous studies, we thereby show that NGS from FFPE-MS tissues is feasible [12,13]; however, as our study exceeded previous analyses in genes and cases analyzed, we were furthermore able to extend the insight into the spectrum of mutations occurring in this AML subform (Figure 1). In more detail, our mutational profiling revealed a median of two mutations per sample (range 1–5) with NPM1, NRAS, and DNMT3A being affected most frequently. Mutations in NPM1 were present in five out of 18 (28%) cases, which further confirms their frequent occurrence in extramedullary AML reported previously [2,12,14]. This is also in agreement with previous *in vitro* data, where ectopic overexpression of mutated NPM1 increased the adhesive, migratory and invasive potential of AML cells [14]. Mechanistically, these effects were mediated by activation of the RAS-MAPK/ERK pathway and consecutive upregulation of matrix metalloproteases. This essential role of RAS signaling in MS development is also in line with the high rate of NRAS mutations observed within our study, which was 28% (five of 18 cases affected) as well. Importantly, in case mutations that directly affect the RAS pathway in general were considered, this rate was even higher with ten out of 18 patients (56%) being affected. Finally, also mutations in DNMT3A occurred with a high frequency (5/18, 28%). Although this observation is novel, a link between mutated DNMT3A and extramedullary manifestation of AML has been shown previously [15]. Xu et al. used *in vitro* and *in vivo* approaches to study the role of mutated DNMT3A in the development of MS. They could further show that this was mediated via upregulation of TWIST1, a critical inducer of epithelial-mesenchymal transition. Sequencing of larger MS cohorts will be needed to further validate these findings and to clearly establish a genomic landscape of MS.

In a final approach, we focused on the eleven patients with simultaneous manifestation of systemic AML and MS, respectively, and aimed to delineate whether molecular profiling of MS specimens might affect the BM-derived risk stratification. Interestingly, the molecular makeup of leukemic AML-BM did not differ from simultaneously obtained MS specimens in these analyses, suggesting that the risk profile obtained from leukemic BM might be sufficient and not altered by additional analysis of MS specimens. This is in line with a recent article by Ganzel

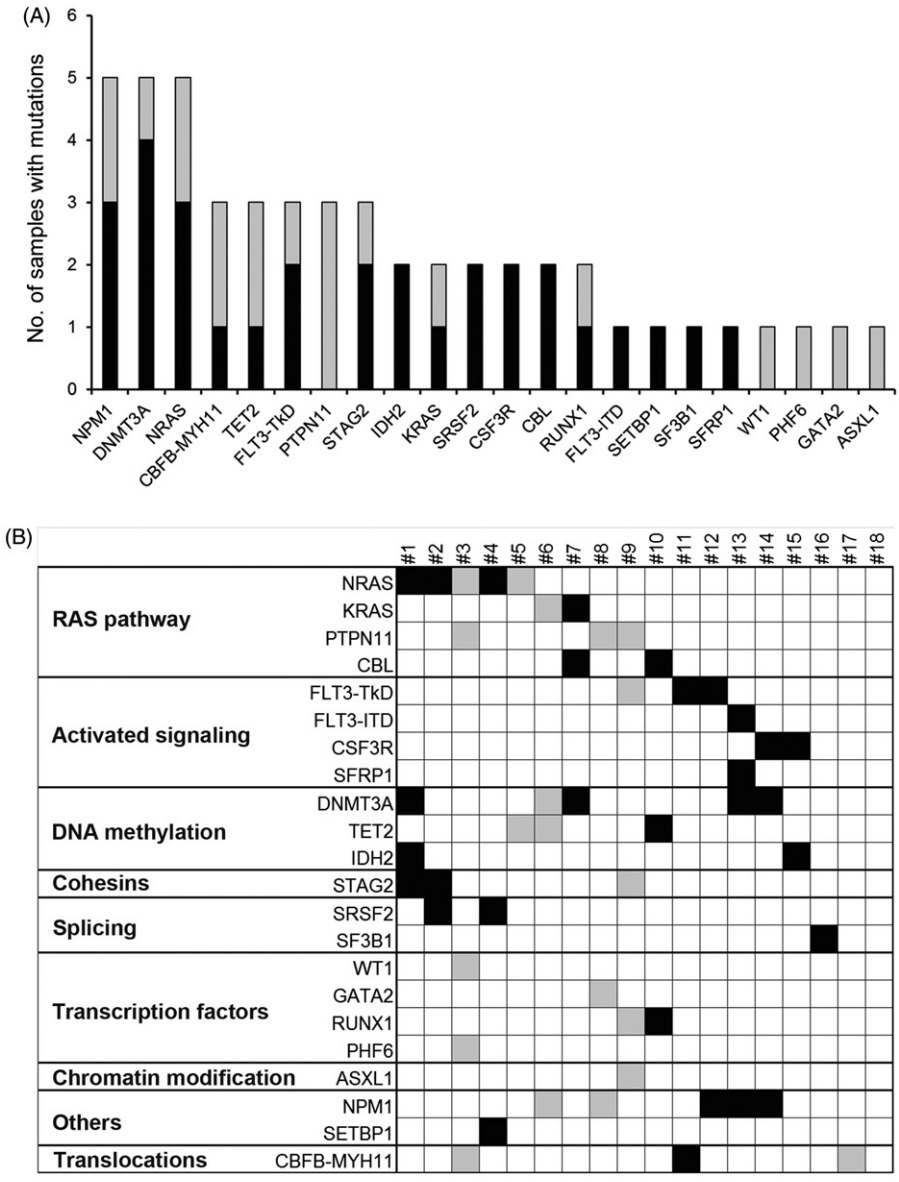


**Figure 1.** Risk stratification in MS. (A) Frequency of mutations/translocations in 39 genes with recurrent mutations in myeloid neoplasias. Eighteen patients with MS have been analyzed, comprising seven cases of isolated MS (gray) and 11 cases of MS simultaneously arising with AML-BM infiltration (black). (B) Heatmap showing the distribution of mutations/translocations in these patients. Again, aberrations in cases with isolated MS are depicted in gray, whereas aberrations in cases with MS simultaneously arising with AML-BM infiltration are displayed in black.

et al., who failed to observe a prognostic effect of extramedullary manifestation(s) in AML patients treated with standard chemotherapy, thereby concluding that additional biopsy of suspected MS sites might be dispensable in case AML was already diagnosed via the BM [4]. However, these data are in contrast to a previous study by Pastoret et al., who analyzed five paired leukemic BM/MS samples and detected a geographic clonal heterogeneity at the stage of relapse in one case, with a TP53 mutation detectable in MS specimens only [13]. Analysis of larger cohorts specifically addressing this issue will therefore be needed to clarify this issue.

In conclusion, we demonstrate that comprehensive AML risk stratification is possible in FFPE specimens of MS, even if only limited material is available. We therefore suggest that it should ideally be performed in every patient with isolated MS to help clinicians in selecting optimal therapeutic postremission strategies. Analysis of larger cohorts will be needed to unambiguously clarify whether additional risk stratification is necessary in MS biopsies of cases, where MS coincides with systemic AML and where risk stratification is already available from leukemic BM.

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