LETTER TO THE EDITOR

Molecular analysis of RAS-RAF tyrosine-kinase signaling pathway alterations in patients with plasma cell myeloma

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In patients with plasma cell myeloma (PCM), interphase fluorescence in situ hybridization (FISH) detects prognostically relevant genetic alterations, for example, the prognostically adverse t(4;14)(p16q32) or 17p deletions. Furthermore, mutations of the RAS proto-oncogene were suggested to be associated to myeloma pathogenesis. The RAS pathway inhibitor lonafarnib combined with the proteasome inhibitor bortezomib demonstrated synergistic cell death in human myeloma cells in association with downregulation of P-AKT in an in vitro setting. By whole-genome and whole-exome sequencing, Chapman et al. found frequent involvement of genes associated to the nuclear factor-kappaB pathway. Rare PCM cases were positive for BRAF mutations, which had previously been detected in solid tumors (for example, melanoma) and hematological neoplasms, for example, hairy cell leukemia.

Studies combining FISH data and mutation analyses in PCM are rare. We performed amplicon deep-sequencing mutation analyses in 79 patients with PCM or plasma cell leukemia investigating different members of the RAS-RAF signaling pathway, that is, NRAS, Kras, Hras, Braf, Flt3 and, in addition, Tp53. This was combined with FISH and array-based profiling of DNA copy number alterations. There were 29 females and 50 males (median age, 70.8 years; 33.4–85.6 years) at first diagnosis of PCM (n = 73)/plasma cell leukemia (n = 6). Bone marrow samples were sent to the MLL Munich Leukemia Laboratory from December 2006 to November 2011. All patients gave their written informed consent. The study was approved by the Internal Review Board of the MLL and performed in accordance with the Helsinki Declaration.

We performed magnetic-activated cell sorting (MACS) of the CD138-positive plasma cells from bone marrow (RoboSep, STEMCELL Technologies SARL, Grenoble, France). The majority (72/79 patients) were investigated by FISH after MACS including del(13)(q14) (D13S25), del(17)(p13) (TP53), +3 (D3Z1), +9 (D9Z1), +11 (D11Z1), and +15 (D15Z4), t(4;14)(p16q32)/IGH-FGFR3, t(14;16)(q32;q23)/IGH-MAF, and t(11;14)(q13;q32)/IGH-CCND1 (Abbott, Wiesbaden, Germany/MetaSystems, Altlussheim, Germany). Seventeen cases were investigated by array-CGH (4 × 180 K microarrays; Agilent Technologies, Santa Clara, CA, USA). All 79 patients were analyzed for NRAS, KRAS, HRAS, BRAF, FLT3 and TP53 mutations by a deep-sequencing assay (Roche 454, Branford, CT, USA) in combination with the 48.48 Access Array Technology (Fluidigm, South San Francisco, CA, USA).

By FISH, 13q14 deletion was most frequently observed in 48/72 (66.7%) of cases. IGH rearrangements were detected in 37/67 (55.2%); t(4;14); n = 8, t(11;14); n = 17, t(14;16); n = 4, other IGH rearrangements: n = 8. Trisomy 3 was detected in 25/59 (42.4%), +9 in 30/57 (52.6%), +11 in 25/62 (40.3%) and +15 in 17/32 (53.1%), respectively. Moreover, 9/72 (12.5%) cases had a TP53 deletion, but in 9/41 (22.0%) cases FISH revealed a gain in the TP53 gene or in the 17p region, respectively. At least one aberration was detectable in all 72 cases in which FISH data were available (Table 1).

Array CGH analysis allows performing a genome-wide detection of unbalanced chromosomal gains or losses, whereas FISH allows detecting only a limited pattern of genetic aberrations depending on the selection of probes. On the other hand, as array CGH analysis is not able to detect balanced translocations, reciprocal IGH rearrangements in PCM are only detected by FISH. In our cohort, array CGH analysis revealed aberrant karyotypes in the majority of cases (n = 12/17; 70.6%), including trisomies or partial trisomies of chromosomes 3 (n = 6), 9 (n = 5), 11 (n = 5) and 15 (n = 6), and deletion of chromosome 13 (n = 8). Trisomy 19 was detected in 7/17 cases, and five harbored +7. Three cases showed a dup(19)(p11p13.3). In 3/17 cases, a partial duplication of the long arm of chromosome X, and gains and losses of chromosomes 1, 5 and 16 were detected. Aberrations predominantly involved the whole chromosome instead of small regions.

In 44 patients (44/79; 55.7% of the cohort) at least one mutation was identified by the candidate gene mutation analyses (one mutation: n = 38; two mutations: n = 6, Figure 1). Five cases showed two concomitant mutations: KRAS and TP53 (n = 2), KRAS and NRAS (n = 1), NRAS and TP53 (n = 1), and NRAS and BRAF (n = 1). RAS pathway-activating mutations were frequent (38/79; 48.1%), which is in line with recent studies. KRAS was the most frequently mutated gene with 21/79 (26.6%), followed by NRAS (16/79 patients; 20.3%). We identified three BRAFV600E mutations in our cohort (3/79; 3.8%; Figure 1a). This was similar to Chapman et al. discovering BRAF mutations (K601N and V600E) in 4% of PCM patients by sequencing. Mutations affecting NRAS, KRAS or BRAF were predominantly mutually exclusive (P = 0.032), as only two cases with an overlap (one case with an NRAS and BRAF

Table 1. Frequencies of cytogenetic alterations (FISH) and molecular mutations in 79 patients with plasma cell myeloma

| Genetic alteration | Frequency |
|-------------------|-----------|
| **FISH**          |           |
| IGH rearrangements| 37/67 (55.2%) |
| del(13q14)        | 48/72 (66.7%) |
| -3                | 25/59 (42.4%) |
| +9                | 30/57 (52.6%) |
| +11               | 25/62 (40.3%) |
| +15               | 17/32 (53.1%) |
| TP53 deletion     | 9/72 (12.5%) |
| Gain of TP53      | 9/41 (22.0%) |

| Molecular analysis | Frequency |
|--------------------|-----------|
| NRAS               | 16/79 (20.3%) |
| KRAS               | 21/79 (26.6%) |
| HRAS               | 0/19 (0.0%)  |
| BRAF               | 3/79 (3.8%)  |
| FLT3               | 0/51 (0.0%)  |
| TP53 mutations     | 9/79 (11.4%) |
| One mutation       | 38/79 (48.1%) |
| Two mutations      | 6/79 (7.6%)  |

Abbreviation: FISH, fluorescence in situ hybridization.
Mutation, and one case with NRAS and KRAS) were identified (Figure 1b). No mutations were detected in HRAS (0/19) and FLT3 (0/51), also belonging to the RAS-RAF tyrosine-kinase signaling pathway.

By deep-sequencing, the overall TP53 mutation rate was 11.4% (9/79 patients). In these nine patients, in total 10 TP53 mutations (8 missense mutations and 2 frameshift mutations) were detected (Figure 1a). Eight of these 9 TP53-mutated cases were investigated in parallel by FISH for TP53 deletions: 5/8 cases concomitantly harbored a TP53 deletion, whereas three patients with TP53 mutation showed no TP53 deletion (Figure 1b). Thus, TP53 mutations significantly associated with deletions of the remaining...
TP53 allele (P = 0.001; Figure 1b), as this is known in other malignancies, for example, chronic lymphocytic leukemia. However, comparable to CLL, a subset of patients with TP53 mutation shows no TP53 deletion. Therefore, screening for TP53 mutations may identify additional PCM patients with an adverse prognosis who would not be detected by FISH analysis. No further significant correlation between molecular mutations and cytogenetic data was observed in our patients.

Our cohort included six patients with plasma cell leukemia. Two of these cases demonstrated a TP53 mutation, in one case combined with a KRAS mutation, and one other case showed an isolated KRAS mutation. Taken together, in addition to 13q deletions and IGH rearrangements, we could show that RAS pathway-activating mutations have a major role in patients with PCM with 48% of cases being affected by mutations in NRAS, KRAS or—at low frequency—BRAF (Figure 1b). Coincidence of different RAS pathway-activating mutations is infrequent. TP53 mutations are recurrent in PCM and are associated with deletions of the remaining TP53 allele. Although the pathophysiological background of the respective mutations has to be further investigated, amplicon deep-sequencing assays may identify new prognostic parameters and contribute to more diversified therapeutic concepts, for example, by investigation of compounds targeting the RAS pathway in subsets of myeloma patients.

CONFLICT OF INTEREST
CH, SS, WK and TH declare part ownership of the MLL Munich Leukemia Laboratory GmbH. VG, UB, AK and NN work for the MLL Munich Leukemia Laboratory GmbH. VA declared no conflict of interest.

AUTHOR CONTRIBUTIONS
VG, CH and TH performed the design of the study. VA, AK, SS and VG performed the molecular analyses. CH was responsible for cytogenetic analyses. VG, UB, NN and WK analyzed the data. TH performed the classification of cases by morphology. UB and VG wrote the manuscript draft. All authors contributed to the writing of the manuscript, reviewed and approved the final version.

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