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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)(p16;q32) or 17p deletions. Furthermore, mutations of the *RAS* protooncogene were suggested to be associated to myeloma pathogenesis.[1] 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.[2] By whole-genome and whole-exome sequencing, Chapman *et al.*[3] found frequent involvement of genes associated to the nuclear factor-kappaB pathway. Rare PCM cases were positive for *BRAF* mutations,[3] which had previously been detected in solid tumors (for example, melanoma) and hematological neoplasms, for example, hairy cell leukemia.[4]

Studies combining FISH data and mutation analyses in PCM are rare. We performed amplicon deep-sequencing mutation analyes 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)(p16;q32)/*IGH-FGFR3*, t(14;16)(q32;q23)/*IGH-MAF*, and t(11;14)(q13;q32)/*IGH-CCND1* (Abbott, Wiesbaden, Germany/MetaSystems, Altlussheim, Germany).[5] 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.[3] *KRAS* was the most frequently mutated gene with 21/79 (26.6%), followed by *NRAS* (16/79 patients; 20.3%). We identified three *BRAF*V600E mutations in our cohort (3/79; 3.8%; Figure 1a). This was similar to Chapman *et al.*[3] 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	
RAS pathway-activating mutations	
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.

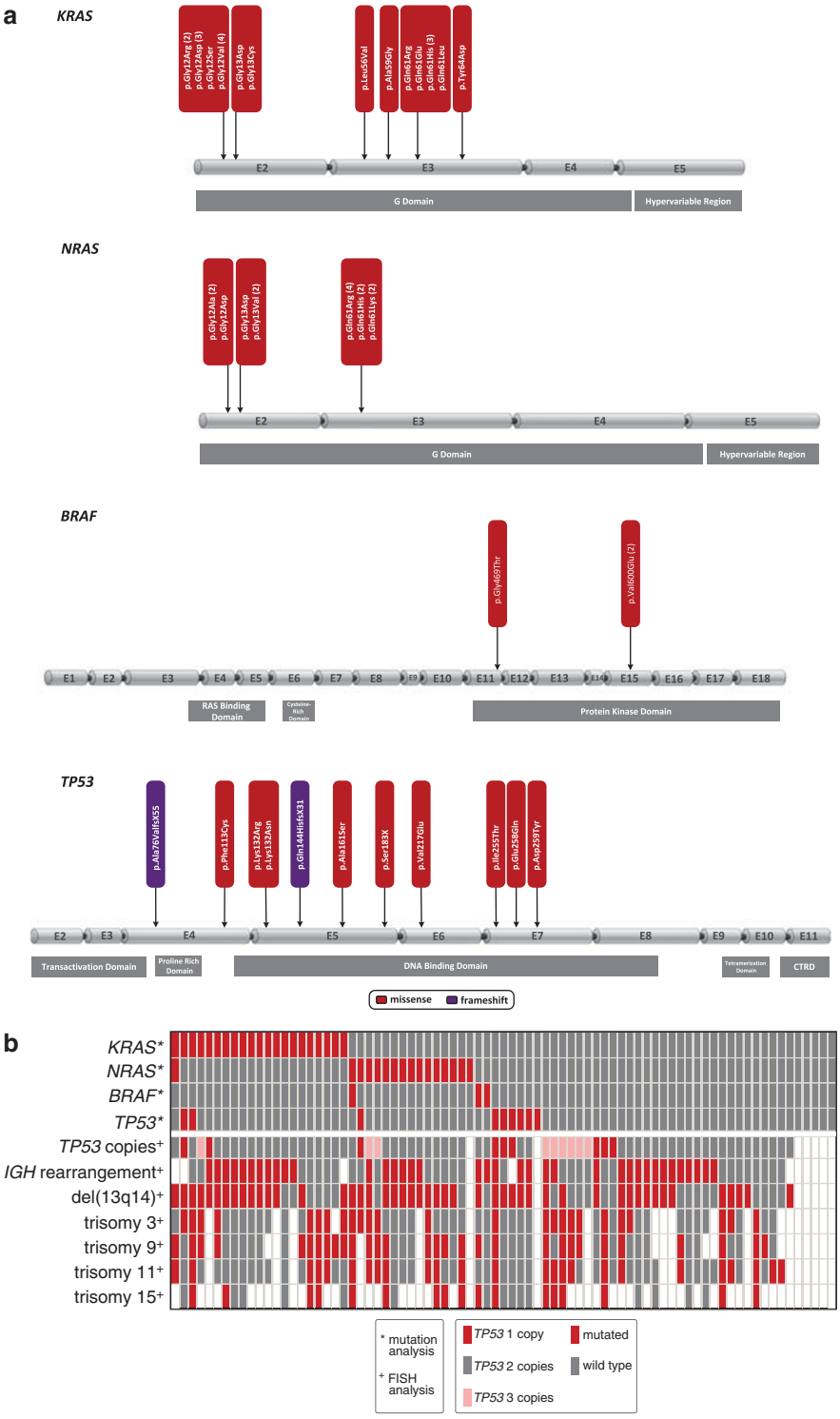

Figure 1. (**a**) Characterization and distribution of the mutations of *TP53*, *NRAS*, *KRAS* and *BRAF* across the functional domains. Missense mutations are shown in red and frameshift mutations in violet. (**b**) Frequencies are given for molecular mutations (*KRAS*, *NRAS*, *BRAF* and *TP53*; upper four lines) and cytogenetic aberrations as detected by FISH (lower seven lines). Red illustrates positivity of the sample for the respective cytogenetic or molecular alteration, gray negativity. White coloring indicates that data are not available. Individual cases are shown in vertical order.

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.[6,7] However, comparable to CLL,[8] 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[2] 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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