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Combining SKY92 gene expression profiling and FISH (according to R2-ISS) defines ultra-high-risk Multiple Myeloma

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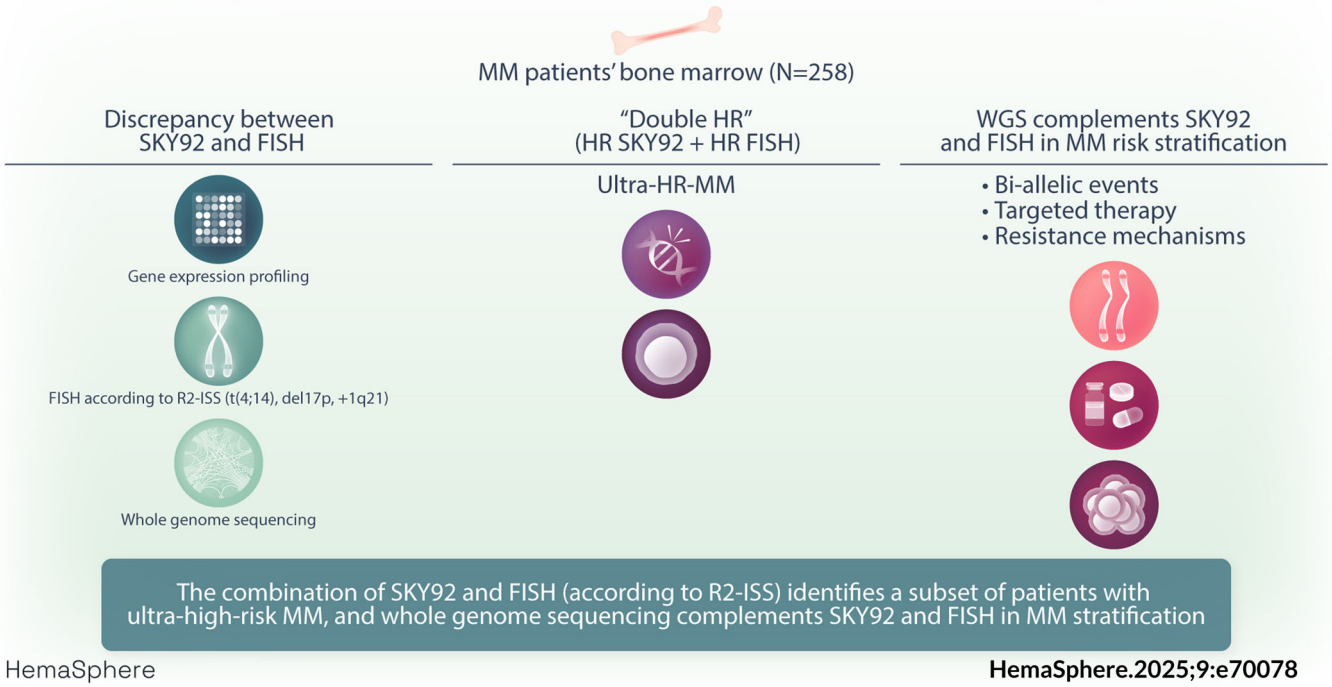
Graphical Abstract



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Abstract

The definition of high-risk (HR) multiple myeloma (MM) is still a matter of debate. We prospectively evaluated the HR detection using FISH in combination with SKY92 gene expression profiling in 258 MM patients (newly diagnosed [ND] MM: $n = 109$; relapsed/refractory [RR] MM: $n = 149$). HR SKY92 was significantly enriched in RRMM (57/121, 47.1%) compared with NDMM (17/95, 17.9%) ($p < 0.0001$). RRMM patients with HR SKY92 showed significantly shorter progression-free survival (PFS) ($p < 0.0001$) and overall survival (OS) ($p < 0.0001$) than SKY92 standard-risk (SR). In NDMM, HR SKY92 also indicated a significantly inferior PFS ($p < 0.0001$) in comparison with SR. We combined SKY92 with FISH (HR: t(4;14), del17p, +1q21 according to R2-ISS) in 181 patients (NDMM: $n = 79$; RRMM: $n = 102$). We found a discrepancy between both risk stratification systems, with only 49 (27.1%) patients being defined as HR by both SKY92 and FISH ("double HR"). In terms of survival outcomes, "double HR" presented a negative prognostic factor for PFS in both NDMM ($p < 0.0001$) and RRMM ($p < 0.0001$). Furthermore, "double-HR" patients showed the worst OS ($p = 0.000\,13$) in RRMM. Additionally, whole genome sequencing (WGS) revealed *CRBN* mutation ($n = 3$) and bi-allelic events (mutation and/or deletion) in *TP53* ($n = 7$) and *TNFRSF17* ($n = 1$). Altogether, we provide the first prospective real-world evidence that the combination SKY92 and FISH (according to R2-ISS) identifies a subset of patients with ultra-HR MM, and WGS complements SKY92 and FISH in MM risk stratification.

INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy in adults, resulting in severe organ damage such as bone lesions, kidney injury, anemia, and hypercalcemia.[1] The prognosis of MM patients has been dramatically improved since the approval of highly potent novel anti-MM drugs; nevertheless, patients with high-risk (HR) disease still face dismal survival.[2–4] Risk-adapted treatment is not yet established in current treatment algorithms. However, clinical studies were able to demonstrate survival improvement by risk-adapted therapy.[5,6] Currently, cytogenetics determined by fluorescence in situ hybridization (FISH) is recommended for risk stratification in MM. In the recently published Second Revised International Staging System (R2-ISS), HR cytogenetics is defined as presence of del17p, t(4;14), and/or +1q21.[7] In addition, gene expression profiling (GEP) has been used to define increased risk in clinical trials such as IFM15, UAMS70/GEP70, UAMS80/GEP80, and SKY92.[8–13] Currently, there are various strategies combining different diagnostic tools to define the ultra-high-risk MM, which may lead to death within 24–36 months of diagnosis and is characterized by co-occurrence of ≥2 HR cytogenetic abnormalities, extramedullary disease (EMD), plasma cell leukemia, and HR GEP.[4,14,15] Here, we provide the first prospective data on combined SKY92 and FISH (according to R2-ISS) risk assessment in the real-world setting,

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suggesting that combined HR profile of FISH and SKY92 defines an ultra-high-risk subset of MM patients.

METHODS

Study population

We prospectively included 258 patients with MM since November 2019, including 109 NDMM and 149 RRMM patients. All patients were treated at the University Hospital of Würzburg, Germany. RRMM was defined as per current IMWG criteria.[16] All procedures were performed in accordance with the Declaration of Helsinki and the national ethical standards. Informed consent was obtained from all patients included in this study. We evaluated the patients' demographics, MM-related data, therapy, and survival outcome. The data cutoff was in December 2023.

Cytogenetics, SKY92, and whole genome sequencing (WGS)

We collected bone marrow (BM) samples from MM patients treated at our institution into ethylenediaminetetraacetic acid-coated tubes, and further analyses were performed on purified CD138-positive cells. Cytogenetics was analyzed by FISH, and HR FISH was defined according to the second revised international staging system (R2-ISS) (del17p, t(4;14), and +1q21)[7] or the revised international staging system (R-ISS) (del17p, t(4;14) and t(14;16)).[17] At the same time point as FISH analysis, SKY92 risk status was determined using MMprofiler gene expression assay,[18] and WGS was performed as previously described.[19] The detailed description of SKY92 and WGS is provided in the Supporting Information Methods.

Statistical analysis

Microsoft Excel 2016 (Microsoft Corp.) was used for descriptive statistics. If not otherwise stated, the data are presented as absolute numbers and percentages or medians and ranges. For comparisons between two different subgroups, we used Mann–Whitney *U* test and Fisher's exact test for continuous variables and percentages, respectively. Survival analysis was performed with the Kaplan–Meier method. Median follow-up was defined as the median time between enrollment and the last follow-up or death of the patients. A $p < 0.05$ was considered to be statistically significant. These analyses were performed with R (version 4.3.2). The data generated in this study are available upon reasonable request from the corresponding author.

RESULTS

Patients' characteristics

In total, we included 258 patients in our study (NDMM: $n = 109$; RRMM: $n = 149$). The majority of the patients were male ($n = 153$, 59.3%). The median age of the patients was 64 years (range 32–86 years), and EMD was found in 27 (10.5%) patients. The median BM infiltration grade was 40% (range 0%–100%). FISH was evaluable in 206 patients (NDMM: $n = 88$; RRMM: $n = 118$). According to the R2-ISS classification (t(4;14), del17p and +1q21), HR FISH was present in 115 (55.8%) patients (t(4;14): $n = 31$; del17p: $n = 38$; +1q21: $n = 88$), with 38 (18.4%) patients exhibiting ≥2 HR FISH markers (Supporting Information S1: Figure S1A). SKY92 status was evaluable in 33 of these 38 patients with ≥2 HR FISH markers, and 10 (30.3%) of them displayed SR in SKY92. The fraction of plasma cells (CD138+) with del17p was higher in RRMM (median: 83%, range 5%–95%) than in NDMM (median: 40%, range 5%–95%) but not statistically significant ($p = 0.17$) (Supporting Information S1: Figure S1B). However, we did not observe a significant prognostic impact of HR FISH as defined by the R2-ISS classification in NDMM patients, and in RRMM, HR FISH showed a trend toward shorter PFS (median: 6.5 vs. 11.6 months, $p = 0.045$) and OS (median: 19.7 months vs. not reached, $p = 0.11$) (Supporting Information S1: Figure S2). The 149 RRMM patients were pretreated with a median of three prior lines of therapy (range 1–14). In RRMM, 147 (98.7%), 138 (92.6%), and 113 (75.8%) patients were exposed to proteasome inhibitors (PI), immunomodulatory drugs (IMiD), and daratumumab, respectively. Among the 109 patients with NDMM, 63 (57.8%) patients were eligible for and treated with high-dose melphalan and autologous SCT, and these patients showed significantly better OS compared to transplant-ineligible patients ($n = 46$, 42.2%) (Supporting Information S1: Figure S3). Moreover, 114 (76.5%) and five (3.4%) patients underwent autologous and allogeneic SCT, respectively. The patients' characteristics and prior treatments in the RRMM subgroup are summarized in Tables 1 and 2.

HR SKY92 status indicates adverse survival outcome in MM

Overall, the SKY92 status was available for 216 (83.7%) patients, and the samples of the remaining 42 (16.3%) patients did not meet the SKY92 quality control criteria (Supporting Information S1: Table S1). The failure rates of SKY92 were similar in NDMM (14/109, 12.8%) and RRMM (28/149, 18.8%) ($p = 0.23$). Noteworthy, the patients with invalid SKY92 results showed significantly lower BM infiltration grade than the remaining patients did (median: 20% vs. 50%, $p = 0.006$) (Figure 1). In the entire group, HR SKY92 status was significantly more common in patients with EMD (12/20, 60.0%) compared to those without EMD (62/196, 31.6%) ($p = 0.01$, Supporting Information S1: Figure S4A). Importantly, HR SKY92 was significantly more frequent in patients with HR FISH (49/99, 49.5%) than with standard-risk (SR) FISH (18/82, 21.9%) as per the R2-ISS classification ($p = 0.0002$, Supporting Information S1: Figure S4B). We then compared the frequency of HR SKY92 in NDMM versus RRMM and found an enrichment of HR SKY92 status in RRMM (57/121, 47.1%) compared with NDMM (17/95, 17.9%) ($p < 0.0001$, Supporting Information S1: Figure S4C). In RRMM patients, HR SKY92 was significantly more frequent in patients with ≥4 prior lines of therapies (32/52, 61.5%) than those with <4 therapy lines (25/69, 36.2%) ($p = 0.009$, Supporting Information S1: Figure S5A). Moreover, in RRMM, regardless of the number of prior therapies, HR SKY92 was enriched in patients who received high-dose melphalan and autologous SCT (48/89, 53.9%) than the remaining patients (9/32, 28.1%) ($p = 0.01$, Supporting Information S1: Figure S5B). These observations suggest that anti-MM therapy, including autologous SCT, may influence the expression levels of various genes and subsequently SKY92 status at relapse, and, on the other hand, this phenomenon may also be a general feature of disease evaluation. We analyzed the survival outcome in patients with HR SKY92 versus SR SKY92 after a median follow-up time of 10.2 months. In RRMM, HR SKY92 indicated a significantly shorter progression-free survival (PFS) (median: 3.7 vs. 15.8 months, $p < 0.0001$) and overall survival (OS) (median: 16.9 months vs. not reached, $p < 0.0001$) than SR (Figure 2C,D). In NDMM, patients with HR SKY92 showed a significantly inferior PFS (medians not reached, $p < 0.0001$) in comparison with SR, while there was still

TABLE 1 Patients' characteristics in the entire group.

Parameter	
Patients, *n*	258
Gender, *n* (%)	
Male	153 (59.3)
Female	105 (40.7)
Age at diagnosis, years, median (range)	60 (30–86)
Age at sampling, years, median (range)	64 (32–86)
Subtype, *n* (%)	
IgG	137 (53.1)
Non-IgG	68 (26.4)
LC	33 (12.8)
Nonsecretory	3 (1.2)
N/A	17 (6.5)
EMD at sampling, *n* (%)	27 (10.5)
Bone marrow infiltration at sampling, %, median (range)	40 (0–100)
Cytogenetics, *n* (%)	
High risk[a]	115 (44.6)
Standard risk	92 (35.6)
N/A	51 (19.8)
High-risk cytogenetic markers, *n* (%)	
t(4;14)	31 (12.0)
t(14;16)	14 (5.4)
del17p	38 (14.7)
+1q21	88 (34.1)
SKY92 status, *n* (%)	
High risk	74 (28.7)
Standard risk	142 (55.0)
N/A	42 (16.3)

Abbreviations: EMD, extramedullary disease; LC, light chain; N/A, not available.
[a]Defined as presence of at least one of the following: t(4;14), del17p, and +1q21.

TABLE 2 Prior treatments in RRMM.

Parameter	
Patients, *n*	149
Prior lines of therapy, median (range)	3 (1–14)
Drug exposure at sampling, *n* (%)	
IMiD, *n* (%)	138 (92.6)
Lenalidomide	129 (86.6)
Pomalidomide	72 (48.3)
Thalidomide	34 (22.8)
PI, *n* (%)	147 (98.7)
Bortezomib	143 (95.9)
Carfilzomib	82 (55.0)
Monoclonal antibody, *n* (%)	28 (18.8)
Elotuzumab	113 (75.8)
Daratumumab	
Prior SCT, *n* (%)	114 (76.5)
Autologous SCT	5 (3.4)
Allogeneic SCT	

Abbreviations: IMiD, immunomodulatory drug; N/A, not available; PI, proteasome inhibitor; RRMM, relapsed/refractory multiple myeloma; SCT, stem cell transplant.

no difference in OS between the subgroups HR versus SR SKY92 (medians not reached, $p = 0.31$) (Figure 2A,B).

The combination of SKY92 and FISH as defined in the R2-ISS classification identifies ultra-HR MM

We combined the SKY92 GEP with traditional FISH as defined in the R2-ISS classification (del17p, t(4;14) and +1q21) in 181 patients with evaluable data (NDMM: $n = 79$; RRMM: $n = 102$). Patients with missing data (either SKY92 or FISH) were omitted for subsequent analyses. In the entire cohort, we noticed a discrepancy between both risk stratification systems, with 67 (37.0%) and 99 (54.7%) patients being classified as HR by SKY92 and FISH, respectively (Figure 3A). Overall, 13 (16.4%) NDMM and 36 (35.3%) RRMM patients were defined as HR in both SKY92 and FISH ("double HR") (Figure 3B,C). The discrepancy between the risk classification systems enabled the combination of SKY92 and FISH to stratify the patients more precisely. Indeed, patients with "double HR" showed the worst PFS in both NDMM ($p < 0.0001$) and RRMM ($p < 0.0001$) patient groups (Figure 4A,C). Moreover, "double HR" also indicated the worst OS in RRMM patients ($p = 0.000\,13$) (Figure 4D). Furthermore, NDMM patients with "double HR" showed only a statistically insignificant trend toward a worse OS compared with all other patients ($p = 0.052$), probably due to the short follow-up period in our study and the improved survival outcome of NDMM patients (Figure 4B). Noteworthy, in both NDMM and RRMM, patients with "single HR" ("only HR SKY92 but SR FISH" or "only HR FISH but SR SKY92") showed similar PFS and OS as those with SR in both SKY92 and FISH (Figure 4D). We then analyzed the survival outcome of patients with "single HR" ("HR SKY92 but SR FISH" vs. "HR FISH but SR SKY92"). Of note, RRMM patients who exhibited only HR SKY92 but SR FISH showed significantly shorter PFS (medians: 8.8 vs. 15.8 months, $p = 0.015$) and OS (medians not reached, $p = 0.029$) compared to those with only HR FISH but SR SKY92, suggesting that SKY92 could better identify the patients with unfavorable prognosis than FISH (Figure 5A,B). We did not perform this comparison in NDMM due to the lack of survival events in this subgroup.

Since +1q21 is a new HR genetic marker added to the R2-ISS compared with R-ISS, we defined HR FISH using R-ISS (del17p, t(4;14) and t(14;16)) instead of R2-ISS and repeated the above-mentioned survival analysis to investigate if the differences between R2-ISS and R-ISS might impact the risk stratification when combined with SKY92 ($n = 181$; NDMM: $n = 79$, RRMM: $n = 102$). HR cytogenetics according to R-ISS was present in 27 (34.2%) and 49 (48.0%) patients with NDMM and RRMM, respectively, indicating a discrepancy between R-ISS and R2-ISS (Supporting Information S1: Figure S6). As expected, RRMM patients with "double HR" displayed the worst PFS ($p < 0.0001$) and OS ($p < 0.0001$) among all patients (Supporting Information S1: Figure S7C,D). Among the NDMM patients, "double HR" was associated with the worst PFS ($p = 0.0096$) but had no impact on OS ($p = 0.53$) (Supporting Information S1: Figure S7A,B). In addition, RRMM patients with "only HR SKY92" showed significantly shorter PFS (8.8 vs. 15.8 months, $p = 0.015$) compared to those with "only HR FISH" (Supporting Information S1: Figure S8A,B). In NDMM, due to the lack of survival events, we did

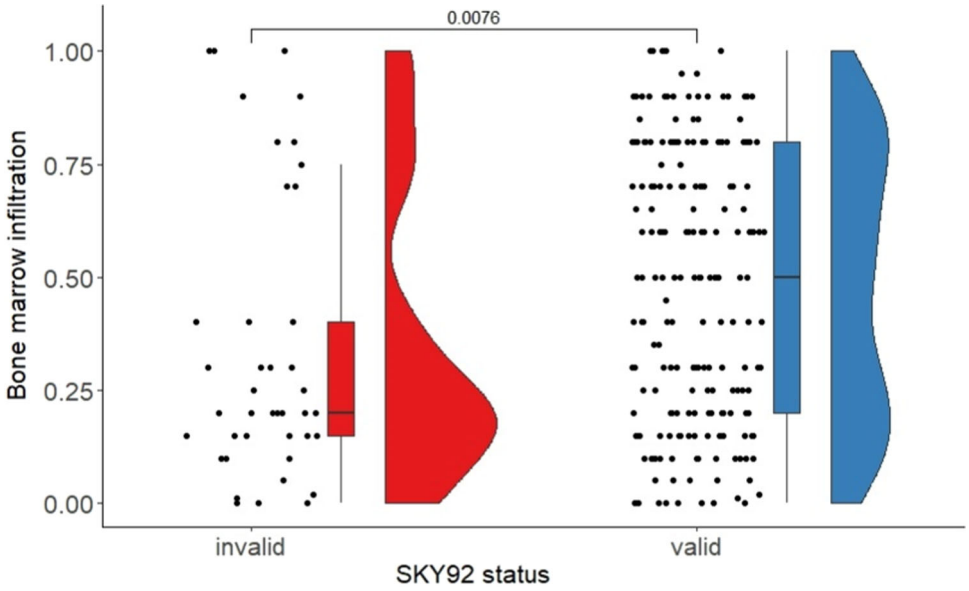

FIGURE 1 **Bone marrow (BM) infiltration grade in patient with valid versus invalid SKY92 results**. BM infiltration grade was significantly lower in patients with invalid SKY92 results than the remaining patients (median: 20% vs. 50%, $p = 0.0076$).

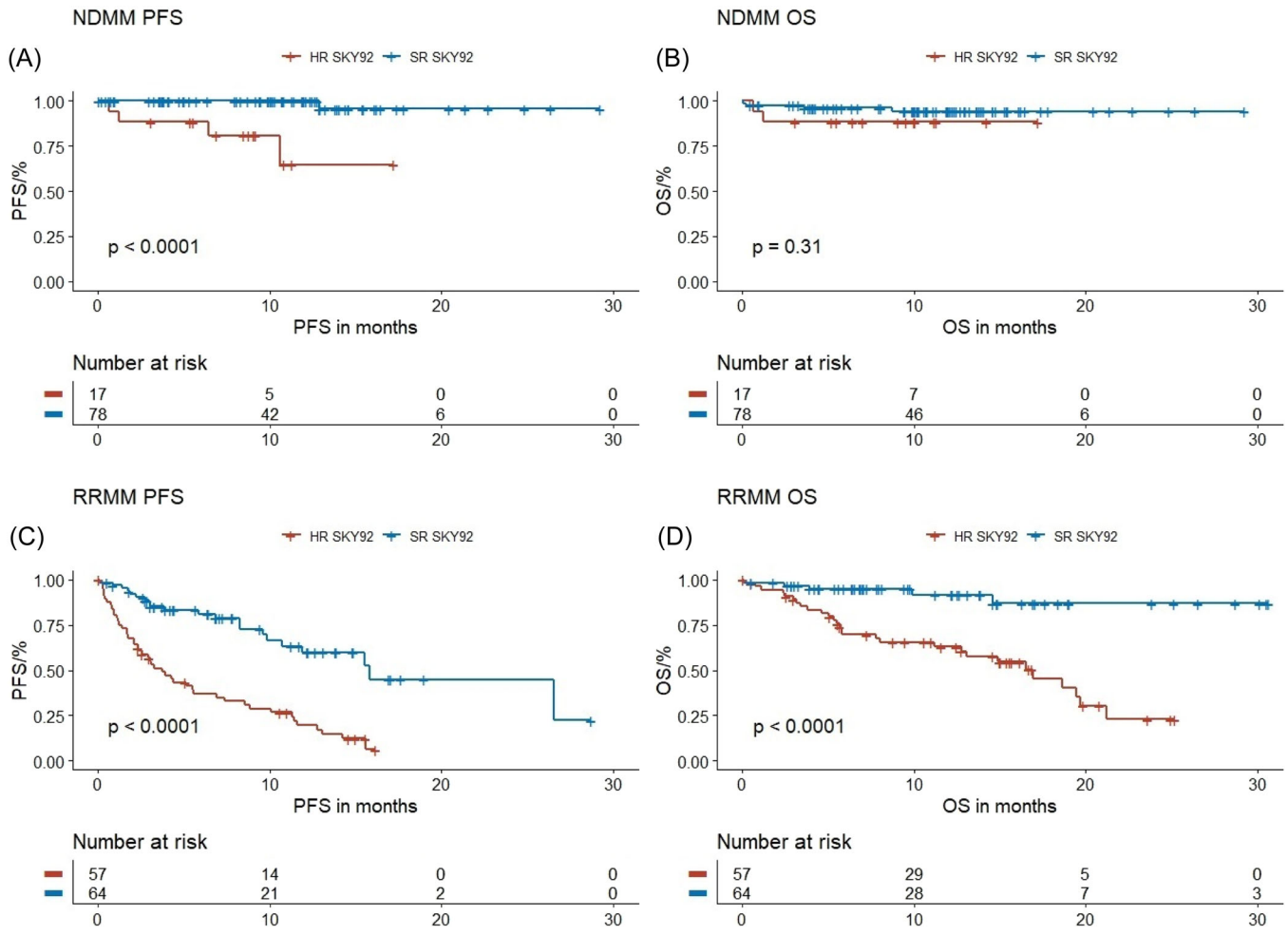

FIGURE 2 **Survival outcome and SKY92 status**. This figure illustrates the PFS and OS in NDMM **(A, B)** and RRMM **(C, D)**. NDMM, newly diagnosed multiple myeloma; OS, overall survival; PFS, progression-free survival; RRMM, relapsed/refractory multiple myeloma. Log-rank *p* values are provided.

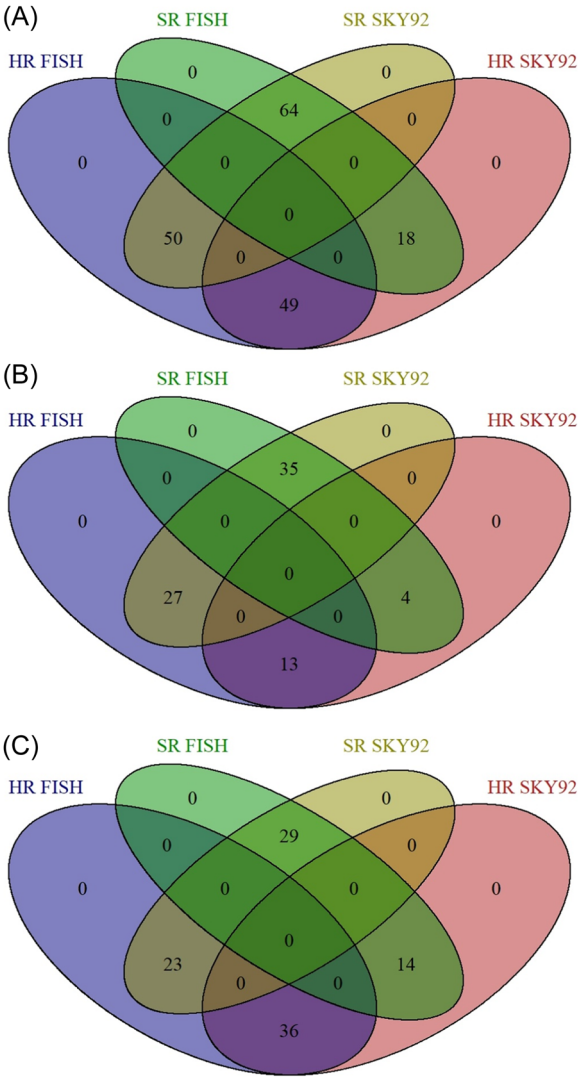

FIGURE 3 **Discrepancy between SKY92 and FISH according to the R2-ISS classification**. The figure exhibits the difference between the risk stratification systems SKY92 and FISH in the entire cohort **(A)**, NDMM **(B)**, and RRMM **(C)**. FISH, fluorescence in situ hybridization; HR, high-risk; SR, standard risk.

not compare the PFS or OS between the groups "only HR SKY92" and "only HR FISH" according to R-ISS. Nonetheless, these observations again highlighted that SKY92 could identify the HR patients more precisely than FISH according to R-ISS.

SKY92 status more predictive of survival than HR cytogenetic marker t(4;14), del17p, or del1p32 alone

We then evaluated the role of t(4;14) or del17p, HR cytogenetic markers described in both R-ISS and R2-ISS classifications, in the combination of SKY92 and FISH for MM risk stratification. In the entire group, 10 patients harbored t(4;14) but showed SR SKY92 (additionally, five patients exhibited +1q21). On the other hand, 48 patients displayed HR SKY92 but were negative for t(4;14), while 31 out of these 48 patients were HR in FISH according to R2-ISS due to other HR cytogenetic markers, that is, del17p ($n = 16$) and/or +1q21 ($n = 24$). As expected, patients with both HR SKY92 and t(4;14) demonstrated the worst survival outcome in terms of PFS ($p < 0.0001$) and OS ($p < 0.0001$) in RRMM (Supporting Information S1: Figure S9A,B). Moreover, the co-occurrence of t(4;14) and HR SKY92 indicated the worst PFS ($p = 0.0013$) and a trend toward inferior OS ($p = 0.07$) in NDMM (Supporting Information S1: Figure S9C,D). Of note, in RRMM, patients with HR SKY92 but negative for t(4;14) showed significantly shorter PFS (medians: 4.3 vs. 15.8 months, $p = 0.007$) and a trend toward inferior OS (medians: 18.7 months vs. not reached, $p = 0.08$) compared with patients being t(4;14) positive but SR in SKY92 (Supporting Information S1: Figure S10A,B). In addition, in our cohort, 12 patients displayed del17p in FISH but were SR in SKY92 (additionally, five and three patients exhibited +1q21 and t(4;14), respectively), while 44 patients showed HR SKY92 but without del17p in FISH. Among these 44 patients, 24 and 13 patients presented +1q21 and t(4;14), respectively, resulting in HR FISH in 28 patients as per R2-ISS. The co-occurrence of both HR SKY92 and del17p showed the shortest PFS ($p < 0.0001$) and OS ($p < 0.0001$) in RRMM (Supporting Information S1: Figure S11A,B), as well as the worst PFS ($p = 0.0003$) and a trend toward inferior OS ($p = 0.09$) in NDMM (Supporting Information S1: Figure S11C,D). Interestingly, in RRMM, patients with HR SKY92 but no del17p demonstrated significantly inferior PFS (medians: 6.9 months vs. not reached, $p = 0.04$) and a trend toward shorter OS (medians: 19.7 months vs. not reached, $p = 0.11$) than patients who were SR in SKY92 but presented del17p (Supporting Information S1: Figure S12A,B). However, in NDMM, we did not observe any significant differences in PFS and OS between the both groups mentioned above due to the short follow up period (Supporting Information S1: Figure S12C,D). In addition, we investigated the impact of del1p32 on MM risk assessment in our cohort. As anticipated, in RRMM, the co-occurrence of both HR SKY92 and del1p32 indicated significantly worse PFS and OS outcomes (both $p < 0.0001$) (Supporting Information S1: Figure S13A,B). However, in NDMM, the patients with HR SKY92 but without del1p32 showed the worst PFS ($p < 0.0001$) and OS ($p = 0.00014$), suggesting that del1p32 failed to identify the patients with the worst survival outcomes (Supporting Information S1: Figure S13C,D). In both RRMM and NDMM, we found no significant difference in PFS and OS between the subgroups "HR SKY92 but no del1p32" and "SR SKY92 and del1p32," possibly due to the low number of this kind of patients in our cohort (Supporting Information S1: Figure S14A–D). Our observations underline that SKY92 can identify the HR MM patients more precisely than t(4;14), del17p, or del1p32 in isolation, and the combination of different HR markers in FISH and SKY92 may provide useful information for the detection of ultra-HR MM.

WGS complements FISH and SKY92 in detection of HR MM

To elucidate the discrepancy between FISH and SKY92 risk classifications, we performed WGS in 45 patients (NDMM: $n = 12$, RRMM: $n = 33$) with available SKY92 and FISH data. HR FISH was defined as the presence of del17p, t(4;14), and +1q21 according to the R2-ISS classification. The single nucleotide variations (SNV), structural variations (SV), and copy number variations (CNV) related to adverse prognosis are presented in Figure 6.

Among the patients who were determined as HR in both SKY92 and FISH ($n = 18$), *TP53* inactivation (deletion and/or mutation) ($n = 13$, 72.2%) and +1q21 ($n = 16$, 88.9%) represented the most common genomic alterations, including five (27.8%) patients with bi-allelic *TP53* inactivation (deletion and mutation). One RRMM patient with HR SKY92 and HR FISH showed bi-allelic events in all

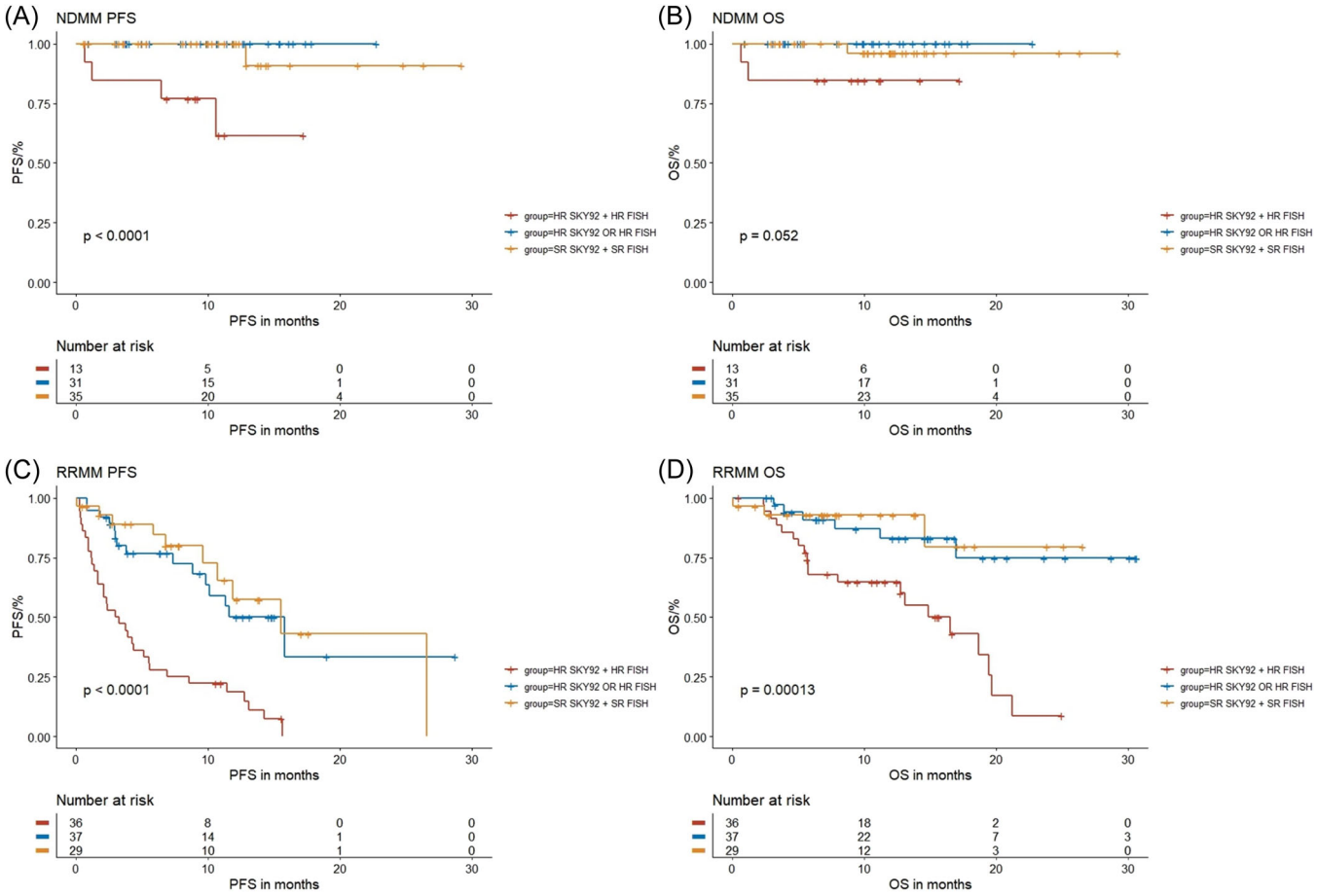

FIGURE 4 **Combination of SKY92 and FISH according to the R2-ISS classification (del17p, t(4;14) and +1q21) in the risk stratification in MM**. This figure shows the survival outcome in NDMM **(A, B)** and RRMM **(C, D)**. FISH, fluorescence in situ hybridization; HR, high risk; MM, multiple myeloma; NDMM, newly diagnosed multiple myeloma; OS, overall survival; PFS, progression-free survival; RRMM, relapsed/refractory multiple myeloma; SR, standard risk.

TNFRSF17, *TP53*, and *RB1* (deletion and mutation). This patient presented with extremely aggressive disease and died 2 months after enrolment. In four out of 11 patients with SR in both SKY92 and FISH, +1q21 ($n = 2$, NDMM) and *TP53* mutation ($n = 2$, RRMM) were revealed only by WGS. Importantly, both RRMM patients with *TP53* mutation showed aggressive disease and short PFS of 0.1 and 6.8 months, respectively, and both FISH and SKY92 falsely assessed them to be SR. In contrast, both NDMM patients harboring +1q21 were still in remission at the last follow-up. Nonetheless, these findings suggested that WGS could complement the FISH and SKY92 analyses in HR detection.

We focused on the 16 patients with HR status either only in SKY92 ($n = 7$) or only in FISH as defined by R2-ISS ($n = 9$). Surprisingly, one NDMM patient with bi-allelic *TP53* inactivation (deletion and mutation) and eight patients (NDMM: $n = 3$, RRMM: $n = 5$) harboring +1q21 were determined as SR by SKY92 but as HR by FISH. The median PFS was not reached after a median follow-up of 11.3 months in these nine patients regardless of subgroups NDMM or RRMM. The one NDMM patient with bi-allelic *TP53* inactivation was treated with high-dose melphalan and autologous SCT, and this patient was still in remission after a follow-up of 20.0 months. This observation suggests that HR FISH (even bi-allelic *TP53* inactivation) does not necessarily translate into aggressive disease with extremely short PFS if the patients concurrently have SR in SKY92. In addition, four out of seven patients with only HR SKY92 but SR FISH showed +1q21, which was detected only by WGS, and del1p32 was found in two patients. Noteworthy, *CRBN* mutation was found in three out of seven patients with only HR SKY92 but SR FISH, and, indeed, all these three patients presented with RRMM resistant to IMiD. The remaining one patient with HR status only in SKY92 did not show any known HR genomic alterations in WGS.

DISCUSSION

To the best of our knowledge, this is the first prospective study combining SKY92 GEP with FISH according to the R2-ISS classification for MM risk stratification in the real-world setting. In general, SKY92 performs well for HR detection in MM patients in the clinical routine. In addition, "double-HR" (HR SKY92 and HR FISH) patients display the worst PFS and/or OS if compared to other patients from the NDMM and RRMM subgroups.

In recent years, various GEP approaches have been developed for risk stratification, but only SKY92 MMprofiler (formerly known as EMC92) and MyPRS (UAMS GEP70) have been validated in clinical trials and are commercially available.[8–10,20–22] SKY92 GEP was initially developed and validated in NDMM patients from various clinical trials (HOVON-65/GMMG-HD4, TT2, TT3, etc.), and HR SKY92 was reported to be associated with significantly shorter OS compared to SR SKY92 patients.[8] More recently, SKY92 has been combined

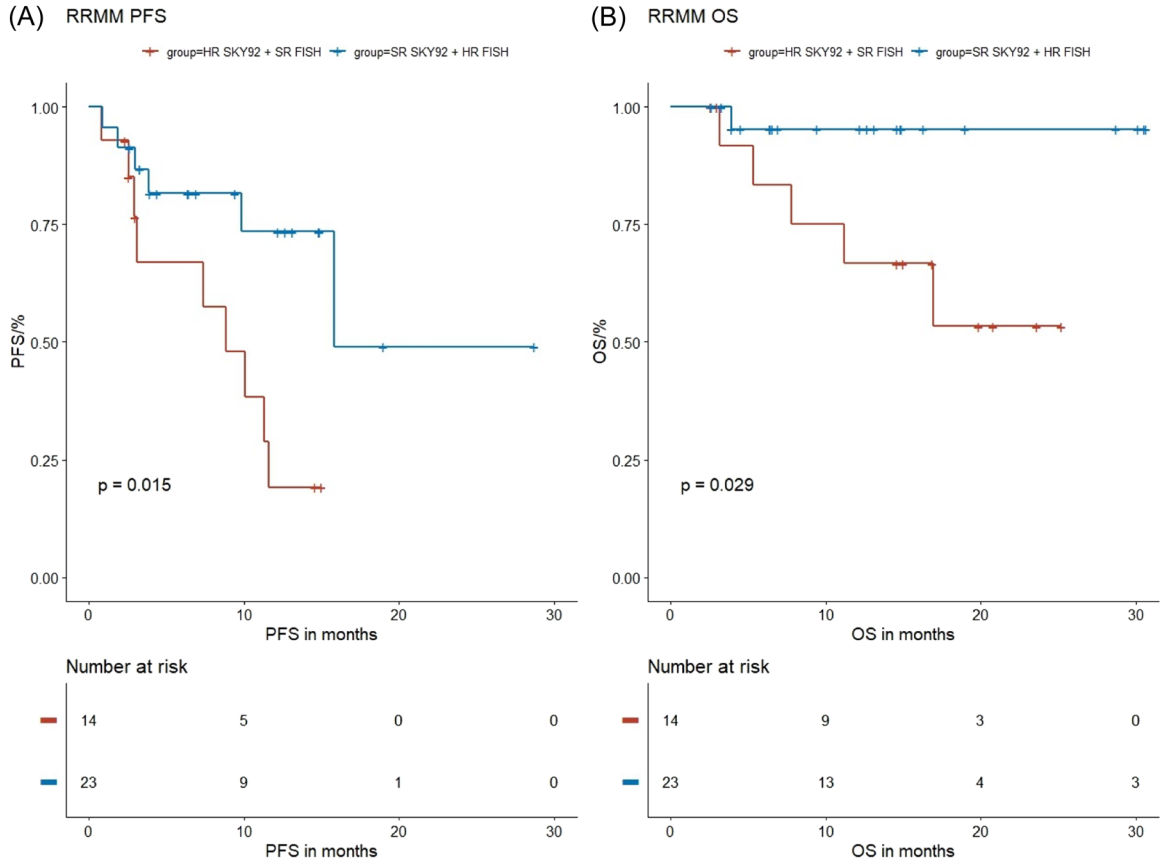

FIGURE 5 **Survival outcome of RRMM patients with either only HR SKY92 or only HR FISH according to R2-ISS classification (del17p, t(4;14) and+1q21).** This figure displays PFS **(A)** and OS **(B)** of RRMM patients with "HR SKY92 but SR FISH" versus "SR SKY92 but HR FISH." FISH, fluorescence in situ hybridization; HR, high risk; OS, overall survival; PFS, progression-free survival; RRMM, relapsed/refractory multiple myeloma; SR, standard risk.

with ISS for NDMM risk stratification purposes in clinical trials, dividing the patients into three groups, that is, low-risk (SR SKY92 and ISS I), HR (HR SKY92 and any ISS) and intermediate-risk (all others), and the HR patients displayed the worst OS.[5,9,23] In our current study, HR SKY92 was associated with unfavorable PFS and OS in both NDMM and RRMM patient groups in the real-world setting, underlining the utility of SKY92 for MM risk stratification in routine clinical practice.

In recent years, several risk classification systems, which are based on cytogenetics, have been developed in MM. Alterations such as t(4;14), t(14;16), t(14;20), del17p, del13q, and +1q21 have been considered as HR cytogenetics.[7,17,24,25] These HR markers can be combined with SKY92 for more accurate risk stratification in MM. For instance, Shah et al. combined SKY92 with chromosomal aberrations t(4;14), t(14;16), t(14;20), +1q21, and del17p for MM risk prediction in NDMM patients from the NCRI Myeloma XI trial.[20,26] The authors demonstrated that patients with ≥2 abovementioned chromosomal HR markers and concurrent HR SKY92 predicted the shortest PFS and OS, and these patients were unlikely to benefit from the lenalidomide maintenance therapy after initial autologous SCT.[20] In another study, SKY92 was combined with R-ISS in NDMM patients from the HOVON-87/NMSG-18 trial,[27] generating a novel prognostic score "SKY-RISS." The SKY-RISS III (HR SKY92 and R-ISS II/III) patients presented with significantly shorter PFS and OS than the other patients.[28] Most recently, D'Agostino has published the R2-ISS classification, which has replaced the R-ISS and represents the up-to-date MM risk stratification system.[7,17] Therefore, in our study, we combined SKY92 with FISH according to the most recently published R2-ISS classification for MM risk stratification in NDMM and RRMM in the clinical routine.[7] We did not find any significant prognostic impact of HR FISH (t(4;14), +1q21 and del17p) on PFS and OS in NDMM or RRMM. This may be due to the high prevalence of isolated +1q21, which not necessarily confers a poor prognosis of the patients.[29] However, despite the short follow-up period, if we combined SKY92 with FISH, the worst survival outcome was shown in NDMM as well as RRMM patients, who displayed HR SKY92 and HR FISH according to R2-ISS (t(4;14), +1q21 and del17p). Moreover, we found similar results with the R-ISS classification (del17p, t(4;14) and t(14;16)), confirming that the combination of SKY92 and FISH was able to identify patients with ultra-HR MM regardless of whether R-ISS or R2-ISS was applied.[30] In addition, in both R-ISS and R2-ISS classifications, t(4;14) and del17p are considered HR cytogenetic markers,[7,17] and del1p32 represents an additional HR FISH marker in MM.[31] In our study, patients with HR SKY92 but negative for t(4;14) or del17p showed significantly shorter PFS compared to the patients being SR in SKY92 but harboring t(4;14) or del17p. There was no significant difference in PFS or OS between the subgroups "HR SKY92 but no del1p32" and "SR SKY92 and del1p32." More importantly, the widely used ISS is based on laboratory results (albumin, β2 microglobulin, and lactate dehydrogenase), which fluctuate continuously and therefore lead to very frequent changes of ISS stage in a single patient. Utilizing more advanced techniques, our study provides a novel strategy for MM risk stratification without fluctuating laboratory values. Taken together, our findings again highlight that SKY92 can detect HR MM patients more

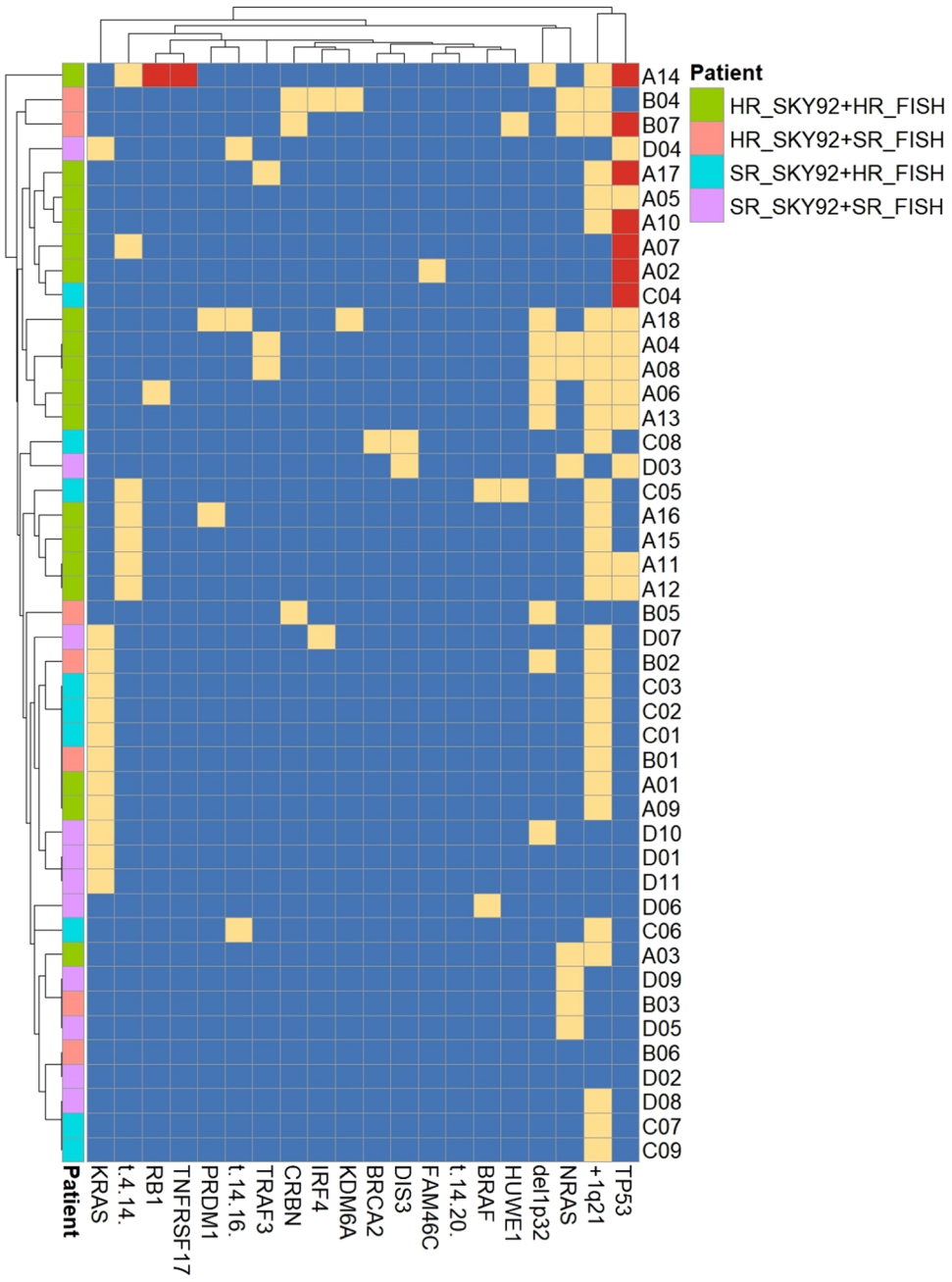

FIGURE 6 **Genomic alterations revealed by whole genome sequencing**. This figure exhibits genomic alterations including single nucleotide variations (SNV), structural variations (SV), and copy number variations (CNV) related to adverse prognosis in MM. Each row represents a patient, and each column shows a single SNV, SV, or CNV. Blue and gold encodes for the absence and presence of genomic alterations, respectively. Specifically for *TP53*, *RB1*, and *TNFRSF17*, red indicates bi-allelic events (deletion and/or mutation), and gold for mono-allelic *TP53* alterations (deletion or mutation). The different patient groups are annotated by a second color code. HR, high risk; SR, standard risk.

precisely than using single HR FISH markers in isolation, and the ultra-HR MM can be identified by combining multiple HR markers in FISH and SKY92.

Currently, WGS is increasingly being used in MM patients to discover genomic markers, especially bi-allelic loss of immunotherapy targets or tumor suppressor inactivation, and to elucidate the mechanisms of relapse and drug resistance.[32–35] In our study, we used WGS to evaluate the discrepancy between SKY92 and FISH. Importantly, some clinically relevant markers, for example, +1q21, *TP53*, and BCMA loss, were exclusively found by WGS, suggesting that WGS could complement SKY92 and FISH in MM HR detection. Surprisingly, eight patients with +1q21 and one patient harboring bi-allelic *TP53* inactivation (deletion and mutation) were determined as SR by SKY92 but as HR by FISH as per the R2-ISS classification. However, patients with isolated HR SKY92 showed significantly shorter PFS and/or OS compared to those with HR FISH but SR SKY92. Moreover, we found *CRBN* mutations in three IMiD-resistant MM patients, who exhibited HR SKY92 but SR FISH. These findings highlight again the advantages of SKY92 over FISH in MM HR detection. The underlying mechanisms for discrepancy between SKY92 and FISH are incompletely understood,

as we have not found any known HR genomic markers, even by WGS, in a patient with HR SKY92 only. Single-cell RNA sequencing (scRNAseq), Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITEseq), and epigenetic analysis may provide further mechanistic insights into the discrepancy between WGS and SKY92. In principle, these modern techniques (SKY92 GEP, FISH, WGS, scRNAseq, and CITEseq) complement each other and the combination of them allows the discovery of the MM tumor biology on different levels, that is DNA, RNA, and protein. In addition, these methods assess different, overlapping but still independent qualities of the tumor, and the combination is required to reflect the complexity of MM. Future studies are needed to explore the role of SKY92 in identifying HR patients not captured by WGS or FISH.

In summary, our study highlights the prognostic value of SKY92 GEP in NDMM and RRMM patients in routine clinical practice. Moreover, we provide the first prospective real-world evidence that the combination of multiple diagnostic tools, that is, FISH, GEP, WGS, and clinical HR factors, can be applied to detect ultra-HR MM. In addition, WGS complements FISH and SKY92 in MM HR detection, especially for the identification of bi-allelic events, targeted therapy, and mechanisms of drug resistance.

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AUTHOR CONTRIBUTIONS

Xiang Zhou, Hermann Einsele, Leo Rasche, and K. Martin Kortüm designed the research. Xiang Zhou, Cornelia Vogt, Silvia Nerreter, E.T., Emilia Stanojkovska, Marietta Truger, Mara John, Seungbin Han, and Umair Munawar performed the experiments. Annika Hofmann, Benedict Engel, Xianghui Xiao, Christine Riedhammer, Maximilian J. Steinhardt, Julia Mersi, Johannes M. Waldschmidt, Claudia Haferlach, and Hermann Einsele provided patient samples and clinical data. Xiang Zhou, Leo Rasche, and K. Martin Kortüm wrote the manuscript, which was approved by all authors; all authors analyzed and interpreted the data.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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