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ORIGINAL RESEARCH

Mutations In Thirty Hotspot Genes In Newly Diagnosed Chinese Multiple Myeloma Patients

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Objective: In recent years, whole-genome sequencing and whole-exon sequencing have revealed the spectrum of gene mutations in multiple myeloma (MM). Gene mutations may play an important role in the pathogenesis, progression, and prognosis of this disease. On the basis of these studies, we established a box of mutations in 30 hotspot genes and analyzed the characteristics in newly diagnosed MM patients in China.

Methods: Bone marrow samples were collected. Mononuclear cells were isolated and plasma cells were separated using CD138 magnetic beads. Gene mutations were detected by PCR and Sanger sequencing. Fluorescence in situ hybridization (FISH) was used to analyze 1q21, 17p13.1, 14q32/16q23, 14q32/4p16, and 14q32/11q13.3. In the first part of this study, characterization of 30 genes and FISH analysis were performed in 40 patients. For economic reasons, in the second part of this study, 12 of 30 genes were characterized in another 46 patients.

Results: In the 40 patients of the first part of this study, single nucleotide polymorphisms (SNPs) were detected in 7 genes (*CRBN, ATM, FAT4, FAM46C, RB1*, *NR3C1*, and *SPEN*), while 16 genes were mutated (*ATM, CUL4B, IRF4*, CCND1, KRAS, DIS3, CRBN, TP53, FAT4, NR3C1, VCAN, RB1, SP140, NRAS, EGR1, and *BRAF*). Overall, 83 mutations of 30 genes were identified, including 54 intronic mutations, 18 missense mutations, 6 synonymous mutations, 3 5′/3′-UTR mutations, and 2 deletions mutations. Cytogenetic abnormalities were also screened in the 40 patients assayed, with 50% of the patients having $1q21^{+}$, 12.5% having $17p^{-}$, 15% having t(4;14), and 17.5% having t(11;14). *DIS3* was mutated in 4/40, three of which involved t(4;14) or t(11;14). *TP53* was mutated in two non-$17p^{-}$ patients, one of whom survived only 7 months, while the other survived 13 months. Three genes (*ATM, CUL4B, and IRF4*) with a high mutation rate were analyzed for an association with survival. There was no statistically significant difference in 2-year PFS (progress free survival) and 2-year OS (overall survival) between patients with or without ATM or CUL4B mutation ($P>0.05$). This finding was also obtained for IFR4 mutation, but patients with IFR4 mutation did show trends for longer PFS and OS.

Conclusion: SNPs and other types of gene mutations are common in newly diagnosed Chinese multiple myeloma patients. The genes most commonly featuring SNPs are *CRBN, ATM, FAT4*, and *FAM46C*, while the genes most commonly featuring other mutation types are *ATM, CUL4B*, and *IRF4*. There were differences in the profiles of genes affected by SNPs and by other mutation types. Intronic mutations were the most common mutation type. Gene mutations may differ among patients with different cytogenetic abnormalities. Genetic mutations may be associated with prognosis.

Keywords: multiple myeloma, gene mutation, single nucleotide polymorphism

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Introduction

Multiple myeloma (MM) is an incurable malignancy of plasma cells. Its pathogenesis is only partially understood. Almost all patients harbor chromosomal aberrations; however,

these alone are unlikely to be sufficient for malignant transformation. Risk assessment based on individual cytogenetics has already been established in MM. However, the prognostic indices used in such assessment do not evaluate patients' genomic profiles accurately and comprehensively to guide precision therapy.[1] Recently, large sequencing studies have defined the mutation landscape of multiple myeloma.[2,3] Therefore, we generated a mutation box for 30 hotspot genes based on previous research to explore the characteristics and clinical significance of gene mutations in newly diagnosed Chinese MM patients.

Materials And Methods

The Institutional Review Board of Beijing Chao-Yang Hospital, Capital Medical University, approved this study, which was conducted in accordance with the Declaration of Helsinki. On the basis of large sequencing studies, we established a box of mutations in 30 hotspot genes. We identified 40 patients (Box 1) (Table 1) and 46 patients (Box 2) (61 males, 25 females) newly diagnosed with MM. The median age was 61 years (42–79 years). The diagnostic criteria were those defined by the International Myeloma Working Group (IMWG). Bone marrow samples were collected from all of the patients. Mononuclear cells were isolated and plasma cells were separated by CD138 magnetic beads. DNA was extracted. Gene mutations were detected using PCR and Sanger sequencing (ABI 3500DX) by Kindstar Global Company in Beijing. Cytogenetic abnormalities, including 1q21, 17p13.1, 14q32/16q23, 14q32/4p16, and 14q32/11q13.3 were detected by FISH. SPSS 19 statistical software was used for survival analysis. When $P<0.05$, the difference was considered statistically significant.

Box 1 (30 genes)

NEB: Exon78; ***DIS3***: Exon5, Exon8, Exon9, Exon17, Exon18, Exon20, Exon21; ***FAM46C***: Exon2; ***SP140***: Exon2, Exon19, Exon27; ***RB1***: Exon8, Exon19; ***ZFHX4***: Exon11; ***VCAN***: Exon7, Exon8; ***PRDM1***: Exon6; ***CCND1***: Exon1; ***TRAF3***: Exon11; ***FAT4***: Exon1, Exon16; ***SPEN***: Exon11; ***ANK2***: Exon38; ***ATM***: Exon10, Exon41, Exon62; ***EGR1***: Exon1; ***FGFR3***: Exon14; ***PIK3CA***: Exon10; ***NFKB2***: Exon17; ***KRAS***: Exon2, Exon3, Exon4; ***NRAS***: Exon2, Exon3; ***TP53***: Exon5, Exon6, Exon7, Exon8; ***BRAF***: Exon11, Exon15; ***CRBN***: Exon3, Exon6, Exon11; ***DDB1***: Exon2, Exon8, Exon9, Exon24; ***CUL4B***: Exon10; ***IRF4***: Exon3; ***NR3C1***: Exon2; ***XBP1***: Exon2; ***PSMG2***: Exon5; ***PSMB5***: Exon1

Note: Bold text indicates 30 detected hot spot genes.

Table 1 Baseline Characteristics In Newly Diagnosed Patients

	Box 1	Box 2
Median age (years)	59 (42–79)	55 (45–75)
Male/female	28/12	33/13
M protein type		
IgG lambda	8	12
IgG kappa	9	9
IgA lambda	4	5
IgA kappa	3/40	3
Kappa	3	7
Lambda	7	5
IgD lambda	2	3
No secretion	1	0
IgA lambda lambda	1	1
IgG lambda lambda	1	1
IgG lambda IgAlambda	1	0
ISS stage		
I	6	7
II	8	12
III	18	18
R-ISS stage		
I stage	5	3
II	14	8
III	11	5
IMWG		
Low risk	4	2
Mediate risk	19	8
High risk	8	4
Cytogenetics		
$1q21^{+}$	20/40	8/20
t(4;14)	6/40	4/20
t(11;14)	7/40	7/20
$17p^{-}$	5/40	3/20

Results

In the 40 patients who were assayed with Box 1, SNPs were detectable in all patients. In total, 230 missense/synonymous/intronic SNPs were detected. These SNPs were detected in seven genes: *CRBN, ATM, FAT4*, *FAM46C, RB1, NR3C1*, and *SPEN*. SNPs in *CRBN* were detected in 92.5% of the patients,

Box 2 (12 genes)

KRAS: Exon2, Exon3, Exon4; ***NRAS***: Exon2, Exon3; ***TP53***: Exon5, Exon6, Exon7, Exon8; ***BRAF***: Exon11, Exon15; ***CRBN***: Exon3, Exon6, Exon11; ***DDB1***: Exon2, Exon8, Exon9, Exon24; ***CUL4B***: Exon10; ***IRF4***: Exon3; ***NR3C1***: Exon2; ***XBP1***: Exon2; ***PSMG2***: Exon5; ***PSMB5***: Exon1

Note: Bold text indicates 12 detected hot spot genes.

Table 2 SNPs In 40 Newly Diagnosed Patients

SNP	Number	%
CRBN	38	92.5
ATM	24	60
FAT4	15	37.5
FAM46C	14	35
RB1	8	20
NR3C1	3	7.5
SPEN	2	5

those in *ATM* in 60%, those in *FAT4* in 37.5%, those in *FAM46C* in 35%, those in *RB1* in 20%, those in *NR3C1* in 7.5%, and those in *SPEN* in 5% of the patients (Table 2). The numbers of patients with SNPs in 1, 2, 3, 4, and 5 genes were 7, 13, 12, 5, and 3, respectively. Mutated genes were detectable in 87.5% of the 40 patients assayed with Box 1. Sixteen mutated genes and 44 mutation sites were detected (Figure 1), including mutations in *ATM* in 57.5% of the patients, *CUL4B* in 27.5%, *IRF4* in 25%, *CCND1* in 12.5%, *KRAS* in 10%, *DIS3* in 10%, *CRBN* in 7.5%, *TP53* in 5%, *FAT4* in 5%, *NR3C1* in 5%, *VCAN* in 5%, *RB1* in 2.5%, *SP140* in 2.5%, *NRAS* in 2.5%, *EGR1* in 2.5%, and *BRAF* in 2.5% of the patients (Figure 2). A total of 83 mutations were detected in the 40 patients who were assayed with Box 1, including 54 intronic mutations, 18 missense mutations, 6 synonymous mutations, 3 5′/3′-UTR mutations, and 2 deletion mutations (Figure 3). In this group, the numbers of patients with mutations in 1, 2, 3, 4, or 5 genes were 13, 11, 4, 4, and 2, respectively. No mutated genes were detected in six patients. Multiple mutations in a single gene, namely, *CCND1*, *NR3C1*,

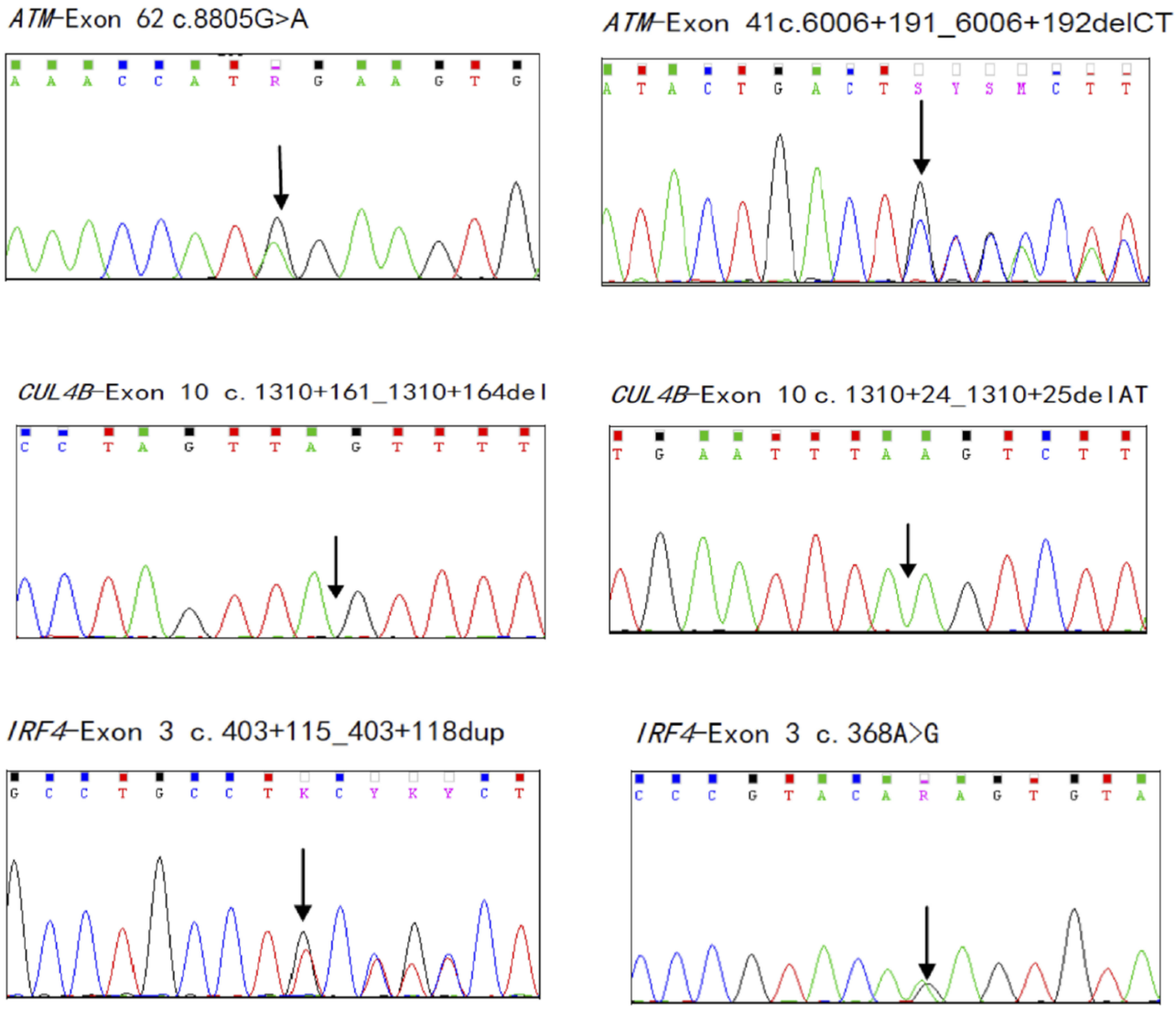
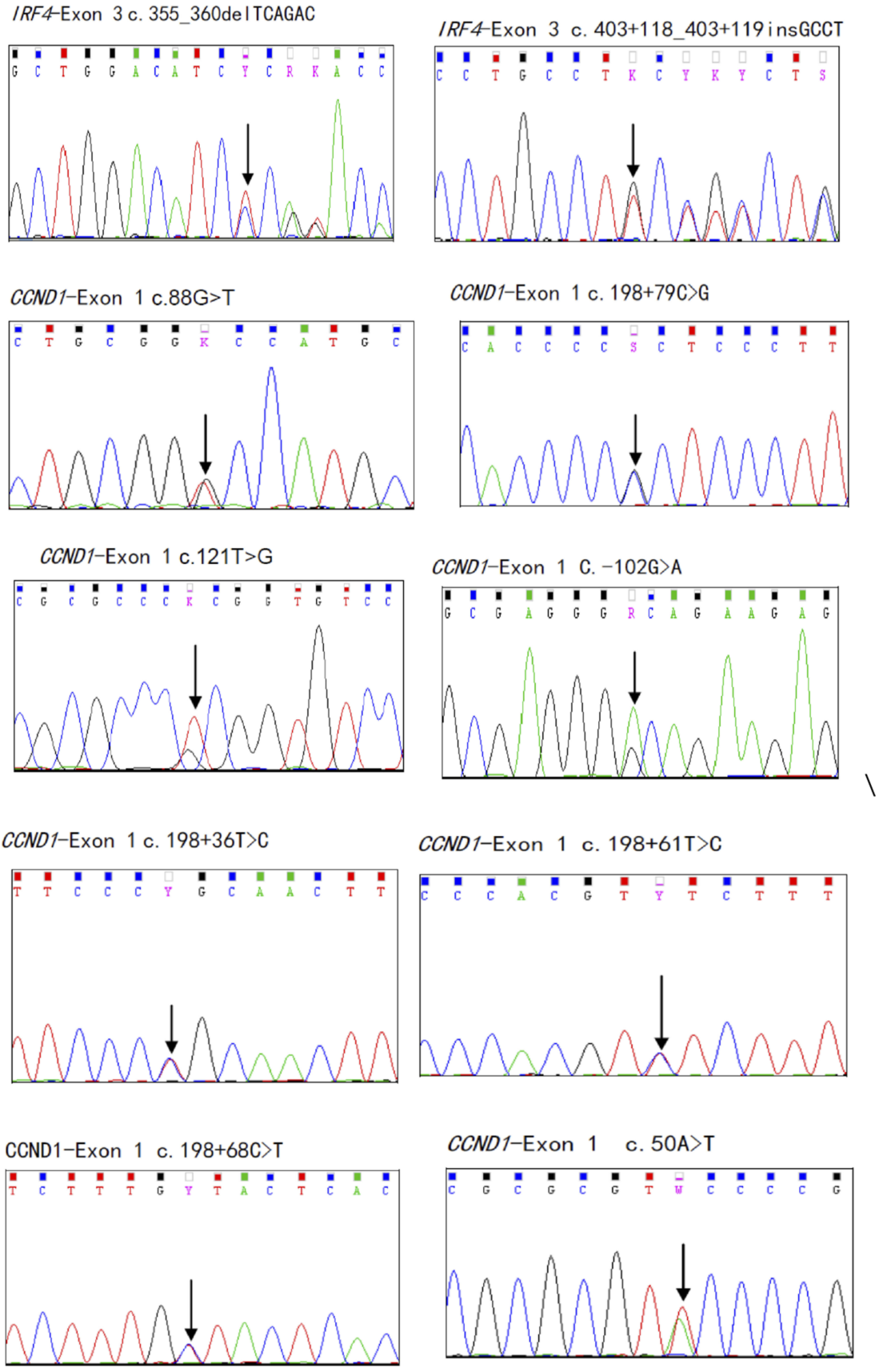


Figure 1 (Continued).



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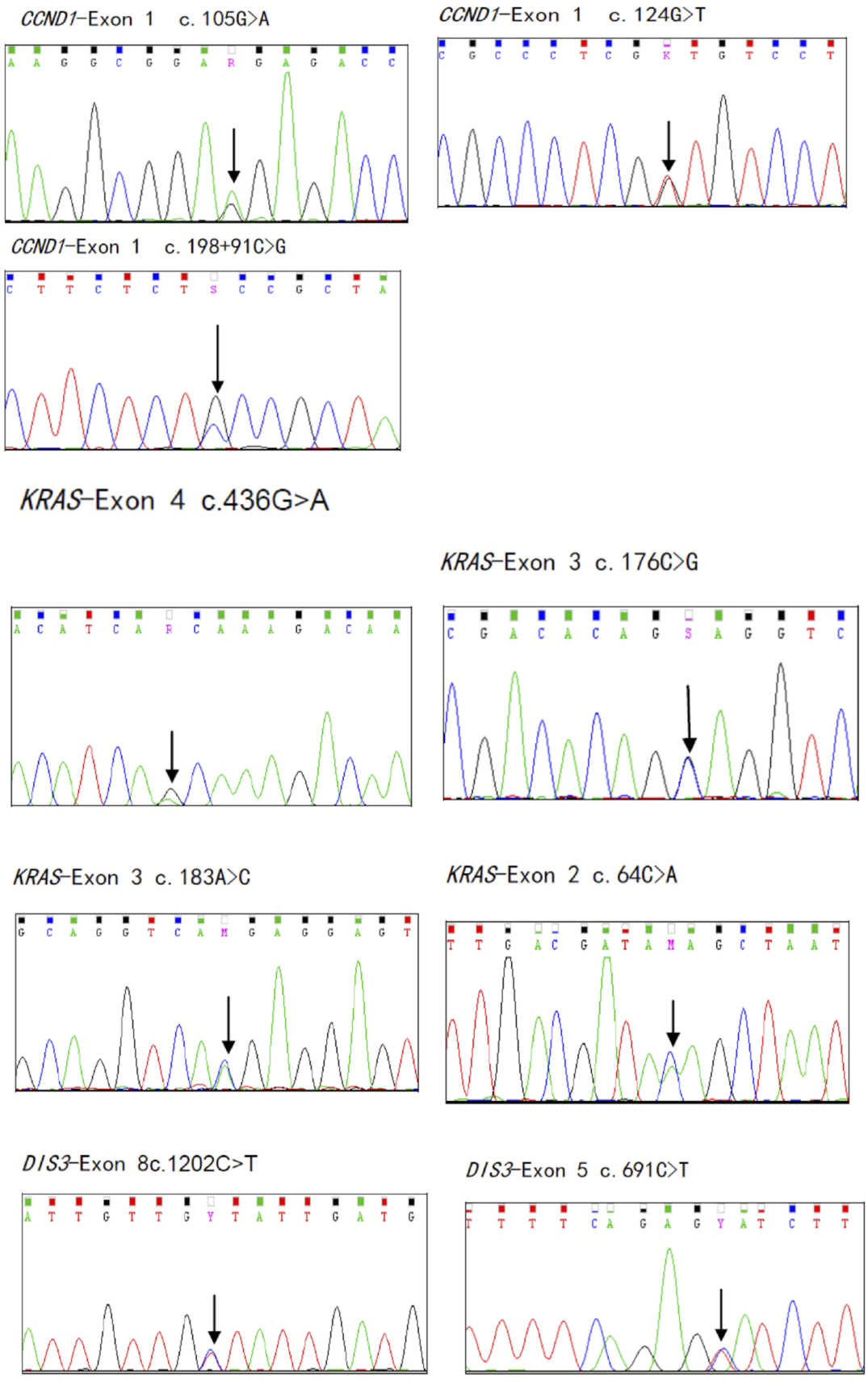

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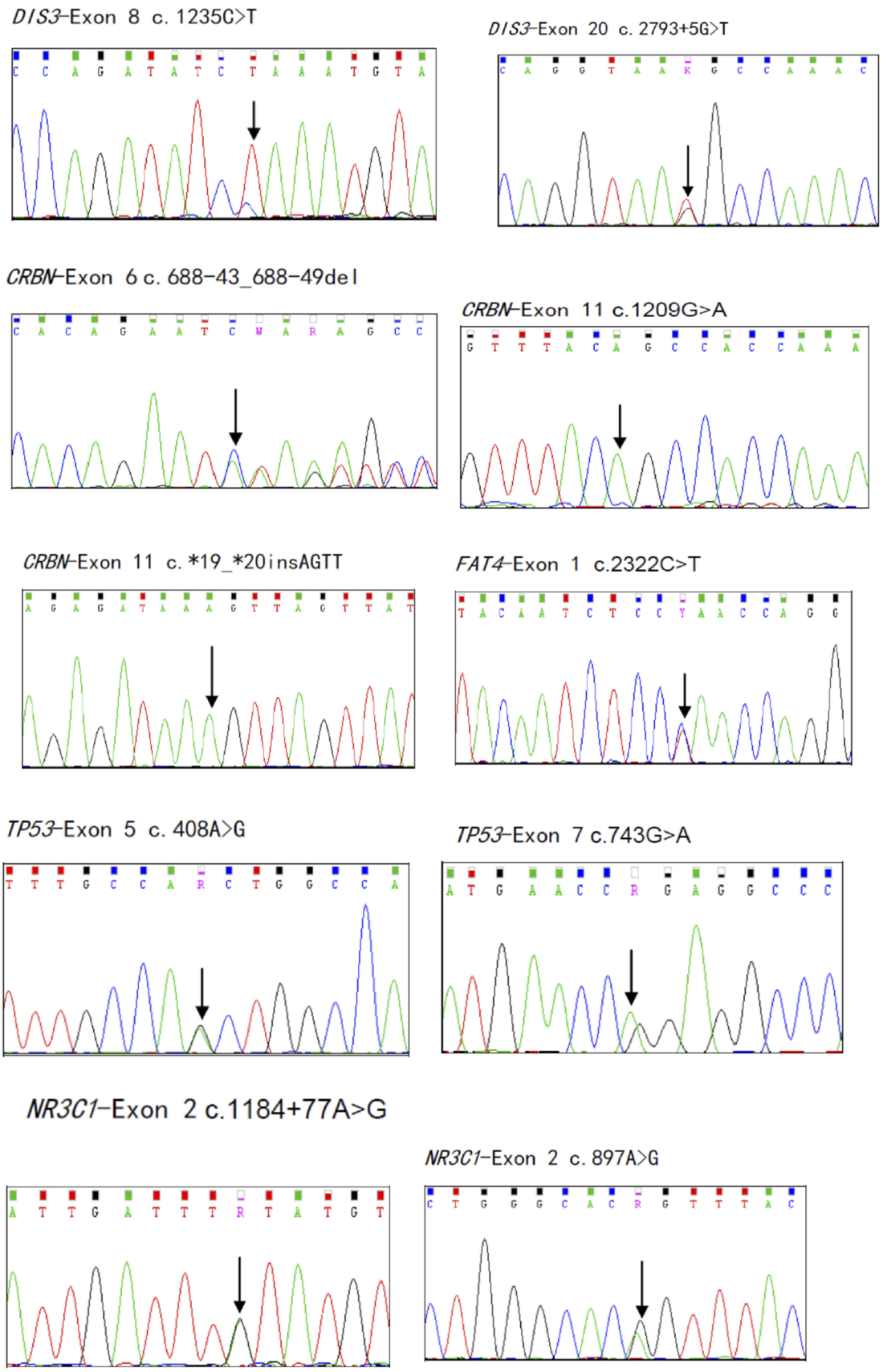

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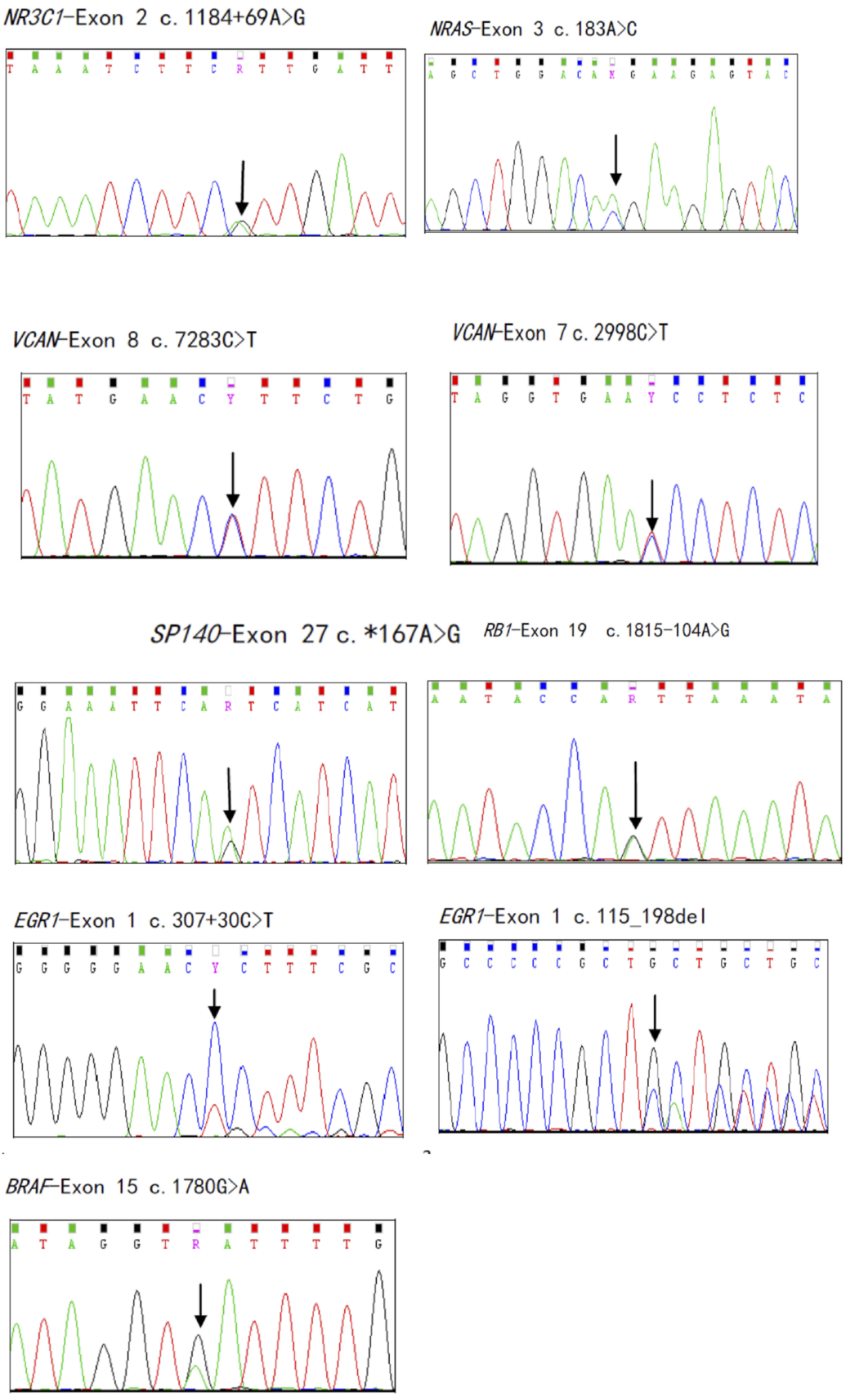

Figure 1 Mutation sites of 16 genes in 40 newly diagnosed multiple myeloma patients. Arrows indicate mutation sites.

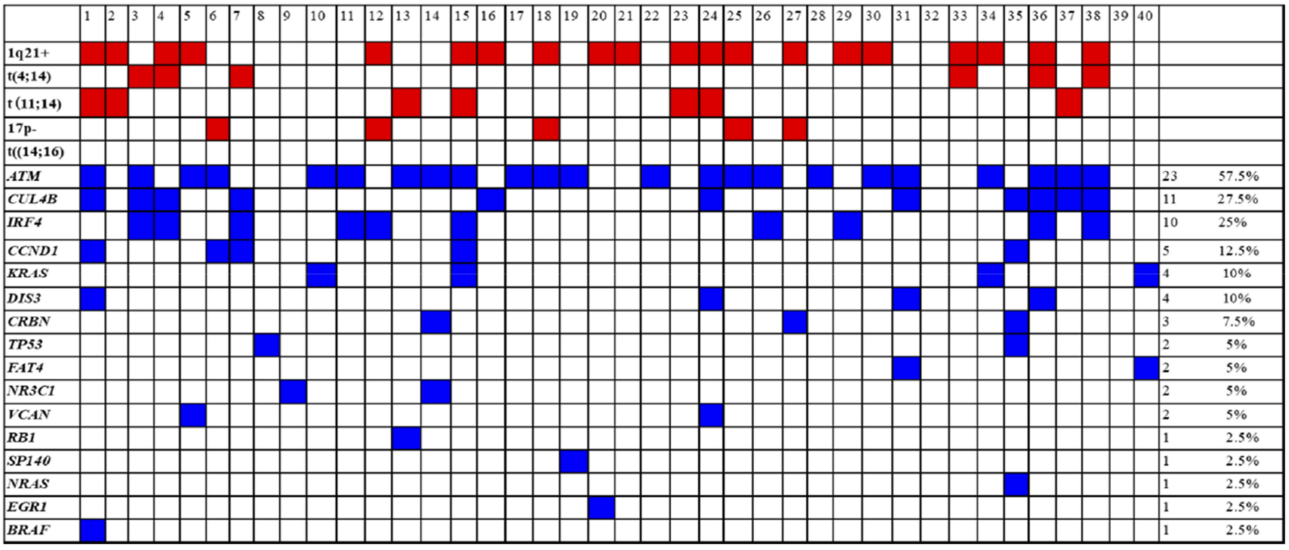

Figure 2 Gene mutations in 40 newly diagnosed multiple myeloma patients with Box 1.

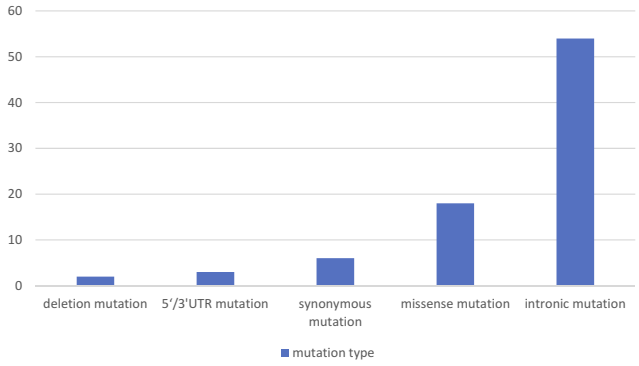

Figure 3 Mutation types of 16 genes in 40 newly diagnosed multiple myeloma patients.

and *IRF4*, were detected in one patient each. Cytogenetic abnormalities were also screened in the 40 patients assayed with Box 1, with 50% (20/40) of them having 1q21^{+}, 12.5% (5/40) having 17p^{-}, 15% (6/40) having t(4;14), and 17.5% (7/40) having t(11;14). *ATM, CCND1, CUL4B, DIS3, BRAF, IRF4, KRAS*, *EGR1, VCAN*, and *CRBN* mutations were characterized in patients with 1q21^{+}. *ATM, CUL4B, IRF4, CCND1*, and *DIS3* were detected in patients with t(4;14). *ATM, CUL4B, DIS3, CCND1, BRAF, KRAS, IRF4, VCAN*, and *RB1* were detected in patients with t(11;14). In addition, *ATM, IRF4, CCND1*, and *CRBN* were detected in patients with 17p^{-}. *DIS3* was mutated in 4 of 40 patients, three of whom had t(4;14) or t(11;14). *TP53* was mutated in two non-17p^{-} patients, one of whom survived only 7 months while the other survived 13 months. For economic reasons, a further 46 newly diagnosed patients were analyzed only with Box 2 (12 of 30 genes) (Figure 4). *NRAS* and *KRAS* were mutated in 7 and 9 of the total of 86 patients, respectively. All patients received bortezomib-based induction chemotherapy. Next, patients younger than 65 received autologous hematopoietic stem cell transplants (ASCT). An analysis of associations with survival was performed on genes with a high mutation frequency, namely, *ATM, CUL4B*, and *IRF4*. For this, all 86 patients were followed for 11 to 35 months. There was no significant difference in 2-year PFS between patients with or

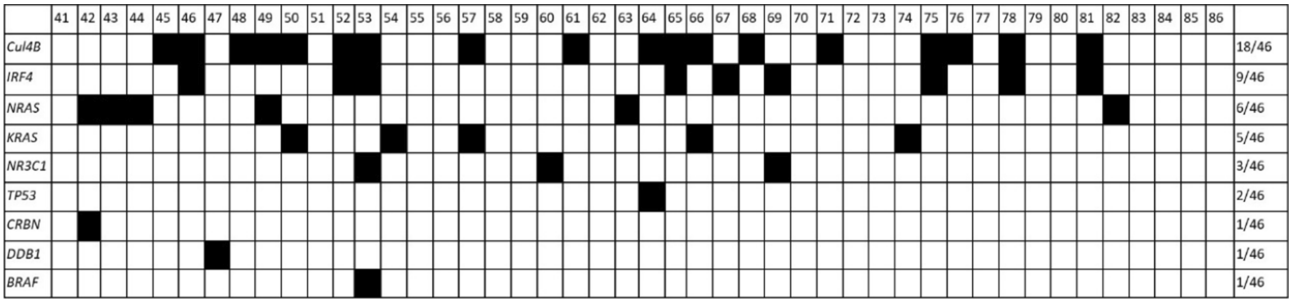

Figure 4 Gene mutations in 46 newly diagnosed multiple myeloma patients with Box 1.

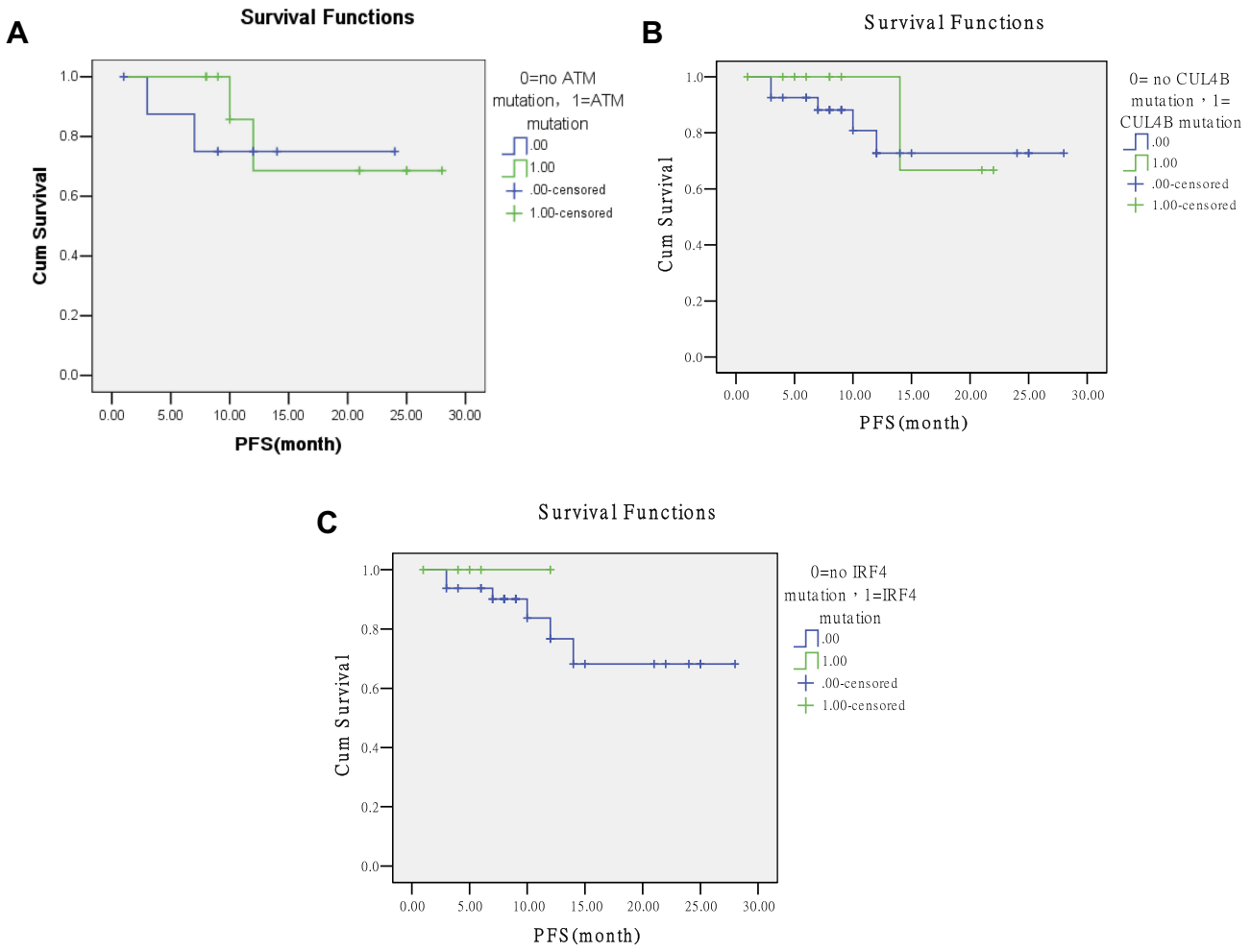

Figure 5 Two-year PFS between patients with or without (A) *ATM*, (B) *CUL4B*, and (C) *IRF4* mutations.

without *ATM* (data from 40 patients), *CUL4B* (data from 86 patients), and *IRF4* (data from 86 patients) mutations (P>0.05). However, patients with *IRF4* mutations had a tendency toward longer PFS (Figure 5). There was also no significant difference in 2-year OS between patients with or without *ATM* (data from 40 patients), *CUL4B* (data from 86 patients), and *IRF4* (data from 86 patients) mutations (P>0.05). However, patients with *IRF4* mutations had a tendency toward a longer OS time (Figure 6).

In conclusion, SNPs and other types of mutation are common in newly diagnosed Chinese multiple myeloma patients. In the first part of this study, SNPs were detected in 100% of 40 patients. Genes with other types of mutation were found in 87.5% of 40 patients and such mutations were found in 53.3% of 30 analyzed genes. The genes most often containing SNPs were *CRBN, ATM, FAM46C*, and *FAT4*. The genes most often exhibiting other mutation types were *ATM, CUL4B*, and *IRF4*. *TP53* was mutated in two non-17p$^-$ patients, who had very short survival times. In contrast, *IRF4* mutations had a tendency to be associated with longer PFS and OS. However, there was no significant difference in PFS and OS in patients with or without mutations in *ATM* and *CUL4B*.

Discussion

In our study, 230 missense/synonymous/intronic SNPs were detected. The genes most often featuring SNPs were *CRBN, ATM, FAM46C*, and *FAT4*. Multiple SNPs were also found simultaneously in a single patient. In Kortüm et al's study, 123 nonsynonymous missense/nonsense SNPs were detected in newly diagnosed patients with 17p^{-1}. SNPs are common in multiple myeloma, but few studies on their roles in this disease have been performed and their clinical significance remains to be determined. In our study, mutated genes were found in 87.5% of the patients and mutations were found in 53.3% of the 30 analyzed genes. Kortüm et al found gene mutations in 78% of newly diagnosed patients

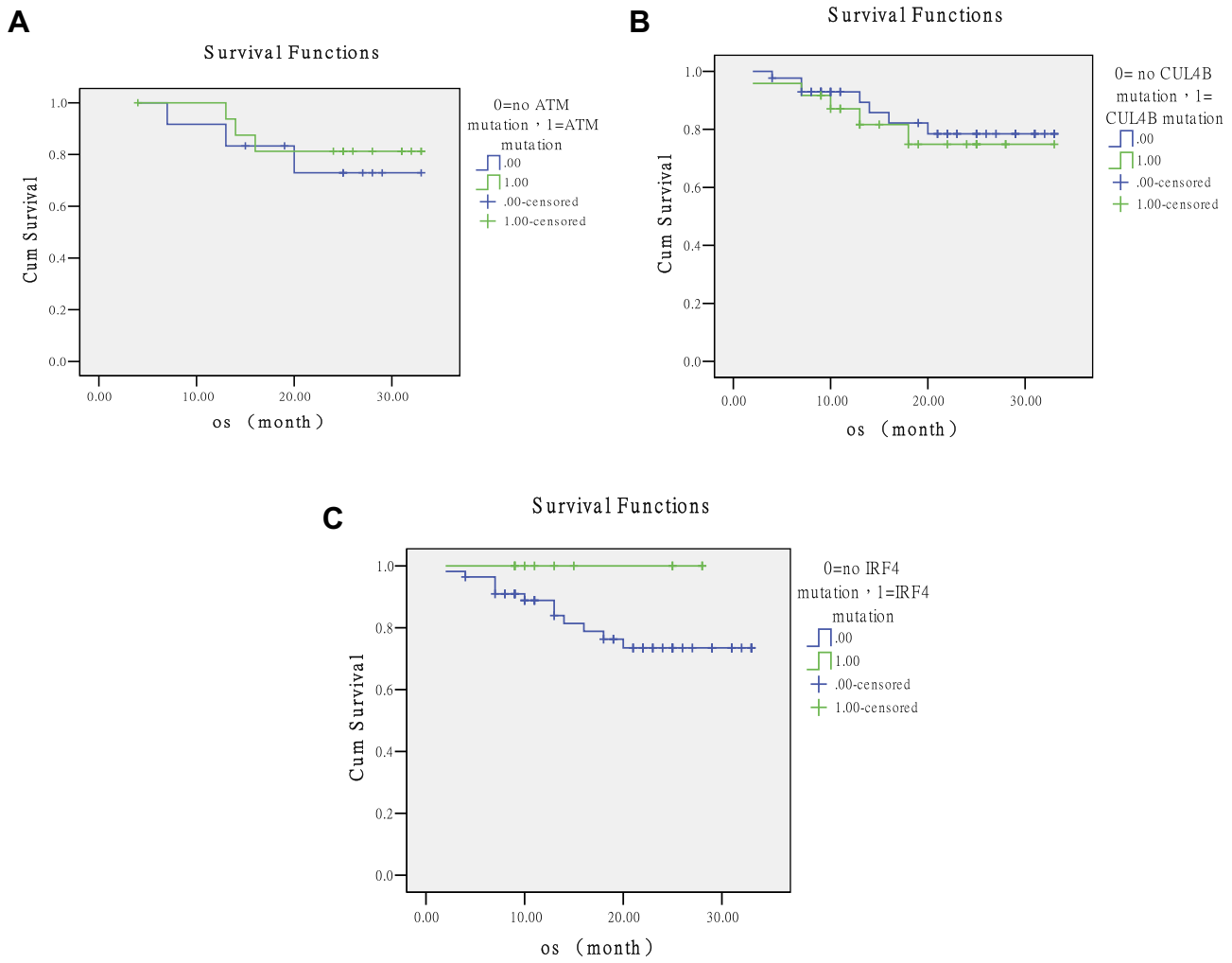

Figure 6 Two-year OS between patients with or without (A) *ATM*, (B) *CUL4B*, and (C) *IRF4* mutations.

with 17p$^-$ and revealed mutations in 66% of the 47 analyzed genes.[1] Moreover, in a study of genetic heterogeneity in multiple myeloma by Lohr et al, the most commonly mutated genes were *KRAS, NRAS, FAM46C, DIS3*, and *TP53*,[2] while in our study the most commonly mutated genes were *ATM, CUL4B, IRF4, KRAS*, and *NRAS*. It is possible that the genes most commonly mutated in newly diagnosed Chinese MM differ from those in patients from other countries. In our study, *NRAS* and *KRAS* were commonly mutated in newly diagnosed patients, while Lohr et al instead reported that they were more often mutated in relapsed patients.[2] *TP53* has been reported to be the most frequently mutated gene in 17p$^-$ patients;[1] however, in our study, *TP53* was mutated in two non-17p$^-$ patients who survived for a very short period, suggesting that *TP53* mutation may also occur in patients without 17p$^-$ and be predictive of a poor prognosis. Bolli et al also reported that *TP53* mutation was associated with impaired EFS (event free survival) and OS.[3] Moreover, *DIS3* mutations have been reported to be exclusively present in t(4;14) and t (11;14) patients.[4] In our study, *DIS3* was mutated in four patients, three of whom had t(4;14) or t(11;14). These results suggest that certain gene mutations may be associated with specific cytogenetic changes. Genes that are members of the *FAT* family have been shown to have a significant number of mutations in chronic lymphocytic leukemia.[5] *FAT4* was mutated in two patients in our study, although the clinical significance of such mutations remains to be determined. *FAM46C* is often mutated in MM, but there is at present a very limited understanding of the function of this particular gene.[6] Moreover, no *FAM46C* mutation was seen in our study. However, *CUL4B* and *IRF4* were commonly mutated in our study. *IRF4* has been reported to be a factor associated with the survival of MM.[7] *CUL4B* and *IRF4* both affect the *CRBN* pathway, a potential source of IMiD (immunomodulatory drug) resistance.[1] *CRBN*

SNPs were common in our patients, but *CRBN* gene mutations were found in only three patients. There are limited data available on the effects of *CRBN* gene mutations. One study identified a *CRBN* truncating mutation and a *CRBN* point mutation in 1 of 30 MM patients, and two *CRBN* SNPs in 24 newly diagnosed patients.[8] Furthermore, *EGR1* was found to be mutated in our study, which was also previously reported to play a role in resistance to MM therapy.[9] *SP140* has also recently been described as having a possible role as a tumor suppressor in MM.[10] A single *BRAF*-mutant MM patient was recently reported to show a durable response to a BRAF inhibitor.[11] So, the role of these mutations needs to be clarified by further research. In our study, multiple mutations of the same gene were detected in the same patient. In addition, mutations of different genes were detected in the same patient. In line with this, Lohr et al observed multiple significant mutations in the same tumor sample: some patients had mutations in two of three oncogenes (*NRAS, BRAF*, and *KRAS*) or had two mutations in *KRAS*.[2] Moreover, Walker et al reported that *FGFR3* was only mutated in the subgroup of patients with t(4;14), and that *CCND1* was significantly more frequently mutated in the t(11;14) subgroup, while t(11;14) was also associated with *KRAS* and *IRF4* mutations.[12] However, in our study, no *FGFR3* mutations were detected in the t(4;14) group, *CCND1* was mutated in different groups with abnormal cytogenetics, and *KRAS* mutations were detected in the $1q21^{+}$ and t(11;14) groups. However, all of these issues require further study. In our study, *ATM*, *CUL4B*, and *IFR4* were the most frequently mutated genes, so survival analyses of these three genes were carried out. Mutations in *IRF4* were previously found to have a positive impact on survival, with a trend toward an improvement in OS (2-year OS, 100% vs 79%, P=0.05).[12] In the current study, similar findings were obtained, in that patients with *IRF4* mutations had a tendency toward longer PFS and OS. Mutations in *ATM* were also shown to be associated with a trend toward impaired OS (2-year OS, 50% vs 80.3%, P=0.01).[12] However, in our study, there was no significant difference in ATM and OS between patients with and without *ATM* mutation.

In conclusion, SNPs and other mutation types are common in newly diagnosed Chinese multiple myeloma patients. The genes most commonly exhibiting SNPs and other types of mutation may differ between Chinese patients and those from other countries. It is not certain that gene mutations in newly diagnosed MM vary between patients with different cytogenetic abnormalities. Genetic mutations may be associated with prognosis. More cases need to be accumulated and longer follow-up is needed.

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Disclosure

The authors report no conflicts of interest in this work.

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