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BRAF and DIS3 Mutations Associate with Adverse Outcome in a Long-term Follow-up of Patients with Multiple Myeloma

Authors

Eileen M. Boyle[1,2,3]*, Cody Ashby[1]*, Ruslana G. Tytarenko[1], Shayu Deshpande[1], Hongwei Wang[4], Yan Wang[1], Adam Rosenthal[4], Jeffrey Sawyer[1], Erming Tian[1], Erin Flynt[2], Antje Hoering[4], Sarah K Johnson[1], Michael W. Rutherford[1], Christopher P Wardell[1], Michael A. Bauer[1], Charles Dumontet[2], Thierry Facon[6], Sharmilan Thanendrarajan[1], Carolina D. Schinke[1], Maurizio Zangari[1], Frits van Rhee[1], Bart Barlogie[6], David Cairns[8], Graham Jackson[9], Anjan Thakurta[4], Faith E Davies[3], Gareth J Morgan[3], and Brian A. Walker[1,10]

*These authors contributed to this work equally

1. Myeloma Center, Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA.
2. INSERM 1052/CNRS 5286 Cancer Research Center of Lyon, Lyon, FRANCE
3. Myeloma Research Program, New-York University, Langone, Perlmutter Cancer Center, New York, NY
4. Cancer Research and Biostatistics, Seattle, WA
5. Celgene Corporation, Summit, NJ
6. Service des maladies du sang. Hôpital Claude Huriez, Lille University Hospital, Lille, France
7. Department of Hematology and Medical Oncology, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY
8. Clinical Trials Research Unit, Leeds Institute of Clinical Trials Research
9. Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK
10. Division of Hematology Oncology, Indiana University, Indianapolis, IN, USA

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Corresponding Author: Brian A Walker

Address: Division of Hematology Oncology, Indiana University, 975 W Walnut Street, Indianapolis, IN, USA

Email: bw75@iu.edu

Telephone: 317-278-7733

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Identifying diagnostic, prognostic, and theragnostic factors is key in the modern management of cancer patients, including myeloma. Here we designed a next-generation sequencing targeted capture over 125 myeloma specific genes and the canonical translocation loci in order to identify the mutations, copy number, and translocation makeup of newly diagnosed myeloma patients and use this to identify independent features associated with outcome. Using this approach, we were able to identify different markers as well as their interaction with one another and both confirm previous findings and identify new changes associated with outcome. The originality of this dataset resides in the extensiveness of features analyzed and the long-term follow up of this trial population. Therefore, the novel markers identified will help add precision to the management of newly diagnosed myeloma patients treated in an intensive setting.

Abstract (250)

Purpose: Copy number changes and translocations have been studied extensively in many datasets with long term follow-up. The impact of mutations remains debated given the short time to follow-up of most datasets.

Methods: we performed targeted panel sequencing covering 125 myeloma-specific genes and the loci involved in translocations in 223 newly diagnosed myeloma samples recruited into one of the Total Therapy Trials (TT).

Results: As expected, the most commonly mutated genes were *NRAS*, *KRAS*, and *BRAF* making up 44% of patients. Double-Hit, *BRAF* and *DIS3* mutations had an impact on outcome alongside classical risk factors in the context of an intensive treatment approach. We were able to identify both V600E and non-V600E *BRAF* mutations, 58% of which were predicted to be hypoactive or kinase dead. Interestingly, 44% of the hypoactive/kinase dead *BRAF* mutated patients showed co-occurring alterations in *KRAS, NRAS* or activating *BRAF* mutations suggesting they play a role in the oncogenesis of multiple myeloma (MM) by facilitating MAPK activation and may lead to chemo resistance.

Conclusion: Overall, these data highlight the importance of mutational screening to better understand newly diagnosed MM (NDMM) and may lead to patient specific mutation-driven treatment approaches.

Introduction

Multiple Myeloma (MM) is a hematological malignancy of plasma cells that afflicts around 30,000 people in the US per year with a five-year survival rate of 47%.[1] High risk MM (HRMM) is seen in up to 30% of newly diagnosed cases whose outcome, in contrast to the majority of MM cases, has seen very little improvement over the past 15 years[2] with a median progression free survival (PFS) of 1.8 years and overall survival (OS) of 2.6 years.[3] There is, therefore, a clear need to identify these patients in order to apply relevant new approaches in their management.

The study of MM has identified many genetic events that are associated with event free survival (EFS) and overall survival (OS). Some of these features occur with a greater frequency in HRMM cases, including translocations into the immunoglobulin (Ig) loci involving chromosomes 4 and 16, which define two etiological subgroups [t(4;14), 15%, and t(14;16), 5%].[4] Other instability mechanisms associated with HRMM include additional structural variations such as del(1p), and del(17p), jumping translocations of 1q and secondary translocations to *MYC* at 8q24.[5–7]

A great deal is known about the genetics of MM with over 800 genomes and 2000 exomes sequenced.[4,6,8–13] However, the prognostic impact of mutations has not been widely evaluated and available datasets have generally had a relatively short follow-up ranging from 22 to 25 months, with one dataset being up to 5.4 years.[4,11] These analyses have identified a diverse range of mutations that are associated with outcome, making it important to extend these observations over time in larger studies with robust diagnostic technologies.

Despite falling prices, it is not feasible to run whole exome sequencing (WES) for every MM patient, and even then, many translocations outside of the capture region would be missed, requiring a combination of fluorescence in situ hybridization (FISH) or gene expression profiling. Since this approach is time and cost prohibitive it has led to the adoption of targeted sequencing to generate data in a timely, cost-effective manner. This led us to design a custom myeloma targeted panel, which provided rapid, fiscally responsible characterization of patient subgroups.

We evaluated this panel on 223 newly diagnosed MM (NDMM) patients included in the Total Therapy (TT) trials and correlated results to both gene expression and clinical data, with the ultimate aim of this work being to effectively characterize NDMM patients and identify the impact of mutations long-term.

Methods

Patients and Samples

A total of 223 previously untreated NDMM patients recruited to the TT trials between February 2004 and August 2017 were included after written informed consent. The sample collection protocol was approved by the UAMS Institutional Review Board (protocol #2012-12). The TT trials are a series of phase II and III, alkylator heavy, double transplant-based clinical trials for first line myeloma treatment that all include both proteasome inhibitors and immunomodulatory drugs (IMiDs). A summary of the treatments received may be found in **Supplemental Figure 1**. Eighty-five of these samples were previously used as a validation of the Double-Hit model.[3] Sample

processing may be found in the **Supplemental Methods.** This study was performed in accordance to the 1964 Helsinki Declaration.

Sequencing

Panel design: Genes and chromosomal regions relevant to the biology, prognosis and treatment of MM were identified. This information was used to design and implement a targeted panel to identify common and important genomic abnormalities in MM. Probes capture exonic regions for the relevant genes (n~125) including +/- 10 base pairs (bp), to include splice site variants, **Supplemental Table 1**. Single nucleotide polymorphisms (SNPs) with a minor allele frequency >0.35 were captured in regions of interest to infer copy number using allelic imbalance combined with read depth ratio. In this way both deletions and gains were confidently assayed. SNPs in GC-rich regions were avoided to prevent hybridization artefacts and low depth problems. To identify Ig translocations the V, D and J segments along with entire constant region were tiled (81.8-90.2 Mb, 17.4-32.6 Mb,106.0-107.3 Mb for *IGK*, *IGL*, and *IGH* respectively.[4,14,15] *MYC* translocations were also detected by tiling 2 Mb upstream and downstream of *MYC (*126.3-130.8 Mb).

Targeted sequencing: The panel was divided into a translocation panel and a mutation/copy number panel to provide high depth coverage for mutation analysis (0.6 Mb), whilst providing lower depth sequencing of translocation regions (4.2 Mb).

Each patient had their tumor DNA from bone marrow and control DNA from peripheral blood sequenced, to identify somatic mutations, copy number changes and translocations. 50 ng of DNA was used to prepare libraries using the HyperPlus kit

(Kapa Biosystems) and split for hybridizing to both mutation and translocation captures (SeqCap EZ target enrichment; Nimblegen), after which mutation and translocation captures were combined. The HiSeq 2500 or NextSeq500 (Illumina, San Diego, CA, USA) were used for sequencing with 75 bp paired-end reads. The median value of the mean coverage of each sample was 135x and 452x for translocations and mutations, respectively.

Data analysis

bcl2fastq was used for demultiplexing and BWA mem (v. 0.7.12) for alignment to Ensembl (GRCh37/hg19) human reference genome. Strelka (v.1.0.14) was used for variant calling and single nucleotide variants (SNVs) were filtered using fpfilter (https://github.com/ckandoth/variant-filter). Indels were filtered using a 10% variant allele frequency (VAF) cut-off. Variants were annotated using Variant Effect Predictor (v.85). To determine copy number, a normalized depth comparison between tumor and control samples was used and segments of SNP variance were utilized to identify regions of chromosomal deletion and gain. Copy number was manually normalized based on the ratio and SNP allele calls using the best fitting chromosomes with the least variance (usually chromosome 2 or 10). Data were visualized using a custom built R-Shiny application. Intra- and inter-chromosomal rearrangements were called using Manta (v0.29.6) with default settings and the exome flag specified. QC metrics estimated the cross-sample contamination of samples using homozygous SNPs in the germline with 95% or higher VAF examined in the tumor sample. A VAF density plot on those SNPs was generated, as well as reporting the minimum, maximum and median of their values in the germline and tumor, **Supplemental Figure 2.**

Validation and comparison datasets

SNVs: SNVs were compared and validated using seven samples (Horizon Diagnostics) with known SNVs and VAF. The VAF of mutations found in the validation samples matched those found on the panel with r^2=0.93, **Supplemental Figure 3**.

FISH: Copy number data generated from the sequencing panel were validated against existing FISH data for del(1p) (1p13 FISH vs. 1p12 (*FAM46C*) seq.), gain (3 copies) /amp (4 copies or more) (1q21), del(13q) (*D13S31* vs. *RB1*), and del(17p) (*TP53*). Plots of comparisons between FISH and sequencing data are shown with specificities and sensitivities of each region at the 20%, 25%, 40%, and 50% FISH cut-off, **Supplemental Figure 4 and Supplemental Table 2**. An additional comparison for *TP53* was made using the prognostic cut-off 55%.[16] All deletions identified by FISH were identified using the targeted panel. Five additional deletions were called using the panel, 3/5 of them having a del(17p) in at least 20% of cells by FISH, **Supplemental Figure 5 and 20.**

Comparison dataset. Gene mutations were compared to the MGP dataset (n=1273)[3] available in the European Genomic Archive under accession numbers EGAS00001001147, EGAS00001000036 and EGAS00001002859, or at dbGAP under accession number phs000748.v5.p4.

Gene expression profiling

Total RNA from plasma cells was used for gene expression profiling (GEP) using U133 Plus 2.0 microarrays (Affymetrix). Raw signals were MAS5 normalized using the

Affymetrix Microarray GCOS1.1 software. GEP70, TC classification and molecular clusters were derived as previously published.[17]

BRAF mutation analysis

The predicted functions of *BRAF* mutations were determined using the Clinical Knowledgebase (CKB) database[18] using the mutations present in the MGP dataset (n=103)[8] and this dataset (n=26).

Data Availability

Sequencing data and expression data have been deposited in the European Genomic Archive under the accession numbers EGAS00001003223 and EGAD00001004117.

Statistical analysis and additional methods may be found in **Supplemental methods**.

Results

Patients characteristics

A total of 223 patients were sequenced and included in the study. Overall, they were representative of a fit-newly diagnosed population. The median follow-up time from diagnosis was 8.14 years (95% CI 7.39-9.02). The median OS was not met at the time of analysis and the 8-year OS was 61% (95% CI 54-69%). The median EFS was 6.16 years (95% CI 5.18-7.75). A summary of patient characteristics may be found in **Table 1.**

The incidence of translocation and copy number were in keeping with previously published data. *MYC* translocations were identified in 26% of cases: they involved the Ig locus in 39% of cases and non-Ig partners in 61% of cases. The breakpoints were

within the previously published hotspots **Supplementary Figure 6A**. *MYC* deletions and gain were seen in 16% and 28% of patients, respectively. An example may be seen in **Supplementary Figure 7**. Overall, *MYC* events were seen in 47% of patients. A summary of the translocations and comparison to the MGP[3] data may be found in **Supplemental Table 11**.

The most commonly mutated genes were *KRAS* (23% of patients), *NRAS* (17% of patients) and *BRAF* (12% of patients), **Figure 1A,** in keeping with previously published datasets. The incidence of mutations was similar to the MGP study, **Supplemental Table 4**.

Exome sequencing identifies an APOBEC-derived mutational signature in approximately 80% of t(14;16) samples.[19] We performed nNMF analysis on our targeted sequencing in order to determine if we can identify an APOBEC signature, which was seen in seven patients (3.2%), five of which had a t(14;16) and one a t(14;20) translocation, **Figure 1C and Supplemental Figure 8.** Both frequency and enrichment for the MAF subgroups were in keeping with previous reports.[19]

Interactions between genomic abnormalities and Double-Hit myeloma

Pearson's correlation identified a significant correlation between *CYLD* mutations and deletions ($r=0.31$, $p=2.07\times10^{-7}$), *TRAF3* mutations and deletions ($r=0.34$, $p=1.5\times10^{-7}$), and *TP53* mutations and deletions ($r=0.32$, $p=1.12\times10^{-6}$), as previously reported.[4,8] *ATM* mutations were positively correlated with the t(14;16) subgroup ($r=0.40$, $p=3.33\times10^{-7}$) and the presence of an APOBEC signature ($r=0.62$, $p=8.66\times10^{-10}$). There was no

significant negative correlation between *DIS3* mutations and hyperdiploidy (HRD) (r=-0.11, p=0.09) in this dataset although they were positively correlated to the presence of t(4;14) (r=0.21, p=0.0005). On the other hand, del(13q) was negatively correlated to HRD (r=-0.26, p=0.0001), **Figure 1B.**

We identified 8.1% patients with Double-Hit (ISS III plus amp(1q) or biallelic inactivation of *TP53*) which is not significantly different to the 6.1% of patients previously described. Double-Hit was associated with both an adverse EFS (median: 24.6 months (95% CI 10.6-42.7) versus and 6.77 years ((95% CI 5.60- ∞), p<0.0001) and OS 37.3 months ((95% CI 32.3-∞) versus 64% at 8 years ((95% CI 57%-73%), p<0.0001). Interestingly, when analyzing the impact of Double-Hit in the TT population, which received two autologous stem cell transplants (ASCTs), the impact of Double-Hit was still significant. This is of particular interest in a double-ASCT population, **Supplemental Figure 9**, identifying a population who still has a dire outcome despite intensive treatment.

Survival analysis identifies that *BRAF* and *DIS3* mutations are associated with an adverse outcome with long-term follow-up.

Univariate analysis

Power estimation was performed, **Supplemental Figure 10**. The results of univariate analyses for EFS and OS for molecular features are shown, **Figure 2**. Overall, this dataset behaved as expected with del(12p), del(17p), gain/amp(1q), and del(1p) being significantly associated with both adverse EFS and OS. Trisomy(9) and trisomy(19) were associated with a better EFS and OS and trisomy(2) and trisomy(5) resulted in a

better EFS. The other trisomies (3, 15 and 21) had no impact on outcome. Sixteen percent of patients were considered as high risk according to the GEP70 score and they had a worse outcome than standard risk patients both in terms of EFS (HR=2.5 ((95% CI 1.6-3.9), p<0.0001) and OS (HR=3.5 ((95% CI 2.1-6), p<0.0001), **Supplemental Figure 11**. The PR subgroup, was also associated with both short EFS and OS whereas the MF and MS subgroup were associated with a short EFS. Based on their ISS, 26.5%, 43.5% and 30% of patients were considered ISS I, ISS II and ISS III respectively with a HR of death of 2.7 ((95% CI 1.2-5.9), p=0.01) and 6.04 ((95% CI 2.8-13) p<0.0001) for ISS II and III respectively in comparison to ISS I. *MYC* translocations, gains and deletions, were associated with a difference in OS in this dataset, but not EFS **Supplemental Figure 6**. In terms of mutations, *BRAF* mutations were associated with an adverse EFS (HR=2 (95% CI 1.2-3.3), p=0.009) and OS (HR=2.7 (95% CI 1.5-4.7), p=0.0007), **Supplemental Figure 16 A-B**. *TP53* and *DIS3* mutations were associated with a worse EFS (HR=2.3 (95% CI 1.3-4.2), p=0.0065 and HR=2 (95% CI 1.2-3.5), p=0.009 respectively) but not OS (HR=1.5 (95% CI 0.67-3.2), p=0.34 and HR=1.2 (95% CI 0.56-2.4), p=0.68) respectively). We went on to test combinations of markers previously published such as DNA repair pathway mutations[4] and bi-allelic *TP53*[3]. As previously shown[4], DNA repair pathway mutations defined by the presence of an *ATM* or *ATR* mutation were associated with an adverse outcome in terms of EFS (HR=2.1 (95% CI 1.1-4.1), p=0.023) and OS (HR=2.2 (95% CI 1.1-4.6, p=0.033)), as was bi-allelic *TP53* (HR=4.3 (95% CI 2.4-7.7), p<0.0001) and OS (HR=2.8 (95% CI 1.4-5.6), p=0.004). There was no significant impact of IGL translocations on outcome, **Supplemental Figure 12.** These data are summarized in **Supplemental Table 5**.

Multivariate Analysis Identifies Mutations of *BRAF* and *DIS3* As Independently Associated With Prognosis

We went on to perform a multivariate analysis using all the genetic features with $p<0.1$.

For EFS, a protective effect was associated with trisomy(19). An adverse association was seen for Double Hit, del(1p)(*FAF1*), t(4;14), del(12p)(*KDM5A*) and mutations of *BRAF* and *DIS3* (Corrected C-index=0.689). Similarly, for OS, trisomy(19) was associated with a positive effect, whereas an adverse association was seen with Double Hit, del(1p)(*FAF1*), del(12p)(*KDM5A*), gain8q24 (*MYC*) and mutations of *BRAF* (Corrected C-index=0.73). With the long follow-up there was consistency between markers in the multivariate analysis of EFS and OS, with the exception of *MYC* gains and mutation of *DIS3*, indicating a high reliability in the dataset. A summary of the multivariate may be found in **Figure 3 and Supplemental Table 6-7.**

We tested the solidity of this analysis by repeating the analysis using classical risk factors and previously published models such as the IFM2009 model[20] and GEP70[17], **Supplementary Figure 13-15**. *DIS3* mutations and *BRAF* mutations retained their prognostic significance, irrespective of other high-risk features.

***DIS3* mutations and biallelic *DIS3* events are associated with poor prognosis in MM**

In our dataset we identified 21 patients (9.4%) with a *DIS3* mutation. The majority of the mutations were missense and were located throughout the gene suggesting they were inactivating, **Figure 4A,** although a hotspot is present at amino acid R780. Mutations in

DIS3 were associated with a worse EFS (HR=2 (1.2-3.4), p=0.01) but not OS. A similar trend was seen in the Myeloma XI dataset and the MGP, **Supplemental Figure 17**. Biallelic events were seen in 11 (5%) patients, mostly consisting of deletions and mutations (91%, n=10/11). There was no case of biallelic deletion. Bialleleic *DIS3* events had a stronger association with EFS (HR for progression of 3.6 (1.8-7.2), p<0.0001) than monoallelic events (HR of 1.2 (0.85-1.8), p=0.27), **Figure 4 B-C.**

***BRAF* non-V600E mutations comprise kinase dead variants which were associated with adverse outcome, and may lead to increase MAPK activation through CRAF via co-occurring *KRAS* and *NRAS* mutations**

BRAF mutations were associated with an adverse outcome in this cohort, **Supplementary Figure 16 A-B**. Forty-six percent (n=12/26) were at the classical V600E hotspot, **Figure 5A**. When comparing the impact of the non-V600E versus the V600E patients, for EFS especially, most of the prognostic impact appeared to driven by non-V600E mutations, (1.3 years (0.58-∞) versus 5.6 years (2.46-∞), p=0.02 for EFS; 3.12 years (1.21-∞) versus 8.62 years (5.73-∞), p=0.08 for OS), **Supplementary Figure 16 C-F**.

From a functional perspective, *BRAF* mutations can be sub-divided into activating or non-activating, based on information from other cancers. Using the CKB database, we were able to dissect the non-V600E mutations into activating (n=5), inactivating (n=8), and unknown (n=1) **Supplementary Table 8.** The outcome of patients with the inactivating mutations was worse (HR=6.4 (2.74-15), p<0.0001) than those who had an activating mutation (HR=2.1 (1.05-4.2), p=0.04), which in turn was worse than those who did not have a *BRAF* mutation, **Figure 5 B-C**. Similar trends were confirmed in the

MGP dataset subset of patients who received an autologous stem cell transplant (OS, p=0.008), **Supplemental Figure 17**.

To explore this further, we expanded this analysis (n=26) using the MGP dataset (n=103). Combined, forty-three percent (56/129) were V600E mutations, 11% (14/129) were predicted to be activating, 8.5% with hypoactive (11/129), 25% kinase dead (32/129) and 12.5% unknown (16/129).

In melanoma inactivating mutations often co-occur with other MAPK alterations such as *NRAS or KRAS* mutations*, NF1* biallelic inactivation or *PTPN11* mutations, and contribute to increased MAPK signaling through enhanced binding and recruitment of CRAF.[21] We hypothesized that the adverse outcome associated with inactivating *BRAF* mutations in myeloma is due to increased MAPK signaling, in which case co-occurring *NRAS* or *KRAS* mutations would need to be present in the same clone.

NRAS, *KRAS*, and *BRAF* mutations are believed to be mutually exclusive[4,8] in MM. In most cases, these three mutations were also mutually exclusive (**Supplemental Figure 18 A-B**), however, inactivating *BRAF* mutants co-occurred more frequently with *NRAS*, *KRAS*, or activating *BRAF* mutations than expected, reaching 44% of patients with inactivating *BRAF* mutations (p=0.0018), **Figure 5E**. To determine if the co-occurring mutations were present in the same clone we calculated the cancer clonal fraction (CCF) of the mutations and, based on the resulting proportions, assessed whether they were in the same clone. We identified that of the 68% (n=13/19) of samples with an inactivating *BRAF* mutation had a co-occurring *NRAS/KRAS* mutation in the same clone and 32% (n=6/19) could not be determined from the data, **Figure 5F and Supplemental Figure 18 C-D.**

Discussion

The incorporation of the complete spectrum of genomic lesions from translocation to mutations including copy number changes is required to gain insight and accurately predict outcome in MM. Using multiple techniques to determine these factors is both labor intensive, time consuming, and yields high failure rates given the amount of tumor cells required.[22] Next-generation sequencing has helped unravel the genetic complexity of MM but cost and time are often setbacks in a clinical setting. Many targeted approaches have been developed, some specific to MM[23,24] and some applicable to MM,[25] but most do not take into account the Ig loci, thus requiring combinations with other tests such as FISH or GEP to identify translocations. Like Bolli et al,[24] our approach offers a complete view of translocations, CNA, and mutations. The set of genes in this capture largely overlaps the Sanger capture but given our wide *MYC* tiling we offer a better understanding of the complex rearrangements that occur on 8q24. Finally, given the size of this capture, we are also able to identify an APOBEC signature, that has also demonstrated prognostic significance in MM.[19]

Here we show that an increased follow-up of patient outcome data combined with targeted sequencing can identify consistent genomic markers associated with inferior outcome. We identified the classical cytogenetic abnormalities, such as t(4;14), del(1p) and del(12p), as affecting outcome, as well as the more recently defined Double-Hit myeloma. In addition, we identify that mutations in *BRAF* and *DIS3* are prognostically implicated in the outcome of myeloma patients in an intensive setting.

In the MGP dataset we have previously shown that Double-Hit myeloma resulted in a worse outcome for PFS and OS, irrespective of the treatment used, but these patients mostly had a single autologous stem cell transplant (ASCT). ASCT is the standard of care for all NDMM aged ≤65 and before the era of novel agents ASCT proved beneficial on OS.[26] Double transplants were subsequently investigated and deemed safe,[27–51] and two randomized trials confirmed the benefit of double versus single transplant in terms of OS.[26] In this dataset, all patients received a double ASCT, but despite this Double-Hit patients still perform badly, although they have a slightly better outcome than was seen in the MGP data (20.7 months (95% CI 17.4-20.6)), with a 16-month improvement in their outcome.

BRAF mutations were seen in 11% of myeloma patients and were associated with a poor outcome. *BRAF* is mutated in numerous cancers and the substitution of a valine (V) for a glutamic acid (E) residue at position 600 in the kinase domain is the most common *BRAF* mutation.[31] This mutation mimics the phosphorylation of the activation loop, thereby inducing constitutive BRAF kinase activation. $BRAF^{V600E}$ mutations are present in 50% of melanoma patients and 2% of non-small cell lung cancer (NSCLC) patients. In NSCLC, they are associated with a shorter OS and resistance to cisplatinum chemotherapy.[32] The clinical significance of $BRAF^{V600E}$ in multiple myeloma has been characterized in two previous studies where seven myeloma patients with $BRAF^{V600E}$ had significantly shorter OS and an increased incidence of extra medullary disease (57% vs. 17%) compared with wild-type *BRAF.*[33] More recently Rustad *et al.*[34] reported a good response to broad acting drugs and no relation to prognosis among eleven $BRAF^{V600E}$ mutant patients.

Fifty-four percent of the *BRAF* mutations seen in this dataset were non-V600E and is a similar rate to that seen in NSCLC[35,36] and other MM datasets.[37] The biology of these non-V600E mutants is heterogeneous, with some leading to high kinase activity (class I and II) and Ras independence while others are hypoactive or kinase dead (class III) variants but nonetheless these still impact on the MAPK pathway through CRAF heterodimerization.[38,39] In our dataset the hypoinactive/kinase dead variants were more likely to co-occur with a *KRAS* or *NRAS* mutation which has also has been previously described by Lionetti et al.[37] In melanoma, biallelic *NF1* inactivation or *PTPN11* activation have also been linked to MAPK activation through inactive *BRAF* mutants,[37,40] but these are rare events in MM and they were not associated with non-BRAF^{V600E} mutants, **Supplemental Figure 19**.

BRAF^{V600E} mutations in melanoma are sensitive to the BRAF inhibitors vemurafenib and dabrafenib. Case reports[41,42] and clinical trials[43] also support the use of vemurafenib in this setting. These drugs are not effective against non-BRAF^{V600E} mutations, but could be targeted using MEK inhibitors.

In other cancers, the presence of concomitant NRAS/KRAS and BRAF kinase dead mutations results in chemoresistance.[35,44] Identifying these non-BRAF^{V600E} mutants would not only help identify patients who should not receive BRAF inhibitors but also patients who would not benefit from intensive alkylator heavy regimens. The adverse outcome of these patients in this dataset could suggest that heavily treating these patients may be deleterious and may suggest they would benefit from alkylator free regimens, thus explaining some of the outcome discrepancies in the literature.[34]

The prognostic impact of chromosome 13 has been long debated. Forty percent of patients have a del(13q) either by a monosomy 13 (35%) or a simple loss of 13q.[45] Del(13q) was found to be associated with a short outcome in many studies, before the associations between t(4;14) and del(13q) were made.[46] *DIS3* is located on chromosome 13 and as such is frequently deleted, as well as being mutated in MM.[4,8] More recently, *DIS3* germline variants have been described in familial cases of plasma cell disorders.[47] Combined, we saw *DIS3* events in 53% of cases and bi-allelic events in 5%. We identified an association with poor outcome and bi-allelically affected *DIS3*, which may suggest that *DIS3* is a tumor suppressor gene. However, the majority of *DIS3* mutations are missense and not nonsense or frameshift mutations, further the mutations are clustered at particular codons which is not typical of a tumor suppressor gene and may suggest an oncogenic potential for DIS3. In this respect, the mutations may cause a change of function, as has recently been suggested in yeast where point mutations are associated with genome instability.[48] Given the role of DIS3 in RNA processing[49] it is possible that complete inactivation of both alleles is lethal, as has been seen for *SF3B1*.[48]

BRAF and *DIS3* mutations have an impact on outcome alongside classical risk markers in the context of the TT trials. We were able to identify both $BRAF^{V600E}$ mutations and non-V600E *BRAF* mutations, 58% of which were predicted to be hypoactive or kinase dead. Interestingly, 44% of the hypoactive/kinase dead *BRAF* patients showed co-occurring mutations in *KRAS* or *NRAS*, suggesting they play a role in the oncogenesis of MM by facilitating MAPK activation by upstream mutated factors through CRAF.

These data highlight the importance of mutational screening to better understand NDMM and may lead to patient specific mutation-driven treatment approaches.

Contribution of authors

Conception and design: EMB, CA, BAW

Collection and assembly of data: EMB, CA, HW, BAW, VVL, MWR, RGT, YW, PHP, JS, SD, MO, EF, SKJ, CPW, MAB, CD, KRR, TF, ST, CDK, MZ, FVR, BB, AT, FED, GJM

Data analysis and interpretation: EMB, CA, HW, AR, AH, GJM, BAW

Manuscript writing: EMB, CA, BAW

Manuscript reviewing: all authors

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Figure legends

Figure 1: General features of the cohort. A. The proportion of patients with each mutation. B. Correlation plot representing the different significant interactions. C. Dendrogram of the nNMF identifying an APOBEC signature among the 223 TT baseline samples using the targeted panel.

Figure 2: Summary of the univariate analysis. A. EFS. B. OS. Forest plots representing the result of the univariate analysis. In red, those that were significantly associated with outcome at the level of $p<0.05$, in blue the non-significant variables.

Figure 3: Multivariate analysis. Forest plots representing the results of the multivariate analysis for A. EFS and B. OS.

Figure 4: *DIS3* mutations. A. Distribution of *DIS3* mutations throughout the gene. B. *DIS3* mutations are associated with an adverse EFS. C. Biallelic *DIS3* inactivation is associated with a worse outcome than monoallelic inactivation.

Figure 5: Inactivating *BRAF* mutations affect outcome and co-occur with *NRAS* or *KRAS* mutations. A. Stick plot representing the locations of the different *BRAF* mutations in the MGP dataset (above) and this dataset (below). Differential impact of *BRAF* mutations depending on predicted function on EFS (B) and OS (C). D. The spectrum of *BRAF* mutations with co-occurring mutations. E. The proportion of cases with co-occurring MAPK (*NRAS/KRAS*/activating *BRAF* mutations) depending on their predicted *BRAF* function. F. Cancer clonal fraction of MAPK mutations to determine clonality.

Tables

Table 1: Summary of patient's characteristics and comparability to the complete TT trial population.

	223-baseline study	**Combined TT3a-3b-4-4like-5a-5b-6**
Number of patients	223	1039
Inclusion dates	02/2004 to 08/2017	02/2004 to 08/2017
Median Follow Up	8.14 years (95% CI 7.39-9.02)	8.35 years (95% CI 8.00-8.63)
Median EFS	6.16 years (95% CI 5.18-7.75)	4.8 years (95% CI 52%-58%)
8- year OS	61% (95% CI 54-69%)	42% (95% CI 39%-45%)
Median age (years)	59 (range: 30-75)	61 (range: 30-76)
Sex ratio M:F	1.8:1	1.6:1
Ethnicity % - **White** - **African-American** - **Other**	 88% (n=197) 10% (n=22) 2% (n=4)	 86.8% (n=902) 9.7% (n=101) 3.5% (n=36)
ISS % - **I** - **II** - **III**	 26.5% (n=59) 43.5% (n=97) 30.0% (n=67)	 34.0% (n=352) 40.2% (n=416) 25.8% (n=267)
R-ISS % - **I** - **II** - **III**	 17.0% (n=38) 67.7% (n=151) 15.2% (n=34)	
GEP70 high risk %	16.1% (n=36)	15.9% (n=165)

Figure 1.

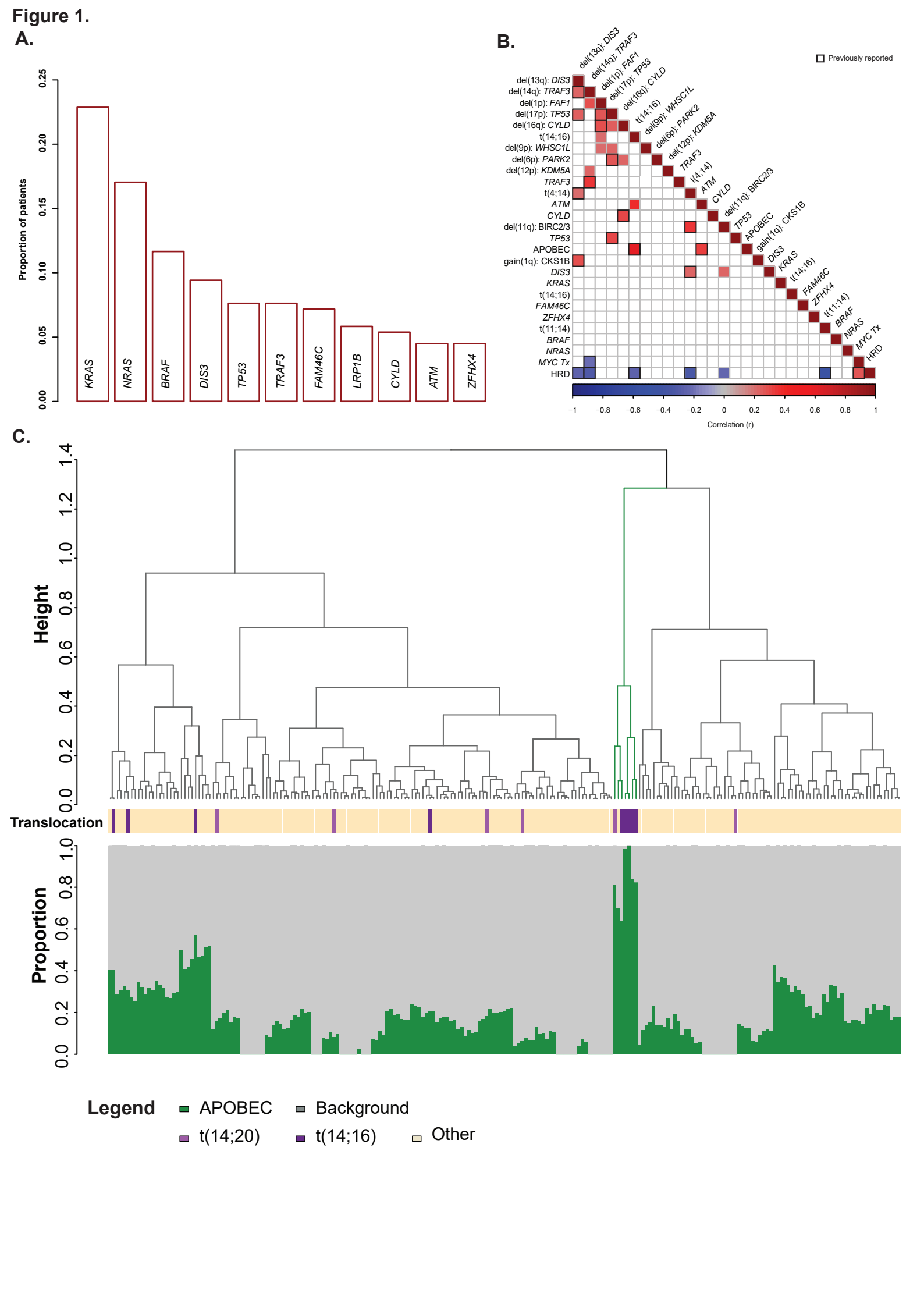
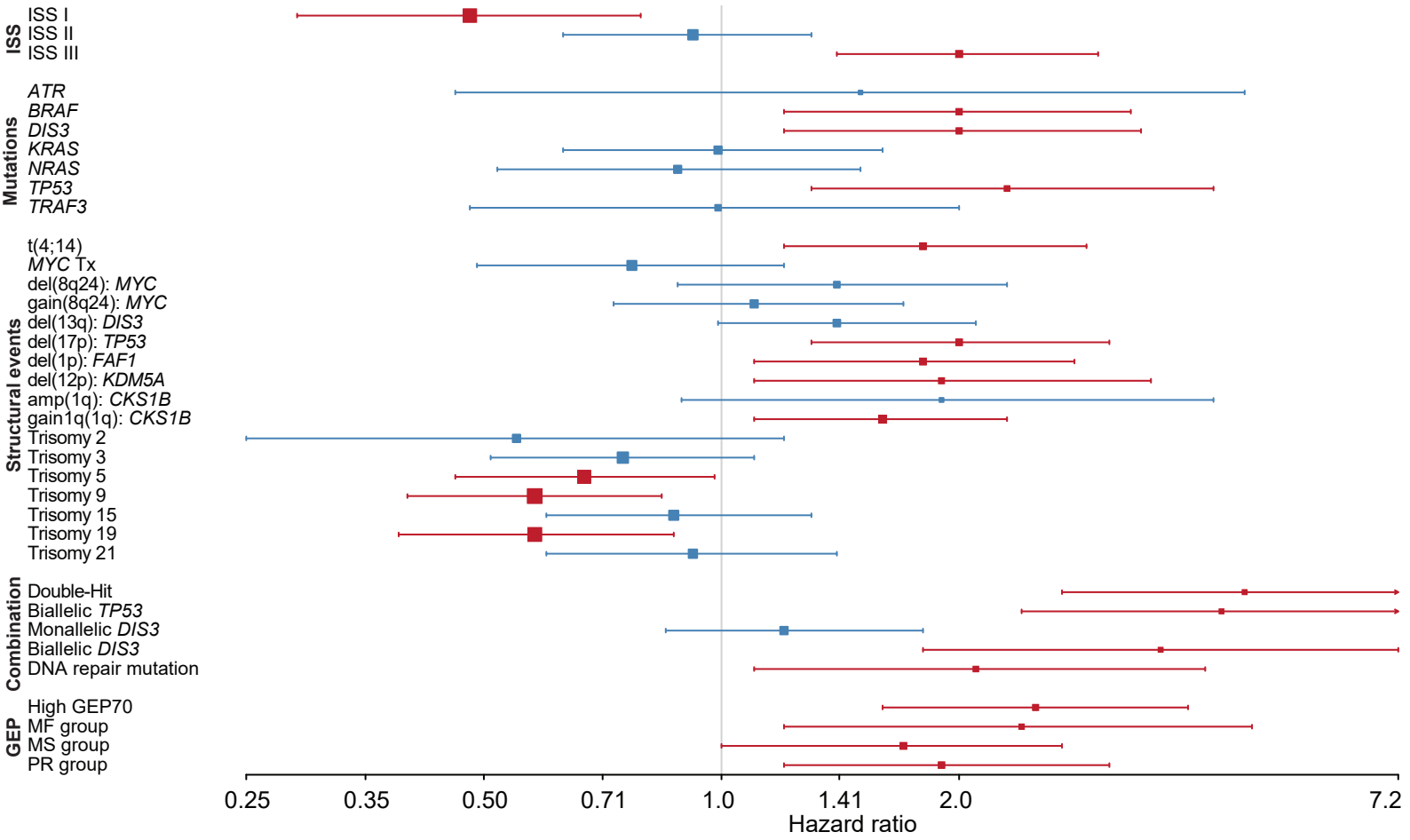

A.
Proportion of patients
0.00
0.05
0.10
0.15
0.20
0.25
KRAS
NRAS
BRAF
DIS3
TP53
TRAF3
FAM46C
LRP1B
CYLD
ATM
ZFHX4
B.
Previously reported
del(13q): DIS3
del(14q): TRAF3
del(1p): FAF1
del(17p): TP53
del(16q): CYLD
t(14;16)
del(9p): WHSC1L
del(6p): PARK2
del(12p): KDM5A
TRAF3
t(4;14)
ATM
CYLD
del(11q): BIRC2/3
TP53
APOBEC
gain(1q): CKS1B
DIS3
KRAS
t(14;16)
FAM46C
ZFHX4
t(11;14)
BRAF
NRAS
MYC Tx
HRD
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1
Correlation (r)
C.
Height
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Translocation
Proportion
0.0
0.2
0.4
0.6
0.8
1.0
Legend
APOBEC
Background
t(14;20)
t(14;16)
Other

Figure 2.

A.


ISS
ISS I
ISS II
ISS III
Mutations
ATR
BRAF
DIS3
KRAS
NRAS
TP53
TRAF3
Structural events
t(4;14)
MYC Tx
del(8q24): MYC
gain(8q24): MYC
del(13q): DIS3
del(17p): TP53
del(1p): FAF1
del(12p): KDM5A
amp(1q): CKS1B
gain1q(1q): CKS1B
Trisomy 2
Trisomy 3
Trisomy 5
Trisomy 9
Trisomy 15
Trisomy 19
Trisomy 21
Combination
Double-Hit
Biallelic TP53
Monallelic DIS3
Biallelic DIS3
DNA repair mutation
GEP
High GEP70
MF group
MS group
PR group
0.25
0.35
0.50
0.71
1.0
1.41
2.0
7.2
Hazard ratio

B.

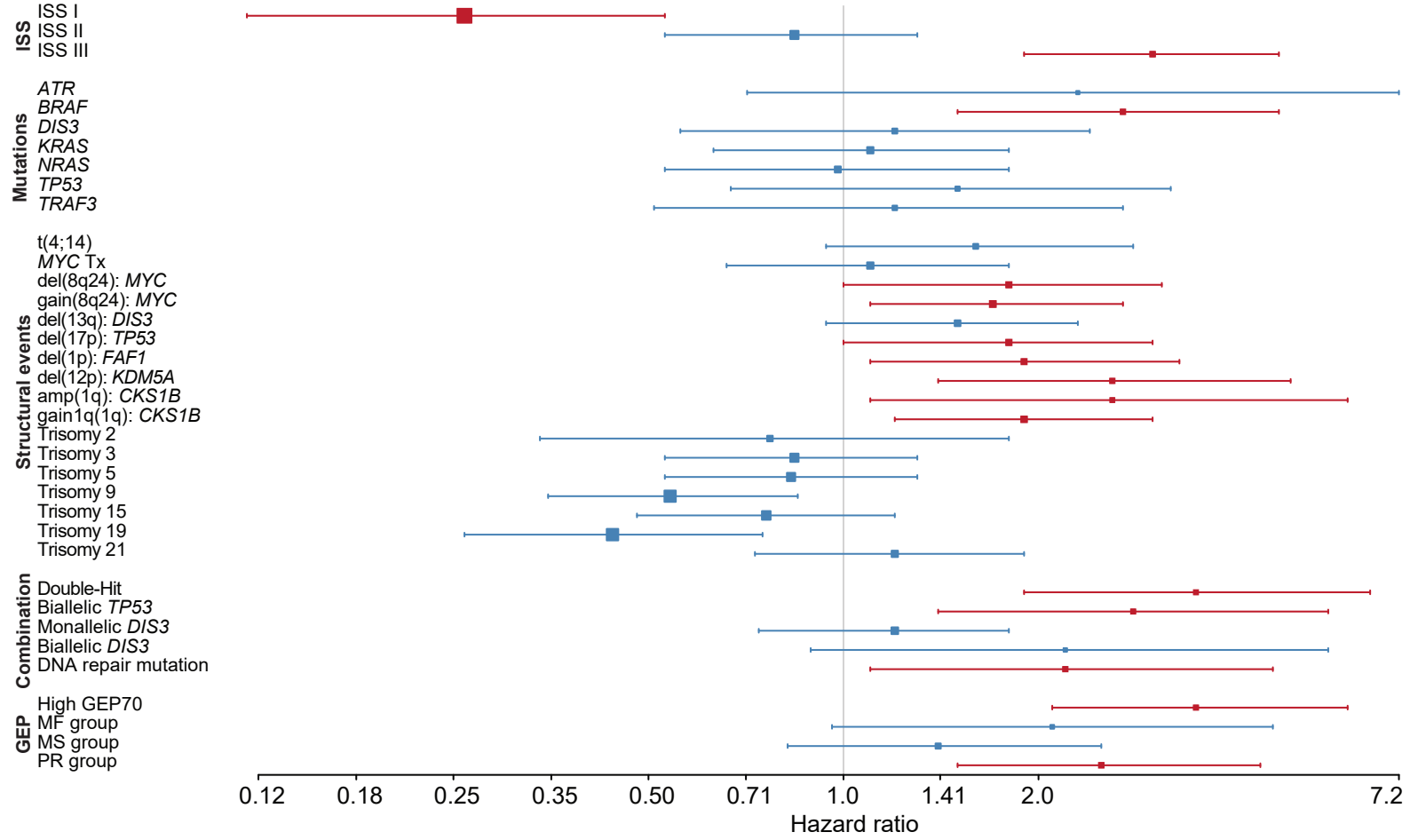
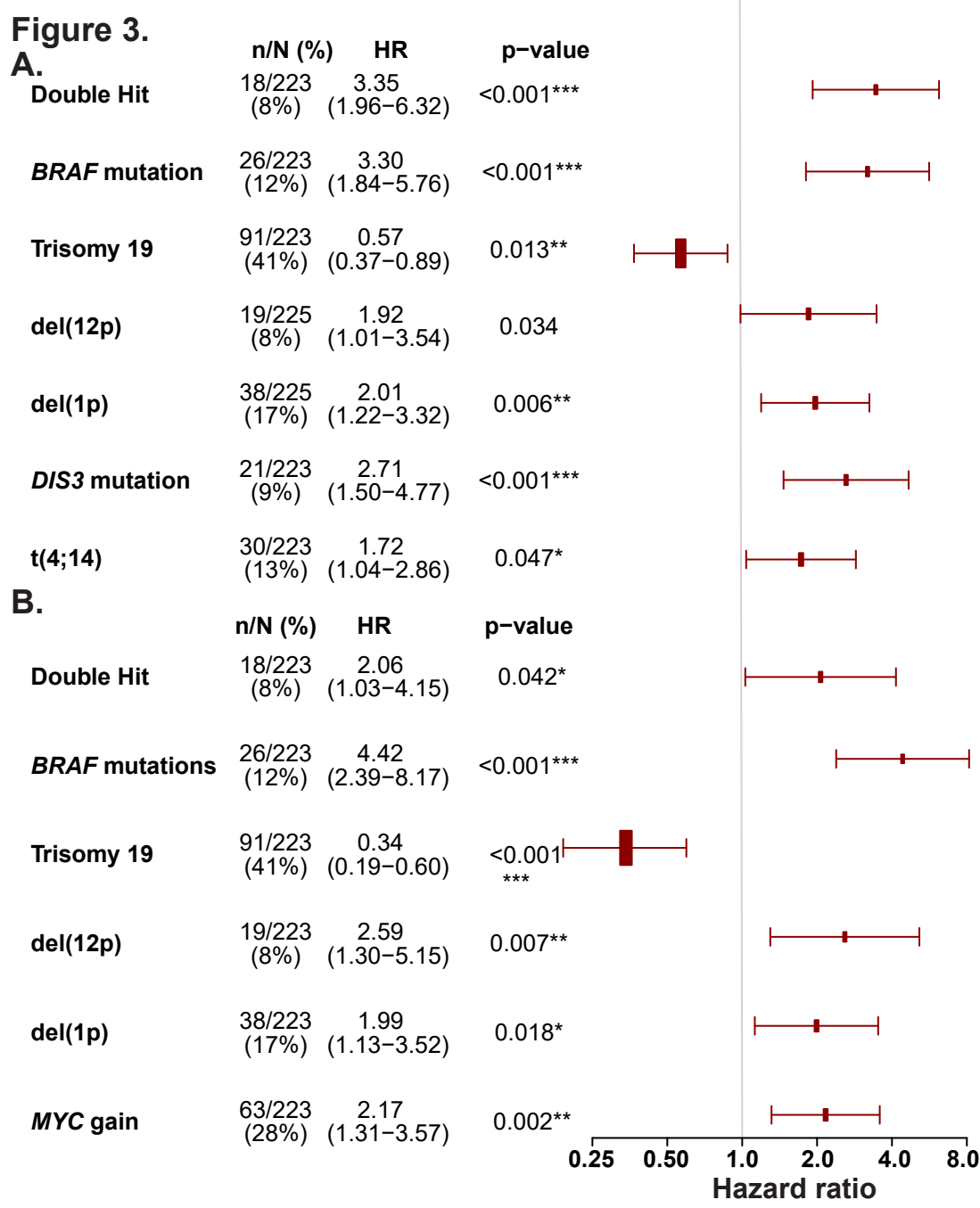
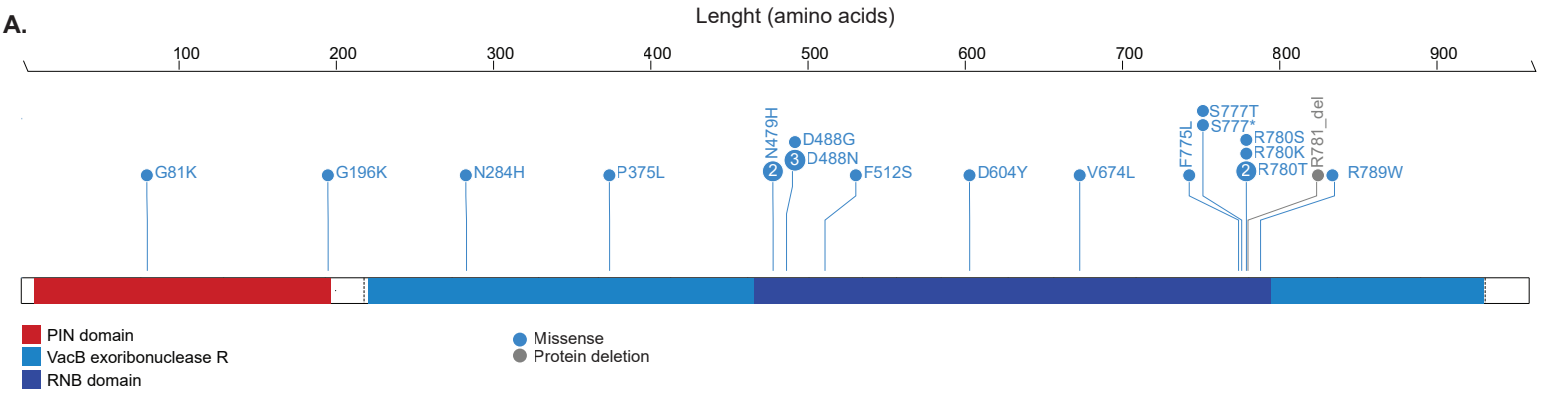
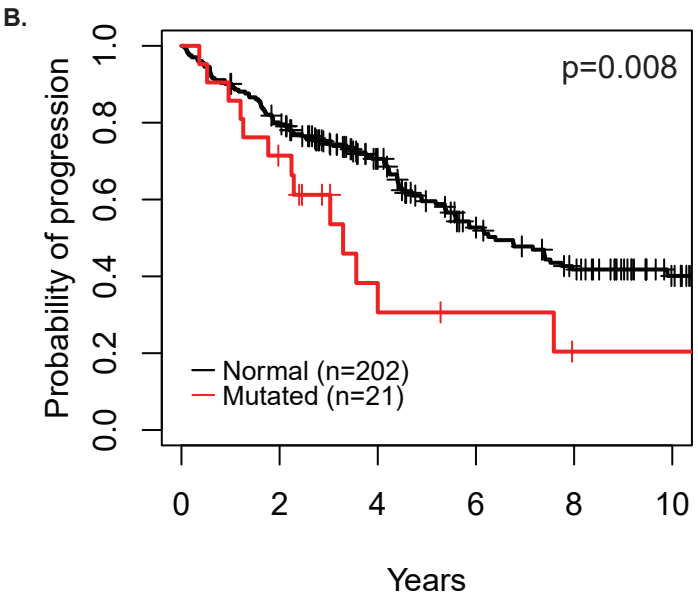

ISS
ISS I
ISS II
ISS III
Mutations
ATR
BRAF
DIS3
KRAS
NRAS
TP53
TRAF3
Structural events
t(4;14)
MYC Tx
del(8q24): MYC
gain(8q24): MYC
del(13q): DIS3
del(17p): TP53
del(1p): FAF1
del(12p): KDM5A
amp(1q): CKS1B
gain1q(1q): CKS1B
Trisomy 2
Trisomy 3
Trisomy 5
Trisomy 9
Trisomy 15
Trisomy 19
Trisomy 21
Combination
Double-Hit
Biallelic TP53
Monallelic DIS3
Biallelic DIS3
DNA repair mutation
GEP
High GEP70
MF group
MS group
PR group
0.12
0.18
0.25
0.35
0.50
0.71
1.0
1.41
2.0
7.2
Hazard ratio

Figure 3.


A.
n/N (%)
HR
p−value
Double Hit
18/223 (8%)
3.35 (1.96−6.32)
<0.001***
BRAF mutation
26/223 (12%)
3.30 (1.84−5.76)
<0.001***
Trisomy 19
91/223 (41%)
0.57 (0.37−0.89)
0.013**
del(12p)
19/225 (8%)
1.92 (1.01−3.54)
0.034
del(1p)
38/225 (17%)
2.01 (1.22−3.32)
0.006**
DIS3 mutation
21/223 (9%)
2.71 (1.50−4.77)
<0.001***
t(4;14)
30/223 (13%)
1.72 (1.04−2.86)
0.047*
B.
n/N (%)
HR
p−value
Double Hit
18/223 (8%)
2.06 (1.03−4.15)
0.042*
BRAF mutations
26/223 (12%)
4.42 (2.39−8.17)
<0.001***
Trisomy 19
91/223 (41%)
0.34 (0.19−0.60)
<0.001***
del(12p)
19/223 (8%)
2.59 (1.30−5.15)
0.007**
del(1p)
38/223 (17%)
1.99 (1.13−3.52)
0.018*
MYC gain
63/223 (28%)
2.17 (1.31−3.57)
0.002**
0.25
0.50
1.0
2.0
4.0
8.0
Hazard ratio


A.
Lenght (amino acids)
100
200
300
400
500
600
700
800
900
G81K
G196K
N284H
P375L
N479H
D488G
D488N
F512S
D604Y
V674L
F775L
S777T
S777*
R780S
R780K
R780T
R781_del
R789W
PIN domain
VacB exoribonuclease R
RNB domain
Missense
Protein deletion


B.
Probability of progression
0.0
0.2
0.4
0.6
0.8
1.0
p=0.008
Normal (n=202)
Mutated (n=21)
0
2
4
6
8
10
Years

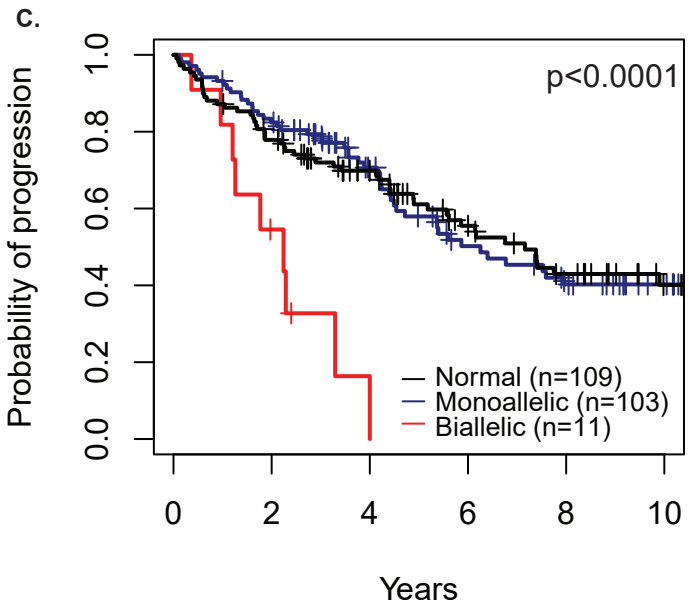
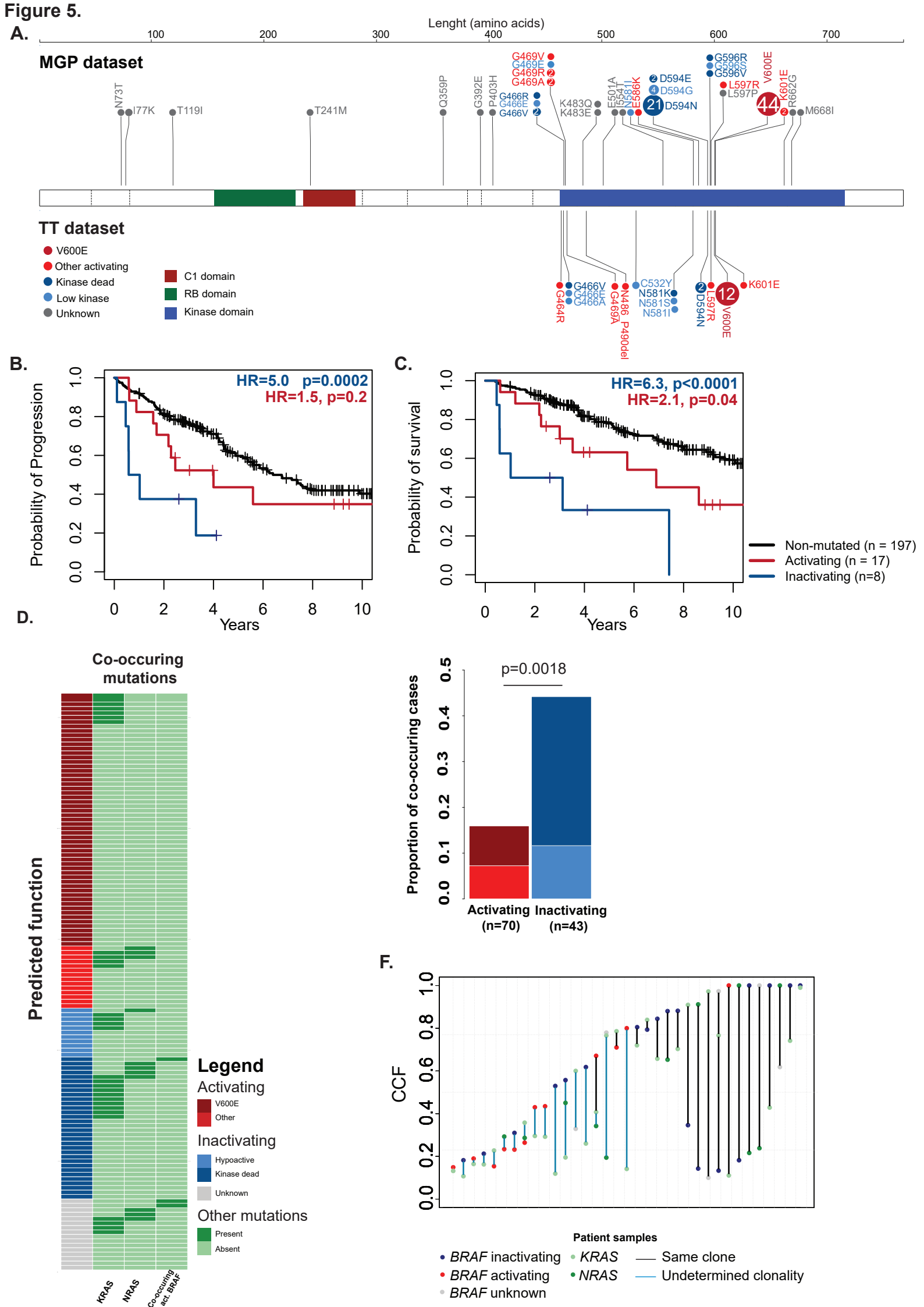

C.
Probability of progression
0.0
0.2
0.4
0.6
0.8
1.0
p<0.0001
Normal (n=109)
Monoallelic (n=103)
Biallelic (n=11)
0
2
4
6
8
10
Years

Figure 5.


A.
Lenght (amino acids)
100
200
300
400
500
600
700
MGP dataset
N73T
I77K
T119I
T241M
Q359P
G392E
P403H
G469V
G469E
G469R
G469A
G466R
G466E
G466V
K483Q
K483E
E501A
E541I
N581I
E586K
D594E
D594G
D594N
G596R
G596S
G596V
L597R
L597P
V600E
K601E
R662G
M668I
TT dataset
V600E
Other activating
Kinase dead
Low kinase
Unknown
C1 domain
RB domain
Kinase domain
G464R
G466V
G466E
G466A
G469A
N486_P490del
C532Y
N581K
N581S
N581I
D594N
L597R
V600E
K601E
B.
Probability of Progression
HR=5.0 p=0.0002
HR=1.5, p=0.2
Years
C.
Probability of survival
HR=6.3, p<0.0001
HR=2.1, p=0.04
Years
Non-mutated (n = 197)
Activating (n = 17)
Inactivating (n=8)
D.
Co-occuring mutations
Predicted function
KRAS
NRAS
Co-occuring act. BRAF
Legend
Activating
V600E
Other
Inactivating
Hypoactive
Kinase dead
Unknown
Other mutations
Present
Absent
p=0.0018
Proportion of co-occuring cases
Activating (n=70)
Inactivating (n=43)
F.
CCF
Patient samples
BRAF inactivating
BRAF activating
BRAF unknown
KRAS
NRAS
Same clone
Undetermined clonality