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Sub-clonal *TP53* copy number is associated with prognosis in multiple myeloma

Vallari Shah[1], David C. Johnson[1], Amy L Sherborne[1], Sidra Ellis[1], Aldridge FM[2], Howard-Reeves J[2], Farzana Begum[1], Amy Price[1], Jack Kendall[1], Laura Chiecchio[3], Suvi Savola[4], Matthew W Jenner[5], Mark T Drayson[6], Roger G. Owen[7], Walter M Gregory[8], Gareth J. Morgan[9], Faith E Davies[9], Richard S Houlston[1], Gordon Cook[10], David A Cairns[8], Graham Jackson[11] and Martin F Kaiser[1] on behalf of NCRI Haematology CSG

[1]Division of Molecular Pathology, Institute of Cancer Research, London, United Kingdom.

[2]Centre for Molecular Pathology, The Royal Marsden Hospital, London, United Kingdom

[3]Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, United Kingdom

[4]MRC-Holland, Amsterdam, Netherlands.

[5]Department of Haematology, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom.

[6]Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom,

[7]Haematological Malignancy Diagnostic Service, St. James's University Hospital, Leeds, United Kingdom.

[8]Clinical Trials Research Unit, Leeds Institute of Clinical Trials Research, Leeds, United Kingdom.

[9]Myeloma Institute, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA.

[10]Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, United Kingdom.

[11]Department of Haematology, University of Newcastle, Newcastle Upon Tyne, United Kingdom.

Correspondence to: Martin Kaiser; Tel: +44(0) 208 722 4166, e-mail: Martin.Kaiser@icr.ac.uk, Postal Address: The Institute of Cancer Research, 15 Cotswold Road, Sutton, London, SM2 5NG, UK.

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Running title: Sub-clonal *TP53* deletion and prognosis in Myeloma

KEY POINTS

1. *TP53* deletion of minor tumour sub-clones is independently prognostic in newly diagnosed multiple myeloma.

2. Assessment of sub-clonal *TP53* deletions by MLPA is readily applicable in standard diagnostics, enabling stratified patient management.

ABSTRACT

Multiple myeloma (MM) is a genetically heterogeneous cancer of bone marrow plasma cells with variable outcome. To assess the prognostic relevance of clonal heterogeneity of *TP53* copy number, we profiled tumours from 1,777 newly diagnosed Myeloma XI trial patients with multiplex ligation-dependent probe amplification (MLPA). Sub-clonal *TP53* deletions were independently associated with shorter overall survival with a hazard ratio of 1.8 (95% CI: 1.2-2.8; *P*=0.01). Clonal, but not sub-clonal, *TP53* deletion were associated with clinical markers of advanced disease, specifically lower platelet counts (*P*<0.001) and increased LDH (P<0.001), and higher frequency of features indicative of genomic instability del(13q)(*P*=0.002) or del(1p)(*P*=0.006). Bi-allelic *TP53* loss-of-function by mutation and deletion was rare (2.4%) and associated with advanced disease. We present a framework for identifying sub-clonal *TP53* deletions by MLPA, to improve patient stratification in MM and tailor therapy, enabling management strategies.

INTRODUCTION

Despite recent improvements in survival, patient outcomes remain variable in multiple myeloma (MM). It is increasingly recognised that tumour heterogeneity is a determinant of patient outcome for many cancers and the identification of sub-clonal driver events is central to better patient stratification[1,2]. Aberrations of *TP53* are recognised to be one of most important markers of poor prognosis in MM[3]. These are secondary driver events with variable sub-clonal distribution with *TP53* typically being deleted and point mutations being relatively rare[4]. Defining the prognostic association of sub-clonal deletion of *TP53* in MM at diagnosis and a cut-off for diagnostic purposes has however been problematic due to the technical challenges of using interphase fluorescence *in situ* hybridisation (iFISH) in MM to quantify sub-clonal populations[5]. To assess the prognostic relevance of sub-clonal *TP53* deletion at diagnosis, we profiled 1,777 MM trial patients using multiplex ligation dependent probe amplification (MLPA), which is readily applicable in diagnostic settings.

METHODS

Myeloma IX and XI trial patients

We studied 1,777 patients with MM enrolled in the UK NCRI Myeloma XI trial and a subset from MRC Myeloma IX and Myeloma XI underwent comparison of MLPA and iFISH **(Supplementary Methods)**.

Copy number, translocation calling and mutation detection

Bone marrow aspirates were processed as detailed in **Supplementary Methods**. Details about iFISH profiling of Myeloma IX and Myeloma XI have been published previously and described in **Supplementary Methods**[6]. Myeloma XI cases were profiled for copy number by MLPA and translocations determined by quantitative PCR as previously reported[7].

The MLPA P425-probemix (MRC-Holland) interrogates *TP53* exons 4, 7 and 10. *TP53* was considered deleted when normalised copy number values of two of three MLPA probes were below the defined cut-off. 1,357 patient tumours were further analysed with probemix X073, covering all exons of *TP53*. Previously published exome-sequencing was available for 463 patients[4].

Statistical analysis

Statistical analyses were performed in R (version 3.4.1) using sub-routines survival, survC1 and survivalROC. Progression-free survival (PFS) was defined as time from randomisation to progression or death and overall survival (OS) as time from randomization to death. To define the optimal prognostic normalised MLPA cut-off value for *TP53* deletion calling, we analysed sub-groups defined by descending (0.05 steps from 1.0 (equivalent to normal diploid copy number)) normalised MLPA value using time-dependent Receiver Operater Curve AUCi estimates for OS for each cut-off[8].

Cox proportional hazards regression was used to estimate univariate and multivariable hazard ratios (HRs) and 95% confidence intervals (CI). Kaplan–Meier survival curves were generated and homogeneity between groups was assessed using the log-rank test. Association between categorical variables was examined using the Fishers exact test and between continuous variables using the Wilcoxon signed-rank test. A two-sided *P*-value <0.05 was considered significant.

RESULTS AND DISCUSSION

To identify the clinically relevant threshold for sub-clonal *TP53* deletions we interrogated step-wise increasing fractions of *TP53* deletion by MLPA using the time-dependent ROC curve analysis method (AUCi) for OS[9]. We identified a normalised *TP53* MLPA value of <0.8 as the cut-off providing optimal prognostic power, identifying 192 of 1,777 (10.8%) tumours as *TP53* deleted **(Supplementary Figure 1)**. These results were consistent in intensively (transplant eligible) and non-intensively treated patients (non-transplant eligible) **(Supplementary Table 1)**. The optimised <0.8 MLPA cut-off is equivalent to 10-20% sub-clonal 17p deletion, lower MLPA levels <0.6 were equivalent to clonal deletions with ≥50% tumour fraction and MLPA values <0.5 were equivalent to fully clonal (95-100%) del(17p) when compared to iFISH in a matched dataset from the Myeloma IX[6] and Myeloma XI trial (**Supplementary Figure 2b**) . The distribution of MLPA normalised values for *TP53* probes across 1,777 Myeloma XI tumours is shown in **Supplementary Figure 2.** Inclusion of sub-clonal deletions by MLPA <0.8 cut-off was confirmed as prognostically most informative by univariate Kaplan-Meier log-rank testing (P=6.7x10^{-15}), Cox regression (Wald P=4.1x10^{-14}) and C-statistics by Uno et al[10] **(Supplementary Table 2)**. A limitation of the study is the lack of a validation trial dataset.

Treatment allocation, key demographics and induction response were comparable between patients with *TP53*-deleted and non-deleted tumours as defined by MLPA <0.8. Patients with *TP53* deletion, however showed features of advanced disease and associated morbidity, specifically reduced platelet counts <150x10^{9}/l (P=5.1x10^{-4}) and poorer performance status (WHO ≥2) (P=0.0012) **(Supplementary Table 3).** Although WHO was independently associated with shorter survival, the association with WHO and *TP53* deletion suggests an inter-relationship with genetic and clinical features that are normally thought of as patient related rather than disease related.

To characterise features of sub-clonal versus clonal deletion, *TP53* deleted tumours were grouped into 3 equal-sized sub-groups based on MLPA values: sub-clonally deleted (n=67; MLPA cut-off ≥0.7<0.8), intermediate clonal (n=64; MLPA ≥0.55<0.7) and clonally *TP53* deleted tumours (n=61; MLPA <0.55). All three groups were independently associated with OS with sub-clonally deleted HR of 1.8 (95% CI: 1.2-2.8; P=0.01) for OS, intermediate deleted HR 2.9 (95% CI: 1.9-4.4; P=5.6x10^{-7}) and clonally deleted HR 2.2 (CI: 1.4-3.2; P=0.0002) **(Figure 1: a and b; Supplementary Table 4)**. Landmarked analyses from autologous stem cell transplant and

lenalidomide maintenance randomisation show consistent results for all three groups **(Figure 1: c and d; Supplementary Table 4)**.

Correlating clinical characteristics, patients with clonal rather than sub-clonal *TP53* deletion were associated with markers of high disease burden, specifically reduced platelet <150x10^9/l (35% vs 9%; *P*=0.00047) and high LDH level >300U/l (57% vs. 32%; *P*=0.012) **(Figure 2b).** Clonal vs. subclonal deletion of *TP53* was associated with higher rates of del(13q) (68% vs. 40%; *P*=0.002) and/or del(1p) (21% vs. 4%; *P*=0.006) **(Figure 2a).** The rate of *TP53* mutations were increased in clonal (3/18) vs sub-clonal deletions (1/21). Although MLPA cannot comprehensively assess clonal architecture, an association between *TP53* deletion clonality with increasing size of del(13q) clone (*P*=0.002) **(Figure 2 c, d; Supplementary Figure 5)** raise the possibility of co-evolution of these lesions. Deletion of *TP53* and *RB1* on chromosome 13q have been shown to be important in cell cycle[11] and senescence[12] suggesting possible mechanisms of how their co-deletion may confer a competitive advantage.

Clonal homozygous *TP53* deletions defined by MLPA values <0.25 were present in nine of 1,777 tumours (0.5%) analysed with the P425 MLPA probemix for exons 4, 7 and 10 of *TP53*. To identify patterns of focal homozygous deletions, all eleven *TP53* exons were analysed using a specifically designed X073 MLPA probe-mix in 1,357 patients. Homozygous deletion frequency was low (0.6%) and deletions were focal and not restricted to the DNA binding domain of *TP53*. Homozygous *TP53* deletion was associated with very short median OS of 22.4 months and a HR for OS of 3.7 (95% CI: 1.5-8.9; *P*=0.004) **(Supplementary Figure 3; Supplementary Table 5)**. Most patients with homozygous *TP53* deletions had markers of clinically and molecularly advanced disease with elevated LDH >300U/l in 67%, reduced platelet counts <150x10^9/l (67%) and del(13q) in 88% of cases. Exome-sequencing data was available for 422 of the MLPA profiled tumours. Of these, ten tumours (2.4%) had bi-allelic (mutation+deletion) *TP53* loss-of-function and 47 tumours (11.1%) mono-allelic loss. Bi- and mono-allelic *TP53* loss were independently associated with inferior survival **(Supplementary Figure 3, Supplementary Table 5)**.

In summary, we demonstrate independent association of sub-clonal *TP53* deletions with MM outcome. Sub-clonal *TP53* deletion detection by MLPA is readily applicable within diagnostic settings and could enable stratified treatment approaches aiming at preventing subsequent rapid disease evolution.

ACKNOWLEDGEMENTS

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AUTHORSHIP CONTRIBUTION

Conception and design: VS, DCJ, RSH, MFK
Acquisition of data: All authors
Analysis of data: VS, DAC, RSH, MFK
Manuscript writing: VS, RSH, MFK

DISCLOSURE OF CONFLICTS OF INTEREST

VS: Sanofi – travel support; Janssen – travel support. SS: MRC Holland - employment; MWJ: Janssen – consultancy, honoraria, travel support, research funding; Takeda – consultancy, honoraria, travel support; Amgen – consultancy, honoraria, travel support; Celgene Corporation – consultancy, honoraria, research funding; Novartis – consultancy, honoraria. MTD: Abingdon Health – equity ownership, membership on board of directors. RGO Takeda – honoraria, travel support; Janssen – consultancy, travel support; Celgene Corporation – consultancy, honoraria, research funding. GJM: Janssen – research funding; Bristol-Myers Squibb – consultancy, honoraria; Takeda – consultancy, honoraria; Celgene Corporation – consultancy, honoraria, research funding. FED Amgen – consultancy, honoraria; AbbVie – consultancy, honoraria; Takeda – consultancy, honoraria; Janssen – consultancy, honoraria; Celgene Corporation – consultancy, honoraria. GC: Takeda – consultancy, honoraria, research funding, speakers bureau; Glycomimetics – consultancy, honoraria; Sanofi – consultancy, honoraria, speakers bureau; Celgene Corporation – consultancy, honoraria, research funding, speakers bureau; Janssen – consultancy, honoraria, research funding, speakers bureau; Bristol-Myers Squibb – consultancy, honoraria; Amgen – consultancy, honoraria, research funding, speakers bureau. DAC: Celgene Corporation, Amgen, Merck Sharp and Dohme – research funding. GJ Roche – consultancy, honoraria, speakers bureau; Amgen – consultancy, honoraria, speakers bureau; Janssen – consultancy, honoraria, speakers

bureau; Merck Sharp and Dohme – consultancy, honoraria, speakers bureau; Celgene Corporation – consultancy, honoraria, travel support, research funding, speakers bureau; Takeda – consultancy, honoraria, travel support, research funding, speakers bureau. MFK Bristol-Myers Squibb – consultancy, travel support; Chugai – consultancy; Janssen – consultancy, honoraria; Amgen – consultancy, honoraria; Takeda – consultancy, travel support; Celgene Corporation – consultancy, honoraria, research funding. The remaining authors declare no conflicts of interest.

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Figure Legends

Figure 1: Association between sub-clonal and clonal *TP53* deletion and survival in newly diagnosed myeloma. Kaplan Meier survival curves showing **(a)** PFS and **(b)** OS of 3 approximately equal sized *TP53* deleted clonal subgroups versus no *TP53* deletion in 1777 patients in the Myeloma XI trial **(c)** OS evaluation of above subgroups in landmarked analysis from time of high-dose melphalan and autologous stem cell transplant **(d)** OS evaluation of above subgroups in landmarked analysis from time of maintenance randomisation.

Figure 2: Relationship between sub-clonal and clonal *TP53* deletion with clinical and genetic characteristics of myeloma. Percentage frequency of **(a)** genetic changes associated with low, intermediate and high deletion of *TP53* clone **(b)** clinical changes associated with low, intermediate and high deletion of *TP53* clone **(c)** MLPA values normalised values for 13q probes in the same patients with low, intermediate and high deletion of *TP53* clone **(d)** MLPA values across subset of patients with del(*TP53*) in patients with increasing size of del(13q) clone. Lower MLPA values represent increasing size of deleted clone.

Figure 1

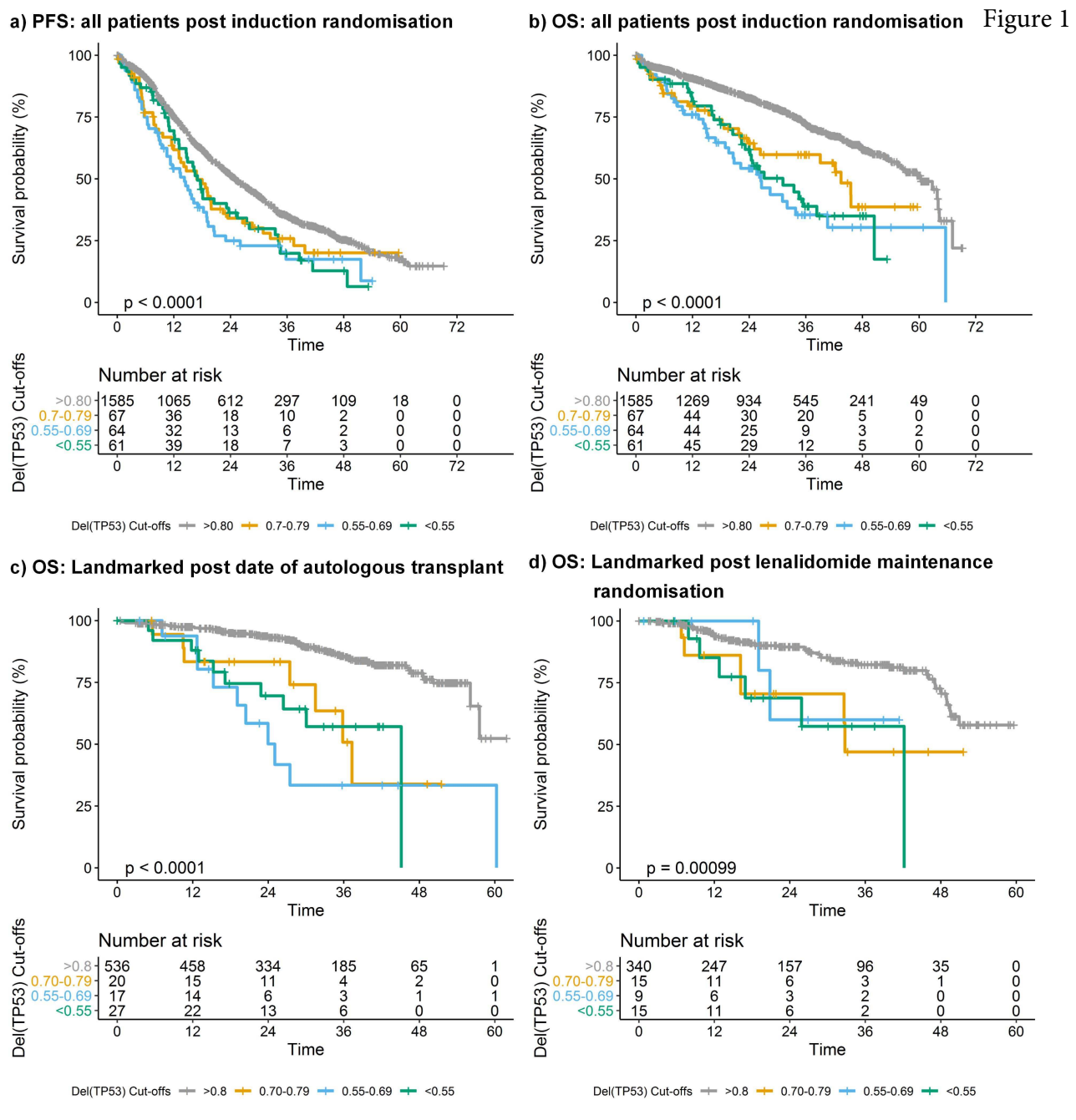
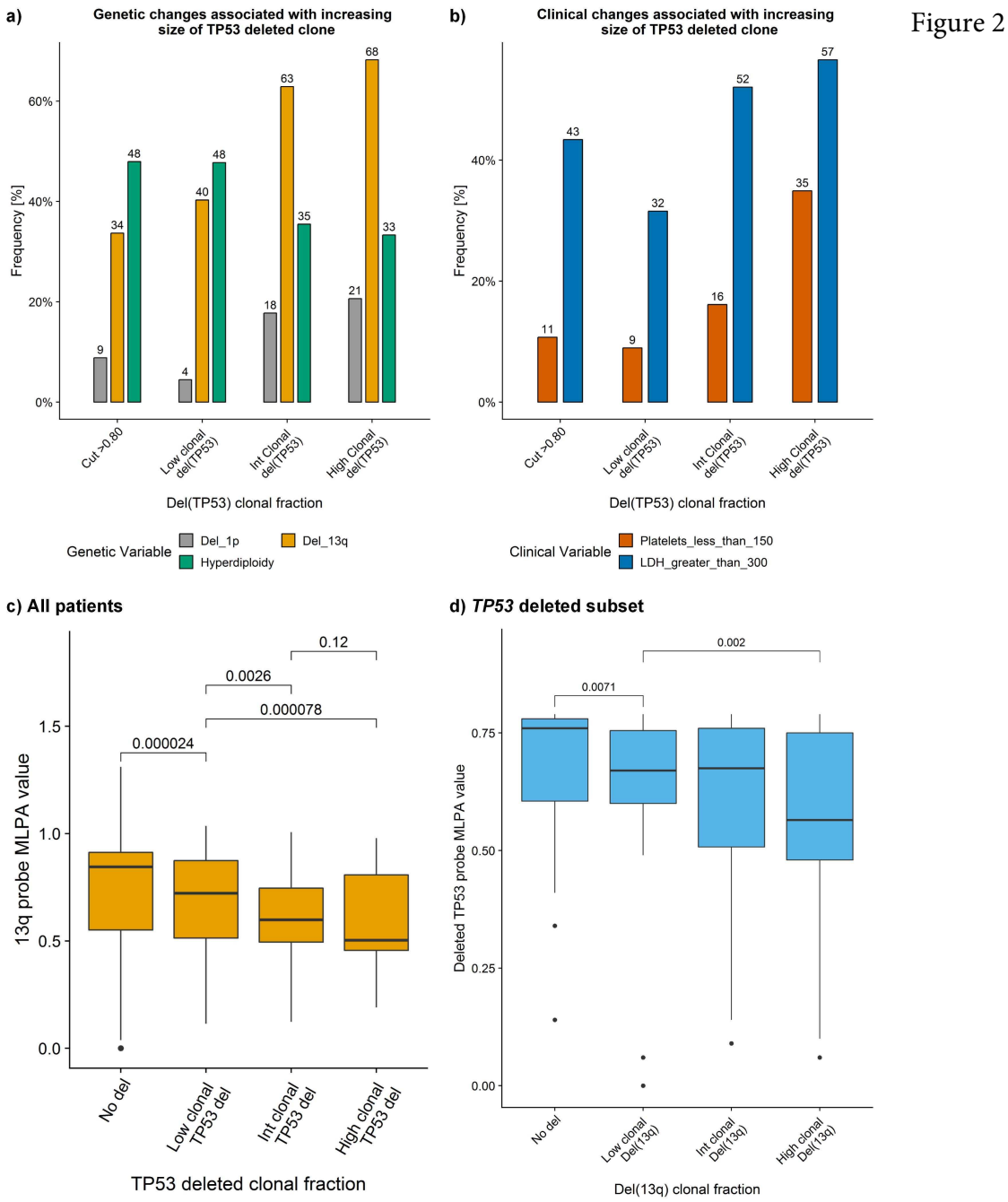

a) PFS: all patients post induction randomisation
Survival probability (%)
p < 0.0001
Time
Number at risk
Del(TP53) Cut-offs
>0.80 1585 1065 612 297 109 18 0
0.7-0.79 67 36 18 10 2 0 0
0.55-0.69 64 32 13 6 2 0 0
<0.55 61 39 18 7 3 0 0
Del(TP53) Cut-offs >0.80 0.7-0.79 0.55-0.69 <0.55
b) OS: all patients post induction randomisation
Survival probability (%)
p < 0.0001
Time
Number at risk
Del(TP53) Cut-offs
>0.80 1585 1269 934 545 241 49 0
0.7-0.79 67 44 30 20 5 0 0
0.55-0.69 64 44 25 9 3 2 0
<0.55 61 45 29 12 5 0 0
Del(TP53) Cut-offs >0.80 0.7-0.79 0.55-0.69 <0.55
c) OS: Landmarked post date of autologous transplant
Survival probability (%)
p < 0.0001
Time
Number at risk
Del(TP53) Cut-offs
>0.8 536 458 334 185 65 1
0.70-0.79 20 15 11 4 2 0
0.55-0.69 17 14 6 3 1 1
<0.55 27 22 13 6 0 0
Del(TP53) Cut-offs >0.8 0.70-0.79 0.55-0.69 <0.55
d) OS: Landmarked post lenalidomide maintenance randomisation
Survival probability (%)
p = 0.00099
Time
Number at risk
Del(TP53) Cut-offs
>0.8 340 247 157 96 35 0
0.70-0.79 15 11 6 3 1 0
0.55-0.69 9 6 3 2 0 0
<0.55 15 11 6 2 0 0
Del(TP53) Cut-offs >0.8 0.70-0.79 0.55-0.69 <0.55

Figure 2


a)
Genetic changes associated with increasing size of TP53 deleted clone
Frequency [%]
60%
40%
20%
0%
9
34
48
4
40
48
18
63
35
21
68
33
Cut >0.80
Low clonal del(TP53)
Int Clonal del(TP53)
High Clonal del(TP53)
Del(TP53) clonal fraction
Genetic Variable
Del_1p
Del_13q
Hyperdiploidy
b)
Clinical changes associated with increasing size of TP53 deleted clone
Frequency [%]
40%
20%
0%
11
43
9
32
16
52
35
57
Cut >0.80
Low clonal del(TP53)
Int Clonal del(TP53)
High Clonal del(TP53)
Del(TP53) clonal fraction
Clinical Variable
Platelets_less_than_150
LDH_greater_than_300
c) All patients
13q probe MLPA value
1.5
1.0
0.5
0.0
0.12
0.0026
0.000078
0.000024
No del
Low clonal TP53 del
Int clonal TP53 del
High clonal TP53 del
TP53 deleted clonal fraction
d) TP53 deleted subset
Deleted TP53 probe MLPA value
0.75
0.50
0.25
0.00
0.002
0.0071
No del
Low clonal Del(13q)
Int clonal Del(13q)
High clonal Del(13q)
Del(13q) clonal fraction