Clonal evolution in myeloma: the impact of maintenance lenalidomide and depth of response on the genetics and sub-clonal structure of relapsed disease in uniformly treated newly diagnosed patients

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ABSTRACT

The emergence of treatment resistant sub-clones is a key feature of relapse in multiple myeloma. Therapeutic attempts to extend remission and prevent relapse include maximizing response and the use of maintenance therapy. We used whole exome sequencing to study the genetics of paired samples taken at presentation and at relapse from 56 newly diagnosed patients, following induction therapy, randomized to receive either lenalidomide maintenance or observation as part of the Myeloma XI trial. Patients included were considered high risk, relapsing within 30 months of maintenance randomization. Patients achieving a complete response had predominantly branching evolutionary patterns leading to relapse, characterized by a greater mutational burden, an altered mutational profile, bi-allelic inactivation of tumor suppressor genes, and acquired structural aberrations. Conversely, in patients achieving a partial response, the evolutionary features were predominantly stable with a similar mutational and structural profile seen at both time points. There were no significant differences between patients relapsing after lenalidomide maintenance versus observation. This study shows that the depth of response is a key determinant of the evolutionary patterns seen at relapse. This trial is registered at clinicaltrials.gov identifier: 01554852.

Introduction

Multiple myeloma (MM) results from the malignant transformation of a normal plasma cell and has a mutational load which lies in an intermediate position between genetically simple malignancies, such as chronic myelogenous leukemia, and the more complex solid cancers.¹² As such, MM provides an excellent model system through which to understand the mutational and evolutionary processes underlying disease relapse. MM is a genetically diverse condition, which is manifested by variations in clinical outcome even in uniformly treated populations.³⁴

The treatment of newly diagnosed (ND) MM has improved over the last decade following the introduction of immunomodulatory drugs and proteasome inhibition, which, together with autologous stem cell transplantation, have increased the median overall survival from three to eight years.²⁷ However, patients frequently
relapse, and to improve outcomes further, strategies have been designed to eliminate the residual clonal cells that mediate relapse.3-10 In this context, an important strategy has been the use of maintenance therapy with lenalidomide.11-13 This agent is well tolerated and has a bifunctional mode of action directly killing the tumor cells and enhancing the immune response to the malignant plasma cells that is mediated by directing Aiolos and Ikaros to the proteasome for degradation.14-17

Understanding the features of residual cells that lead to relapse is an important challenge; however, this is technically difficult, especially in patients who have achieved stringent complete responses. One way of understanding the characteristics of cells in remission is to study the characteristics of cells at relapse. Previous studies of the genetic features of paired presentation and relapse samples have shown that, after intensive chemotherapy, bi-allelic loss of tumor suppressor genes, in particular TP53, and the deregulation of MYC by structural chromosomal changes are important features.18 In addition to mutational change, subclonal structure also varies at relapse and three main patterns have been described; branching, linear and shifting patterns of clonal dominance.19-22 (Table 1). Such analyses have not, however, studied uniformly treated patients for whom the depth of response or treatment information is available. To address the impact of maintenance and response depth on mutational patterns and clonal structure at relapse, we performed whole exome sequencing (WES) on paired presentation and relapse samples from 56 NDMM patients, 30 of whom had received lenalidomide maintenance and 26 who had not. All were treated at first presentation with a triple induction regimen containing an immunomodulatory agent, cyclophosphamide, and dexamethasone, making this the largest analysis of genetic evolution in a uniformly treated series of MM patients to date.

### Methods

Samples were selected from NDMM patients enrolled into the Myeloma XI trial (clinicaltrials.gov identifier: 01554852) for whom adequate DNA volumes were available.23 The study was undertaken after written informed consent from patients and ethical approval was obtained from the Oxfordshire Research Ethics

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**Table 1. Previous studies assessing clonal evolution in myeloma.**

| Study                      | N. of patients | Disease status | Technique                     | Findings                                                                 |
|----------------------------|----------------|----------------|--------------------------------|-------------------------------------------------------------------------|
| Keats et al. Blood 2012    | 28             | Presentation vs. Relapse Array CGH | Three major evolutionary mechanisms identified: 1. Genetically stable 2. Linear evolution 3. Heterogeneous clonal mixtures and shifting predominant clones. |
| Magrangeas et al. Leukaeemia 2013 | 24            | Presentation vs. Relapse SNP array | Branching evolution outlined as a cause for relapse in one-third of patients. The remaining patients displayed linear and stable pathways to relapse. |
| Bolli et al. Nat Commun 2014 | 67             | Presentation vs. Relapse NGS/SNP array and cytogenetics | Linear, branching, stable and differential clonal evolutionary responses described. Description of kataegis and somatic hypermutation. |
| Melchor et al. Leukaeemia 2014 | 6              | Presentation vs. Relapse NGS and single cell analysis | Linear and branching evolution shown at a single cell level. |
| Kortum et al. Ann Hematol 2015 | 25            | Presentation vs. Relapse NGS gene panel | Use of a targeted sequencing panel to demonstrate gain and loss of significantly mutated genes confirming clonal evolution. |
| Weinhold et al. Blood 2016 | 33             | Presentation vs. Relapse NGS and GEP | Mutational load increases at relapse in association with enrichment of driver gene mutations and bi-allelic TSG events. Branching evolution as the main mechanism leading to relapse. |

CGH: comparative genomic hybridization; SNP: single nucleotide polymorphism; NGS: next generation sequencing; GEP: gene expression profiling; TSG: tumor suppressor genes.
Committee (MREC 17/09/09, ISRCTN49407852). A nested case control analysis was performed on 56 patients who received either lenalidomide maintenance (n=30) or were observed (n=26) and subsequently relapsed. All patients included had phenotypical high-risk disease, defined as relapse within 30 months of maintenance randomization, irrespective of classical genetic risk status and best response. The median time from trial entry to relapse was 19 months, notably shorter than the progression-free survival (PFS) reported in the Myeloma XI trial of 35.9 and 32.9 months for patients treated with lenalidomide and thalidomide induction, respectively. Response was determined using International Myeloma Working Group (IMWG) criteria and a near complete response (nCR), defined as no detectable paraprotein, a normal light chain ratio, but where immunofixation and or bone marrow sampling was not performed or if immunofixation was positive. Prior to relapse, 21% (12 of 56) achieved a CR, 21% (12 of 56) a nCR, 42% (23 of 56) a very good partial response (VGPR), and 16% (9 of 56) a partial response (PR). To determine the impact of response on the genetic landscape at relapse we grouped patients according to the best response achieved prior to relapse, complete responders (CR/nCR) and non-complete responders (VGPR/PR). Clinical characteristics were well matched between the maintenance groups (Table 2). Patients’ characteristics according to induction regimen were also well matched (Online Supplementary Table S1).

DNA was isolated from plasma cells following selection using CD138+ MACSorting (Miltenyi Biotech, Bisley, UK) from bone marrow aspirate samples. Control DNA was obtained from peripheral blood samples. Libraries for WES were prepared using the SureSelectQXT sample prep kit and the SureSelect Clinical Research Exome kit (Agilent), with additional baits covering the immunoglobulin and MYC loci, as previously described. Paired-end sequencing was performed to a median sequencing depth of 122x for tumor samples and 58x for controls using a HiSeq2500 (Illumina). Single nucleotide variants, including those of tumor suppressor genes and oncogenes, were determined using Strelka (v.1.0.14) and MuTect. The distribution of mutant alleles determined by the variant allele frequency (VAF) was mapped using the R package SciClone. Cancer clonal fractions (CCF) were calculated for all mutations and plotted using Kernel density estimation to infer clonal structure at presentation and relapse.

Copy number was assessed using both multiplexed ligation...

Figure 1. The mutational profile at presentation and relapse. (A) Recurrent mutations in myeloma and mutations within the genes associated with immunomodulatory agent action. The number of patients with these mutations at presentation, relapse, and at both time points is shown (dotted line denotes level of 10%). (B) Summary of mutations gained and lost at relapse. New mutations at relapse were seen in PROM1, TP53, NF1, TET2, EGFR, MYC, DDB1, CRBN, and FAF (red bars). Loss of mutations in FANCA, DIS3, FAM46C, BRAF, and CDH2 were noted at relapse (blue bars). Mutations in NRAS, KRAS, and SLC16A1 were gained and lost at relapse. (C) Mutational profile for each patient at presentation and relapse. Maintenance strategy and best response prior to relapse is shown. The gain and loss of mutated genes typical of multiple myeloma (MM) was a dominant feature.
dependant probe amplification (MLPA) (SALSA MLPA P425-B1 multiple myeloma probemix, MRC Holland, Amsterdam, the Netherlands) and the bioinformatics assessment tool Sequenza (v.2.1.2). Paired MLPA and Sequenza data was available for 90 of 112 (80%) tumor samples, with a consensus between MLPA and Sequenza being seen in 85 of 90 samples (94%). For the five patients for whom a mismatch was observed, Sequenza was used to call the copy number profile. Translocations were determined using MANTA (v.0.29.3). For 46% (51 of 112) of patient samples, translocations involving the immunoglobulin heavy chain (IGH) were also assessed using multiplexed qRT-PCR. A consensus between MANTA and qRT-PCR was observed in 84% (43 of 51). In the eight patients for whom a mismatch was observed, the integrative genomics viewer (IGV) was used to confirm or exclude the translocation. All suspected bi-allelic copy number abnormalities (CNA) events were confirmed by manual interrogation of BAM files using IGV. Bi-allelic inactivation was also called in patients with evidence of a non-synchronous mutation with mono-allelic loss or a single mutation with a VAF of ≥80%. A summary of the methods, bioinformatics tools and filters used to complete this analysis is available in Online Supplementary Figure S2.

Statistical analysis was performed using GraphPad Prism v.7.01 and R v.3.2.1. P=0.05 (two-sided) was considered statistically significant. Wilcoxon matched-pairs signed rank tests were conducted to determine significance between paired data sets, including the mutational load at presentation/relapse and PFS according to maintenance allocation, depth of response, and induction treatment. Fisher exact test was used to determine differences between two nominal variables, including the change in mutational profile from presentation to relapse and the evolutionary mechanism leading to relapse.

**Results**

Relapse is characterized by a change in mutational spectrum

At presentation, genes mutated in >10% of patients were NRAS (23%, n=13), KRAS (23%, n=13), and DIS3 (13%, n=7) ((Figure 1A and B)). At relapse, TP53 was also seen in >10% of patients (11%, n=6). We examined genes that have previously been shown to be recurrently mutated in myeloma, including tumor suppressor genes, epigenetic modifiers, and genes within the RAS, NFκB and apoptosis pathways, the results of which are summarized (Figure C). Non-synonymous mutations were seen in one or more of these genes in 79% (44 of 56) of patients at presentation and 80% (45 of 56) at relapse. Importantly, gain and/or loss of one or more of these lesions at relapse was seen in 37% of patients (21 of 56), with 21% (12 of 56) of patients gaining a new lesion, 11% (6 of 56) losing a lesion, and 5% (3 of 56) both gaining and losing lesions. The most common new mutations at relapse were KRAS and PRDM1, both seen in 5% of patients (3 of 56), and NRAS and TP53, each seen in 4% (2 of 56). However, mutations in KRAS and NRAS were just as likely to be lost...
at relapse, with 5% (3 of 56) of patients losing KRAS mutations and 4% (2 of 56) losing NRAS (Figure 1B). The most commonly mutated pathway was the RAS-MAPK pathway, with mutations of one or more of NRAS, KRAS, BRAF, NF1, and EGFR being seen in 57% (33 of 56) of patients at some point during the disease course. Loss of one or more of these mutations was noted in 9% (5 of 56) of patients at relapse while new mutations were seen in 13% (7 of 56). The majority of these new mutations were clonal (CCF>80%) and all had a CCF >20% (range, 0.29-1) (Online Supplementary Figure S3). The results of these mutational studies are consistent with there being important changes in subclonal structure at relapse, rather than the simple accumulation of new mutations. The profile of mutations at presentation and relapse in patients who received lenalidomide or thalidomide induction was well matched (Online Supplementary Table S2).

Acquired structural change is a frequent feature of relapse

We interrogated regions of recurrent CNA that are known to be associated with prognosis: 1p32.3 (FAF1/CDKN2C), 1p12 (FAM46C), 13 (RB1), 14q (TRAF3), and 17p (TP53). Copy number loss of ≥1 of these regions was seen in 63% (35 of 56) of patients at presentation and 59% (33 of 56) at relapse (Online Supplementary Figure S4). There was an evident change in the profile at relapse, with regions of loss seen at presentation returning to a diploid status in 9% (5 of 56) of patients while new losses were seen in 13% (7 of 56). Once again, these features are con-

Figure 3. Number of mutational clusters at presentation and relapse. (A) For all 56 patients, the number of mutational clusters was similar at presentation and relapse. The same pattern was seen irrespective of maintenance strategy (B and C) or depth of response (D and E). This suggests that a change in clonal number is not a major factor in disease progression.
sistent with an alteration in subclonal structure and a change in the nature of the dominant clone observed at relapse.

Gain(1q) was the most common new event at relapse, occurring in 13% (7 of 56) patients. Secondary translocations to chromosome 8q, the site of MYC, also occurred more frequently at relapse, increasing from 21% (12 of 56) to 27% (15 of 56) (Online Supplementary Table S3). Consistent with translocations to MYC being late events, five patients had evidence of two separate MYC translocations at relapse. All patients with gain(1q) or tMYC at presentation had evidence of the lesion at relapse, illustrating the driver status.

Bi-allelic inactivation of tumor suppressor genes located at sites of recurrent CNA are likely to be relevant mediators of relapse. We show that bi-allelic inactivation events of RB1, TRAF3, and TP53 are important with 18% (10 of 56) of patients having evidence of bi-allelic inactivation of ≥1 gene at relapse, in comparison to 14% (8 of 56) at presentation. One patient harboring both a TP53 mutation and del(17p) at presentation lost evidence of the del(17p) at relapse, but maintained bi-allelic inactivation via expansion of a different subclone characterized by a TP53 mutation which had a higher CCF at relapse (0.55 vs. 0.83).

**Relapse following a complete response is characterized by a greater mutational load and a change in genetic profile**

Patients achieving a CR had a significantly higher nonsynonymous mutational load at relapse, with a median of 59 mutations compared to 40 at presentation (P<0.001). Non-CR patients had a similar mutational load at relapse, with a median of 39 mutations at both time points (P=0.63). Similar patterns were also seen in patients receiving or not receiving lenalidomide maintenance (Online Supplementary Table S4).

By comparing the profile of known recurrently mutated genes, we show that 67% (16 of 24) of CR cases had a change in mutational profile at relapse compared to only 25% (8 of 32) of non-CR cases (P=0.005) (Figure 2A); these findings were a feature of both the observation and lenalidomide maintenance series (Figure 2B and C). Consistent with the mutational profile changes described, gain and loss of the structural lesions del(1p), del(13), del(14), del(17p), gain (1q), and tMYC at relapse was more common in the CR series, with 42% (10 of 24) of patients having a change in the profile of these aberrations compared to 28% (9 of 32) of non-CR patients (Online Supplementary Figure 5S). The changes in mutational load

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### Table 2. Series characteristics.

|                      | Whole series (% of all) | Maintenance randomization | Observations (% of group) |
|----------------------|-------------------------|---------------------------|----------------------------|
|                      | Lenalidomide (% of group) | Observation (% of group) |
| N. of patients       | 56                       | 30                        | 26                         |
| Median age           | 68                       | 67                        | 69                         |
| Gender               |                          |                           |                            |
| Male                 | 28 (50)                  | 13 (48)                   | 15 (58)                    |
| Female               | 28 (50)                  | 17 (57)                   | 11 (42)                    |
| Pathway              |                          |                           |                            |
| TE                   | 22 (39)                  | 11 (37)                   | 11 (42)                    |
| TNE                  | 34 (61)                  | 19 (63)                   | 15 (58)                    |
| Induction            |                          |                           |                            |
| Thalidomide          | 29 (52)                  | 15 (52)                   | 14 (48)                    |
| Lenalidomide         | 27 (48)                  | 15 (56)                   | 12 (44)                    |
| Best response        |                          |                           |                            |
| CR series (CR/hCR)   | 24 (43)                  | 14 (47)                   | 10 (38)                    |
| Non-CR series (VGPR/PR) | 32 (57)            | 16 (53)                   | 16 (62)                    |
| Median maintenance duration (months, range) | 10 (1-27) | 10 (1-27) | 9 (1-22) |
| Median number of maintenance cycles (range) | n/a                       | 7 (1-28) | n/a    |
| Median PFS (months, range) | 19 (8-51)          | 19 (8-51) | 19 (8-34) |
| Presentation ISS     |                          |                           |                            |
| I                    | 13 (23)                  | 5 (17)                    | 8 (31)                     |
| II                   | 21 (38)                  | 12 (40)                   | 9 (35)                     |
| III                  | 21 (38)                  | 12 (40)                   | 9 (35)                     |
| Missing              | 1 (2)                    | 1 (3)                     |                            |
| Cytogenetic risk*    |                          |                           |                            |
| High risk            | 20 (36)                  | 12 (40)                   | 8 (31)                     |
| Ultra-high risk      | 7 (13)                   | 5 (13)                    | 2 (8)                      |

*High risk defined as one of t(4;14), t(14;16), t(14;20), gain(1q), and del(17p). Ultra-high risk defined as two lesions; TE: transplant eligible; TNE: transplant ineligible; CR complete response; nCR: near CR; VGPR: very good partial response; PR: partial response; PFS: progression-free survival; ISS: International Staging System; n/a: not applicable.
Figure 4. The evolutionary patterns seen leading to relapse. (A) Branching: branching evolution was the predominant mechanism seen and was characterized both by the gain and loss of mutational clusters at relapse. The cancer clonal fractions (CCF) for all coding mutations using kernel density estimation for a typical patient (left) is shown and reveals the presence of a new dominant PRDM1 (CCF 1.0) containing clone at relapse only (each dot represents a mutation). In addition, a clone containing CHD2 (CCF 0.91 presentation only) is lost at relapse while a clone containing NRAS remained dominant at presentation (CCF 1.0) and relapse (CCF 0.99). (Right) Illustration of the branching evolutionary process using the same patient. Prior to treatment, there are a number of competing sub-clones, but as a result of effective therapy, clonal extinction occurs leading to a genetic bottleneck. This leads to the emergence of a new clonal structure at relapse; in this case, the loss of a dominant CHD2 clone, the gain of a PRDM1 clone, and a stable NRAS clone. In addition, the emergence of a new DDB1 mutation was seen within a minor clone with a CCF of 0.21. (B) Linear: linear evolution was seen in 20% of patients, characterized by the gain of mutations at relapse but no evidence of clonal loss. The KDE plot is displayed and shows the emergence of a new clonal PRDM1 mutation at relapse with a CCF of 1.0. (Right) Over time, successive generations of daughter cells acquire aberrations making them genetically distinct; in this example, we see the emergence of a new PRDM1 mutation. (C) Stable progression: KDE plot (left) showing a typical patient with stable progression, revealing a preserved clonal structure at both time points, with CCF values for all mutations remaining consistent at both time points. The CHD2 mutation was present within a dominant clone at presentation and relapse with a CCF of 0.83 and 0.87, respectively. Stable evolution was a characteristic of patients achieving a non-complete remission (non-CR), and in particular a partial remission (PR). These patients appeared to have a treatment resistant disease status and therefore the emergence of the same clonal structure was seen at relapse as had been seen at disease onset; in this case, with a CHD2 dominant clone at both time points (right). (D) Stable with loss was seen in one patient and kernel density estimation (right) revealed the presence of a predominantly preserved clonal structure at relapse with clusters containing TRAF3 and LTB present with similar CCF values at both points. There was evidence of the loss of a cluster of mutations at relapse, suggestive of clone loss (circled). The evolutionary process is shown. Treatment sensitive clone(s) are eliminated but the resistant clone(s) remain and lead to the relapse disease state.
could not be linked to a change in the number of clones predicted by SciClone clustering, where a median number of seven clones was seen at relapse in both the CR and non-CR series (Figure 3). The same findings were noted for all 56 patients and when analyzed according to maintenance strategy. No patient had evidence of the loss or gain of >2 mutational clusters at relapse, further suggesting that a change in the number of clones is not a major cause for change in the mutational load (Online Supplementary Table S5).

Lenalidomide maintenance has no impact on the mutational profile at relapse

There was no specific mutational, copy number or structural feature which characterized patients who received lenalidomide maintenance compared to observation patients. Of patients who received lenalidomide maintenance, 83% (25 of 30) had a mutation in one or more of the recurrently mutated myeloma genes at some point during the disease course compared to 85% (22 of 26) of observation patients. Gain and/or loss of one or more mutation at relapse, including those in genes implicated in the mechanism of action of an immunomodulatory agent, were seen in 43% (13 of 30) of patients receiving lenalidomide maintenance and 35% (9 of 26) of the observation patients (Figure 1B). We did not identify a mutational signal consistent with the selection of clones carrying mutations associated with acquired resistance to lenalidomide. Five patients had mutations in DDB1 (n=2), SLC16A1 (n=2), and CRBN (n=1), but these were not confined to the cases of lenalidomide maintenance, nor were they seen exclusively at relapse (Figure 1B). Mutations in other genes linked to the mechanism of action of lenalidomide, including regulator of cullins 1, cullin-4A, Ikaros, Aiolos, and Basigin, were not found at presentation or relapse in any patient.14,46-48

Mutations in MYC and IRF4, transcription factors known to be down-regulated in response to immunomodulation, were seen in 2% (n=1) and 4% (n=2) of patients, respectively. However, consistent with an acquired mutation having a possible role in drug resistance, we identified one patient who had been exposed to eight months of lenalidomide maintenance and achieved flow cytometric minimal residual disease (MRD) negativity (minimum 5x10^5 cells interrogated) and who then went on to develop a novel CRBN mutation (p.Cys326Gly) at relapse; this is consistent with the emergence of resistance due to mutation at the immunomodulatory molecule binding site. The mutation was also present in a dominant clone, with a CCF of 0.71, further suggesting its presence may have impacted on the fitness of the tumor in relation to pressure from the immunomodulatory treatment (Online Supplementary Figure S6).

Branching evolution is the predominant process leading to relapse, particularly in patients achieving a deep treatment response

We observed three main evolutionary patterns at relapse: branching, linear, and stable. Branching evolution was characterized by both gain and loss of mutational clusters and was the predominant pattern, seen in 66% (37 of 56) of all cases (Figure 4A). Linear evolution characterized only by the gain of mutations at relapse was seen in 20% (11 of 56) of cases (Figure 4B). The remaining 14% (8 of 56) had either the same mutational profile at both time points, and were classified as stable progression (n=7), or displayed a loss of a mutational cluster at relapse, classified as stable progression with clone loss (n=1) (Figure 4C and D). There was no significant impact of induction or maintenance lenalidomide on the evolutionary pattern seen at relapse (Online Supplementary Figure S7).

We show that the depth of treatment response is the most important determinant of the evolutionary pattern seen at relapse. Although branching evolution was domi-
nant in both the CR and non-CR series, stable progression was only seen in the non-CR series, (25%, 8 of 32, vs. 0%, 0 of 25; \(P=0.008\)) (Figure 5A). Breaking down the non-CR series further showed that stable progression was predominantly associated with a PR (56%, 8 of 9 of PR; 13%, 3 of 23 of VGPR patients). No CR or nCR patients (0 of 24) had evidence of stable progression, with all patients showing branching or linear evolution (\(P=0.002\)) (Figure 5B). Consistent with a deep response being synonymous with a change in clonal architecture, 86% (6 of 7) of patients who achieved an MRD-negative state relapsed via a branching mechanism and 14% (1 of 7) via linear evolution. The type of evolution leading to relapse had no impact on the time to relapse or overall survival (Online Supplementary Table S6).

Discussion

This study shows that an increase in mutational load, altered mutational profile, accumulation of deleterious structural lesions [particularly tMYC and gain(1q)], and a change in copy number profile are key molecular features of relapse. The depth of response to treatment has a significant impact on both the genetic landscape and the evolutionary patterns seen at relapse, with an increased mutational load being predominantly associated with the achievement of a CR. We also note that relapse from CR is associated with an altered mutational profile, with 63% (15 of 24) of the CR patients having evidence of loss or gain of known recurrently mutated genes in comparison to only 25% (8 of 32) of non-CR patients (\(P=0.006\)). This pattern was also seen for CNA and structural variants, with del(17p), tMYC, gain(1q), and loss of tumor suppressor gene regions, including CDKN2C, FAI4, FAM46C, RB1, and TRAF3, being seen more frequently in the patients relapsing after CR.

It has been shown that the progression through the multistep transformation of MM is associated with an increased mutational load. This is well illustrated by studies that have compared monoclonal gammopathy of undetermined significance, MM, and plasma cell leukemia. Our results are consistent with this observation, and we clearly show that an increased mutational load is an important factor in early disease progression following a good response to treatment. Similar findings are observed in other cancer types where a greater mutational load is associated with a more aggressive disease status, for example, lung cancer in smokers compared to non-smokers, and in malignant melanoma.

A variety of subclonal patterns, including branching, linear, and stable patterns, are seen at relapse (Figure 4A-D). The pattern of clonal change is most consistent with the hypothesis that branching evolutionary pathways are the predominant mechanism underlying relapse, especially if spatial variation is taken into account. Treatment can be seen as causing an evolutionary bottleneck, particularly in patients who achieve a deep treatment response, providing a selective pressure for the emergence of pre-existent resistant clones. Importantly, these branching patterns and increased genetic damage in the clonal cells are hallmarks of effective treatment and achievement of deep responses. In contrast, the stable patterns seen in non-CR patients are most consistent with microenvironmental change, possibly as a consequence of the presence of treatment resistant dominant clones at disease onset. This stable pattern is reminiscent of the results seen in the progression of smoldering MM to MM, where the emergence of new mutations is infrequent, yet there is a profound change in clinical behavior.

Mechanistically, relapse in the setting of effective therapy that results in the achievement of a CR is occurring either due to the emergence of low-level sub-clones that are undetectable at presentation, but which are selected for by treatment, or as a result of the acquisition of new mutations, albeit less likely given the short duration of remission. It may also be that the differences seen are the result of sampling bias, as previously shown by our group, although this is less likely given that all biopsies were obtained from the pelvis. The pattern of results seen with eradication of dominant clones and emergence of low-level clones with different pattern of mutation, and its association with CR, supports the concept that treatment can result in subclonal eradication. These findings highlight the therapeutic importance of achieving a CR to eradicate dominant clones present at the initiation of therapy. Supporting this as a therapeutic aim, we have recently shown that high-risk patients achieving a molecular CR have very significantly improved 5-year survival rates.

The results show that, even in MRD-negative CR, low-level resistant subclones remain, and therapeutic strategies need to be designed to address them. We believe this work supports the current best practice strategy of using different treatment regimens at successive relapses and shows why this approach is successful. We show that early relapse is either due to innate treatment resistance, requiring no change in the clonal structure, or due to a change of clonal architecture in response to effective therapy. Both mechanisms are consistent with a disease state at relapse that is resistant to the initial therapies used. This knowledge supports the use of combination regimens at relapse, incorporating agents with differing mechanisms of action to those used at presentation. This will be analyzed in a planned comparator series of patients who achieve long-term remission in the trial. Our work here does, however, provide new insights into the mechanisms of early relapsing disease, revealing a different mechanism in those who achieve a deep therapeutic response and those who do not.

Interestingly, a recent study looking at non-small cell lung cancer has shown that mutational load may direct treatment choices, whereby the use of immunotherapy was associated with a longer and durable remission in patients with a greater mutational load at presentation. Therefore, as our understanding of myeloma biology increases, information such as mutational load may guide specific treatment modalities in the future. The use of lenalidomide maintenance is one of the first clinical strategies to address the residual cells that remain following initial treatment. Using this approach, we did not see a signal of any adverse impact on the clonal cells as a result of this therapy. In particular, we did not see a signal for selection of mutations that could confer resistance to lenalidomide either at presentation or at relapse. Previous studies have suggested that such mutations may exist and could be relatively frequent in cell lines and heavily pre-treated patients, but in this study of newly diagnosed patients they were rare. However, we do describe a patient who received eight months of maintenance treatment, achieving an MRD-negative CR who did relapse with a new
CRBN mutation. It is likely that the mutation occurred by chance, but that, in true Darwinian fashion, in the presence of lenalidomide, it conferred a survival advantage to the malignant cells harboring it. Recent analyses indicating that prolonged exposure to lenalidomide is not linked to an increased incidence of second primary hematologic malignancies when used in combinations excluding oral melphalan may be supported by a lack of evidence of mutagenesis leading to treatment resistance in this work, although it is acknowledged that larger analyses using patients with prolonged remissions are required. It is important to note that the patients studied here relapsed early and that they were selected because of this characteristic, as it had previously been suggested that exposure to lenalidomide could enhance progression of such cases post maintenance. However, we did not find any evidence to support such a hypothesis.

It does remain important to evaluate the impact of maintenance on low-risk cases who are long-term survivors, a question not addressed in this study. In addition, we acknowledge that clonal structure may be further assessed using single cell analysis. However, for the moment, the challenges of obtaining individual malignant plasma cells from large series of patients at multiple disease time points has proved to be a significant barrier. In this series of patients, however, we do see evidence of parallel evolution, as previously described using single cell technology, further validating the clonal changes described here. An example is seen in a patient with bi-allelic inactivation of TP53 at presentation characterized by del(17p) and TP53 mutation, but only the presence of a TP53 mutation at relapse. In this case the clone with del(17p) was no longer detectable at relapse, suggesting it was treatment sensitive and/or out-competed by a more aggressive treatment insensitive clone harboring a TP53 mutation that had expanded following treatment, confirmed by a higher CCF at relapse (0.55 vs. 0.83).

In this group of high-risk early-relapsing patients, we show that relapse is a result of the dynamic interplay of evolutionary processes based on the capacity of clonal cells to adapt to their bone marrow microenvironment as a result of new mutations, copy number change, and the selective pressure of the treatment used. Specifically, the depth of response is a critical feature that impacts the evolutionary patterns seen at relapse, rather than the use of lenalidomide maintenance.

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