Title
Microhomology-mediated end joining drives complex rearrangements and over-expression of MYC and PVT1 in multiple myeloma

Running Head
MMEJ drives 8q24 rearrangements in myeloma

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Abstract

MYC is a widely acting transcription factor and its deregulation is a crucial event in many human cancers. MYC is important biologically and clinically in multiple myeloma, but the mechanisms underlying its dysregulation are poorly understood. We show that MYC rearrangements are present in 36.0% of newly diagnosed myeloma patients, as detected in the largest set of next generation sequencing data to date (n=1267). Rearrangements were complex and associated with increased expression of MYC and PVT1, but not other genes at 8q24. The highest effect on gene expression was detected in cases where the MYC locus is juxtaposed next to super-enhancers associated with genes such as IGH, IGK, IGL, TXNDC5/BMP6, FAM46C and FOXO3. We identified three hotspots of recombination at 8q24, one of which is enriched for IGH-MYC translocations. Breakpoint analysis indicates primary myeloma rearrangements involving the IGH locus occur through non-homologous end joining, whereas secondary MYC rearrangements occur through microhomology-mediated end joining. This mechanism is different to lymphomas, where non-homologous end joining generates MYC rearrangements. Rearrangements resulted in over-expression of key genes and ChIP-seq identified that HK2, a member of the glucose metabolism pathway, is directly over-expressed through binding of MYC at its promoter.
Introduction

The genome of multiple myeloma (MM) is characterized by primary translocations in ~40% of newly diagnosed patients that are considered initiating events and involve rearrangements of the immunoglobulin heavy chain (\textit{IGH}) locus on 14q32.\textsuperscript{1} The partners of these rearrangements include 11q (\textit{CCND1}, 15%), 4p (\textit{FGFR3} and \textit{MMSET}, 10%), 16q (\textit{MAF}, 2-3%), 20q (\textit{MAFB}, 1%) and 6q (\textit{CCND3}, 1%). These rearrangements result in placement of the \textit{IGH} super-enhancers next to a partner oncogene, resulting in its over-expression.\textsuperscript{2} The rearrangements predominantly occur in the switch regions 5' of the constant regions in the \textit{IGH} locus, where a high concentration of activation-induced cytidine deaminase (AID) binding motifs are found. Normally, AID binds to the switch regions leading to class switch recombination, resulting in antibody isotype switching.\textsuperscript{3} However, abnormal breaks in the switch regions, resulting from AID activity, results in \textit{IGH} translocations.\textsuperscript{4}

Secondary translocations involving \textit{MYC}, located on 8q24.21, also occur in MM and are associated with disease progression and increased expression of \textit{MYC}.\textsuperscript{5-8} \textit{MYC} encodes a transcriptional regulator and has been shown to be involved in proliferation, differentiation, protein synthesis, apoptosis, adhesion, DNA repair, chromosomal instability, angiogenesis and metastasis.\textsuperscript{9-13} Translocations and high expression of \textit{MYC} are associated with poor outcome, especially in MM where it is a marker of aggressive disease.\textsuperscript{5, 14} \textit{MYC} can be deregulated by a range of different mechanisms including chromosomal rearrangement\textsuperscript{5, 6}, copy-number gain/amplification\textsuperscript{15, 16}, protein stabilization\textsuperscript{17}, via secondary messengers involved in \textit{MYC} transcription\textsuperscript{18} or miRNAs such as \textit{PVT1}.\textsuperscript{19, 20}
The frequency of MYC rearrangements seen in newly diagnosed MM (NDMM) varies from 15-50% and is dependent on the method used to identify it. The data is consistent with MYC rearrangements being rare in the asymptomatic stages such as MGUS and smoldering myeloma, and increases as the disease progresses with a high incidence (>80%) in myeloma cell lines.

MYC rearrangements are not only seen in MM but are also frequent in lymphomas where they have been studied extensively. In Burkitt’s lymphoma and diffuse large B cell lymphoma, t(8;14) rearrangements between IGH and MYC have also been shown to result from abnormal class switch recombination. The relevance of AID in these rearrangements is supported by data from IL-6 transgenic mice which also develop MYC/IGH rearrangements in B cells. Rearrangements, however, do not occur if the mice are also deficient in AID, indicating that class switch recombination via AID is key in generating these rearrangements. In MM, while karyotypic abnormalities similar to that observed in Burkitt’s lymphoma are seen, variant structures can also be detected, suggesting that the mechanism of rearrangement in MM may not be identical to lymphoma. Indeed, MYC rearrangements are not predominantly considered primary translocations in MM, often developing at later stages in the disease, whereas in lymphoma they are considered primary events.

We and others have previously shown that MYC translocations result in the juxtaposition of immunoglobulin loci super-enhancers to MYC resulting in its over-expression. However, the details of breakpoint locations, the presence of copy-number abnormalities and the chromatin landscape of the rearrangement have not been well-characterized. In the present study, we have
analyzed a large dataset of 1267 NDMM patients to determine the genomic architecture of MYC rearrangements and their effect on the expression of this proto-oncogene.

Methods

Patient Samples and Next Generation Sequencing

Total of 1267 NDMM patients were included in this study after informed consent and the study was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. Plasma cell were isolated from bone marrow by magnetic-activated cell sorting using CD138⁺ marker, AutoMACS Pro (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or Robosep (STEMCELL Technologies, Vancouver, Canada). DNA from peripheral blood was used as a control sample for each patient to exclude germline variants. Three paired-end read sequencing platforms were combined without overlapping patients, namely targeted sequencing, whole exome sequencing, and low depth, long insert whole genome sequencing (Supplementary Methods). Additional expression data were available through either gene expression microarrays (Affymetrix, Santa Clara, CA, USA) or RNA-sequencing. An overall summary of methods, number of patients and external datasets are shown in Supplementary Figure 1. Patients’ characteristics are summarized in Supplementary Table 1 and MYC region capture is illustrated in Supplementary Figure 2.
**Patient Derived Xenografts**

Patient derived xenografts were generated by passaging primary patient CD138+ selected cells through the previously described SCID-rab myeloma mouse model. Tumors were dissected from the mouse, and pieces dispersed into a single cell population using a Kontes disposable tissue grinder. Cells were filtered through a 70 μm sterile filter, washed twice in PBS, treated with red cell lysis buffer, washed twice more, and treated immediately with Annexin V coated magnetic beads (Miltenyi Biotec), resulting in a population of cells with a viability >95%, as checked by flow cytometry. Passaged cells underwent CD138+ selection before being processed for 10x Genomics whole genome sequencing, RNA-sequencing, and ChIP-seq.

**ChIP-seq**

ChIP-seq was performed on the myeloma cell lines KMS11 and MM.1S as well as a PDX sample with a MYC rearrangement identified by whole genome sequencing. 1x10^7 cells per mark were fixed in a 1% formaldehyde solution, followed by the addition of glycine to a final concentration of 0.125 M. Cells were washed and resuspended in PBS containing 0.5% lgepal with 1% PMSF, before being pelleted and frozen at -80 °C. ChIP-seq for the histone marks H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K27Ac, and H3K36me3 (Active Motif, Carlsbad, CA, USA), as well as the super-enhancer proteins BRD4 and MED1 (Bethyl, Montgomer, TX, USA), and the transcription factor MYC (Santa Cruz Biotechnology, Dallas, TX, USA) were performed by Active Motif. Controls without antibody input were performed to ensure data quality.
Data Analysis

Data analysis was performed as described previously, with minor differences between sequencing modalities. For details see Supplementary Methods.

Statistical Analysis

Basic statistical analysis was performed using GraphPad Prism 7.01 (GraphPad Software, San Diego, CA, USA), R 3.4.4 and/or RStudio 1.1.442. Fisher’s exact test, the Mann-Whitney U test, Spearman’s rank correlation and Log-Rank test with Benjamini-Hochberg adjustment were used for data analysis and P values ≤0.05 were considered statistically significant.

Data Access

Sequencing data have been deposited in the European Genomic Archive under the accession numbers EGAS00001001147, EGAS00001002859, or at dbGAP under Accession phs000748.v5.p4.
Results

*Myc* Rearrangements Are Usually Present as Inter-Chromosomal Translocations, Co-Occur with Secondary Genetic Events and Are Associated with Shorter Survival in Non-Hyperdiploid Cases.

We examined a set of 1267 NDMM patient samples that had undergone either whole genome sequencing, exome sequencing, or targeted sequencing, of which the latter two methods involved capture of 2.3 Mb and 4.5 Mb, respectively, surrounding the *MYC* locus. Structural abnormalities involving the region surrounding *MYC*, including translocations, inversions, tandem-duplications and deletions, were detected in 36.0% (456/1267) of NDMM samples. Of those 456, 56.6% (258/456) had only a translocation, and 30.0% (137/456) had only an intra-chromosomal rearrangement. In 13.4% (61/456), both translocation and intra-locus rearrangement were present. Non-synonymous *MYC* mutations were rarely detected (0.7%, 9/1264), **Supplementary Table 2**.

The frequency of 8q24 abnormalities was significantly increased across ISS stages (I – 28.6%, II – 37.5% and III – 41.6%, P<0.001), and were higher in the IMWG high-risk (34.6%) and standard-risk (28.1%) groups than in the low-risk group (23.6%, P<0.05). The association of 8q24 abnormalities with these negative prognostic factors may suggest a worse outcome of patients with 8q24 abnormalities, however analysis of this did not confirm the assumption in this dataset (**Supplementary Figure 4A**). Also, 8q24 abnormalities were associated with lower, rather than higher, NF-κB pathway activation (**Supplementary Figure 3**). Additional analysis, however, showed a significant effect of 8q24 abnormalities within the non-hyperdiploid sub-group (**Figure 1A**).
Translocations were found in 25.2% (319/1267) of samples and occurred most frequently as inter-chromosomal translocations involving 2 to 5 chromosomes (90.3%, 288/319), but 4.4% (14/319) were highly complex and involved more than 5 chromosomal loci, Figure 2. Of the remaining cases, 5.3% (17/319) involved a large inversion of chromosome 8, >10 Mb in size. The proportion of MYC translocations involving 2, 3, 4, and 5 loci was 62.1% (198/319), 22.9% (73/319), 8.2% (26/319) and 2.5% (8/319), respectively. However, the number of chromosomes detected as affected by rearrangements involving MYC was dependent on the sequencing capture method used, as rearrangements involving 5 or more chromosomes were detected only by whole genome sequencing, Supplementary Tables 3–4. This data demonstrates that MYC is affected through chromoplexy, where 3 or more loci are involved in rearrangements, in 9.6% (121/1267) of NDMM or 26.5% (121/456) of samples with MYC abnormalities.

**IGH-MYC Translocation Breakpoints Have a Distinct Distribution Compared to Primary Translocations and Involve Recurrent Partners with Known Super-Enhancers.**

A total of 149 chromosomal loci were found to be involved in MYC translocations (Figure 2A; Supplementary Table 5–6). Six translocation partners were found in at least 10 cases and were the Immunoglobulin loci, *IGH* (63/1253, 5.0%), *IGL* (63/1253, 5.0%), *IGK* (26/1253, 2.1%), and also *TXNDC5/BMP6* on chromosome 6 (34/1253, 2.7%), *FAM46C* on chromosome 1 (20/1253, 1.6%), and *FOXO3* on chromosome 6 (14/1253, 1.1%), Supplementary Table 5. Each of these non-Ig loci were confirmed to contain highly-expressed genes in MM using RNA-sequencing data, being present in >95% of patients with log₂ normalized counts >10. All of the loci except for *IGK* had super-enhancers previously identified in the MM.1S cell line. 67.2% (205/305) of cases with
non-complex translocation (5 or less loci involved) had at least one of these super-enhancers involved in the translocation. Another five partners were present in 5–10 cases, three of which overlapped with the highly-expressed genes FCHSD2, FBXW7 and SERTAD2, which are associated with known super-enhancers.\(^{30}\)

Interestingly, 13 samples had complex MYC translocations with more than one of these super-enhancers. Also, eight samples had rearrangements involving IGH, MYC and CCND1, and four samples had rearrangements with IGH, MYC and MAF, indicating that they may occur as primary events early in the disease process. All oncogenes involved in these translocations show high expression (Supplementary Figure 5). This targeting of multiple oncogenes may explain worse patients’ survival with complex MYC translocations (Figure 1B). Ig loci were involved in 47.9% (146/305) of cases with a MYC translocation and were not associated with significantly higher MYC expression (Figure 3B, Supplementary Figure 6B) or patient’s survival (Supplementary Figure 4D) compared to samples involving other super-enhancer-associated genes. In six cases, an IGH translocation occurred together with one of the light-chain immunoglobulin loci, but no sample involved both light chain loci. Within the Ig translocation groups, patients with IGL partners showed significantly worse outcome in comparison to IGH (P<0.05), other non-Ig translocations (P<0.01) and cases without MYC translocations (P<0.001, Figure 1C).

Analysis of the breakpoints at the IGH locus indicated a different pattern of MYC rearrangements to that of the primary Ig translocations. The primary translocations involving t(4;14), t(6;14), t(11;14), t(14;16) and t(14;20) have breakpoints clustered around the constant switch regions where AID motifs are concentrated. However, the MYC translocations do not share this pattern and are dispersed across the constant region, showing no association with AID motif clusters.
This indicates that the MYC translocations are likely independent of AID and occur in a manner that is distinct to that of the primary translocations, Figure 4A–B.

**MYC Breakpoints Show Evidence of Recombination through Microhomology.**

It is known that class switch recombination breakpoints in B cells occur through AID and non-homologous end joining (NHEJ), resulting in blunt ended DNA being ligated together. As the MYC breakpoints identified here do not align to switch regions, and are presumably not mediated by AID, we examined the aligned breakpoints to determine if they were constructed through blunt ended joining or other mechanisms. In comparison to re-aligned t(4;14), t(6;14), t(11;14), t(14;16) and t(14;20) breakpoints, which are mediated by AID and NHEJ, the MYC breakpoints had significantly fewer blunt ended rearrangements (54.1% vs. 27.7%, P<0.001) and significantly more rearrangements with at least two nucleotides of homology (25.4% vs. 45.8%) between the chromosomes, Figure 4C. Homologous sequences between chromosomes of up to 12 nts were found. Representative alignments of rearrangements are shown in the Supplementary Alignments. These homologous sequences are representative of microhomology-mediated end joining (MMEJ), which is a mechanism more common to all secondary translocation events, Figure 4C.

**8q24 Breakpoints Occur in Three Hotspots and Associate with Open Chromatin Markers.**

Breakpoints were determined in a region covering up to 2.5 Mb from MYC and were categorized by the type of rearrangement. Three clusters of chromosomal breakpoints related to
translocations, inversions, deletions and tandem-duplications were identified in the region chr8:126.0–131.0 Mb, Figure 5.

Translocation breakpoint hotspots were located in two 310 kb regions, one around MYC (chr8:128.6–129.0 Mb) and one telomeric of MYC (chr8:129.1–129.4 Mb). When examining all translocations, 28.2% were centered around the first hotspot and 46.6% around the second hotspot. However, there was an enrichment of Ig partner breakpoints at the second hotspot (55.3%) compared to first hotspot (18.9%), which was not so pronounced with non-Ig partners (41.2% vs. 34.0%). There was no evidence of an AID motif cluster at the second hotspot, which could have explained the enrichment for Ig partners and there was no effect of the breakpoint position on patient outcome (Supplementary Figure 4E).

Tandem-duplication breakpoints were enriched at the second hotspot (69.0% of breakpoints), Figure 5 and Supplementary Figures 7–8, as have been noted in MM cell lines previously.34 Conversely, deletion breakpoints were enriched at the first hotspot (30.5%) and at an additional hotspot centromeric of MYC (chr8:126.3–126.4 Mb). Inversion breakpoints were equally spread across all three hotspots.

By examining histone marks from the U266 cell line and four myeloma samples, for which we generated ChIP-seq histone mark data, there was also a link with accessible chromatin marks (H3K4me1, H3K4me3, H3K27ac and H3K36me3), DNaseI hypersensitivity sites and all three
breakpoint hotspots, indicating that rearrangements may be more likely to happen in highly accessible, transcribed regions, Figure 5.

Disruption of Topologically Associated Domains by MYC Rearrangements.

Topologically associated domains (TADs) have been shown to contain DNA elements that are more likely to interact with one another. Disruption of these TADs may bring super-enhancer elements into the same TAD as MYC, resulting in its increased expression. We examined the super-enhancers from the MM.1S cell line and TADs from RPMI-8226 and U266 cell lines and integrated MYC breakpoints.

On the six frequent MYC translocation partner loci, breakpoints were clustered near to the super-enhancer and within the same TAD as the super-enhancer, Figure 6. At 8q24, the translocation breakpoints, at the two hotspots, were clustered within the TAD containing MYC and PVT1. The resulting rearrangements would bring the super-enhancer from the partner loci adjacent to MYC, resulting in the formation of a Neo-TAD (Figure 7B) and over-expression of MYC.

We identified a patient derived xenograft sample with a t(4;8) that resulted in insertion of 3 regions of chromosome 4 next to MYC, Figure 7A. This resulted in the super-enhancer from PCDH10, defined by the presence of H3K27Ac and MED1 marks, being placed next to MYC, resulting in over-expression. This shows for the first time in a patient sample a rearrangement that confirms the importance of placing of a super-enhancer next to MYC.
Lastly, deletions at 8q24 centromeric of MYC are present in 2.9% (36/1249) of samples, Figure 5 and Supplementary Figure 7–8, most frequently result in contraction of the region bringing NSMCE2 into close proximity of MYC, Figure 7C. This interstitial deletion results in TAD disruption bringing the super-enhancer at NSMCE2, present in the cell lines KMS11 and MM.1S, into the same TAD as MYC, resulting in a fused TAD and over-expression of MYC.

8q24 Translocations Result in Increased Expression of MYC and PVT1.

The biological consequence of rearrangements at 8q24 is thought to be increased expression of MYC, so we examined the available CoMMpass study RNA-sequencing data, Figure 3, and a set of microarray data, Supplementary Figure 6, and categorized samples by type and location of breakpoints. In addition to MYC, we examined the expression of other genes in the regions, but only found significant increases in MYC and the non-coding RNA, PVT1, Figure 3A–F, which were associated with particular types of rearrangements. Expression level of these two genes showed a significant but weak correlation (r=0.4, P<0.001).

The six MYC partner loci present in >10 samples (IGH, IGK, IGL, TXNDC5/BMP6, FOXO3 and FAM46C) had significantly higher expression of MYC (P<0.001) and PVT1 (P<0.001) compared to those without rearrangements or less frequent partners, Figure 3B,E. Complex rearrangements involving more than five loci also resulted in higher expression of MYC (P<0.001) and PVT1 (P=0.02) compared to those without rearrangements, at levels equivalent to the frequent translocation partners indicating a selection pressure on these six loci for increased MYC expression. There was no difference in expression between samples with breakpoints at the
hotspot around MYC or telomere of MYC, Figure 3C,F. Expression trends were not different in hyperdiploid (Supplementary Figure 9) and non-hyperdiploid (Supplementary Figure 10) subgroups, but a comparison between specific MYC abnormality groups shows that MYC and PVT1 expression is higher in hyperdiploidy group (Supplementary Figure 11).

Integration of MYC Binding Sites with Over-expressed Genes Identifies Proliferation Markers as Key Targets.

We went on to determine if there is a gene expression signature associated with MYC abnormalities. We compared samples with and without any structural change at 8q24 and adjusted for hyperdiploidy status, as MYC abnormalities were present twice as often in samples with hyperdiploidy (46.0%, 290/630) as compared to non-hyperdiploid samples (22.7%, 102/449; P<0.001). A total of 121 genes (113 protein-coding and 8 non-coding RNA genes) were significantly de-regulated with a fold-change threshold of 1.8, of which 31.4% (38/121) were up-regulated and 68.6% (83/121) were down-regulated, Figure 8A. No significant pathway enrichment was detected by Gene Ontology Consortium\textsuperscript{35} using both PANTHER\textsuperscript{36, 37} and Reactome\textsuperscript{38} pathway analysis (for details of each gene see Supplementary Table 7).

We performed ChIP-seq against c-Myc and determined binding sites in two MM cell lines, MM.1S and KMS11, both of which have a MYC rearrangement. The peaks with a significance P < 10\textsuperscript{-100} using MACS2 in either cell line were considered significant and accounted for 4.7% of peaks (1266/27006), Figure 8B. The peaks were compared to the 121 genes that were significantly changed in expression, Figure 8A. Six genes were in the intersection between over-expressed and significant peaks: HK2, MTHFD1L, SLC19A1, MFNG, SNHG4, GAS5, Figure 8C. Using less
stringent ≥1.3 fold-change cut-off that provided 1801 genes of which 40.8% (735/1801) were over-expressed, the intersection of over-expressed genes and those with a significant MYC binding peak was 25.3% (186/735). At the top of the list of 186 genes ordered by ChIP-seq -log10 P, we detected upregulation of the genes with known or potential oncogenic activity such as genes promoting cell proliferation, tumor growth and/or inhibition of apoptosis (SNHG15, PPAN, MAT2A, METAP1D, MTHFD2, SNHG17), translation factors (EIF3B, EIF4A1, EEF1B2) and genes involved in ribosome biosynthesis (RPL10A, RPL35, RPL23A, RPSA, RPL13, WDR43).

Importantly, we identified HK2 and PVT1 as direct targets of MYC. HK2 is one of the most significant genes detected by ChIP-seq in both cell lines (-log10 P > 200, Figure 8C) as well as having the highest fold-change using RNA-sequencing analysis (Supplementary Table 7). This gene is an interesting direct target of MYC as it is part of the glucose metabolism pathway and would lead to increased energy metabolism and proliferation. PVT1 showed a smaller fold-change by RNA-sequencing analysis (~1.4) but had a significant c-Myc protein binding site identified by ChIP-seq meaning that over-expression of PVT1 is likely a downstream effect of MYC over-expression. This leads to a positive feedback loop and even higher MYC expression as PVT1 positively regulates MYC expression.39

Discussion

We show that MYC breakpoints in myeloma are clustered in three main hotspots on chromosome 8, one of which is associated with Ig translocations and tandem-duplications, another with non-Ig translocations and deletions, and the third with deletions and inversions. All breakpoints
surrounding MYC result in increased expression of the oncogene, but inter-chromosomal translocations result in the largest increase in expression.

In this dataset we have used 1267 NDMM patient samples, of which 36.0% had MYC abnormalities, using next generation sequencing consisting of whole genome, exome and targeted panel data. The frequency of MYC abnormalities reported here is higher than previously seen using other techniques such as karyotyping or fluorescence in situ hybridization (FISH). This is likely due to the increased resolution of sequencing technologies, which can identify small insertions or deletions as well as translocations involving infrequent partner chromosomes. In addition, the complexity of breakpoints at 8q24 makes the placement of FISH probes difficult if all abnormalities are to be detected.

The scale of this analysis has allowed us to define the molecular breakpoints surrounding MYC with unparalleled accuracy and without technical bias. One of the two rearrangement hotspots involved in inter-chromosomal translocations in MM is also seen in other B cell malignancies. In Burkitt’s lymphoma, two breakpoint clusters within exon 1 and intron 1 of MYC were defined, which corresponds in location to the non-Ig rearrangement hotspot in MM. The same cluster is seen in diffuse large B cell lymphoma, where other random breakpoints are also seen scattered both centromeric and telomeric of MYC. Both of these studies looked at relatively small numbers of samples (78 and 17, respectively) and used older techniques, such as long distance PCR and FISH, to detect the breakpoints. It may be that in other B cell malignancies there are also other breakpoint hotspots similar to MM.
The main chromosomal partner to MYC through inter-chromosomal rearrangements is chromosome 14, specifically the IGH locus. In Burkitt’s lymphoma the IGH-MYC breakpoints on this chromosome lie almost exclusively within the switch regions (87%), upstream of the IGH constant regions. The remaining 13% are within the joining region of the locus. These breakpoints are consistent with the IGH-MYC rearrangement being a primary event in Burkitt’s lymphoma, occurring in 70-80% of patients. In contrast, in MM we clearly see that IGH-MYC breakpoints within the IGH locus are not in the switch or joining regions. Instead, they are spread out across the constant regions of the locus. This spread is distinct from the five common primary translocation breakpoints in MM [t(4;14), t(11;14), etc.] which are restricted to the switch and joining regions. Even those with MYC breakpoints within switch regions (6.9% of IGH-MYC rearrangements) also have primary rearrangements or are hyperdiploid. This indicates that the IGH-MYC rearrangements are secondary events in MM and probably occur through a different molecular mechanism to the primary translocation events. It is known that the primary translocations in MM, and the IGH-MYC primary events in Burkitt’s lymphoma, are mediated by AID and class switch recombination. Therefore, the IGH-MYC rearrangements may occur through an as yet unknown, AID-independent, mechanism.

The mechanism driving MYC rearrangements is likely not to involve NHEJ, which would result in blunt ended rearrangements. We have shown that MYC rearrangements are more likely to have short homologous sequences in common to both partner chromosomes, which is not seen as frequently in the primary IGH translocations. Short homologous sequences are indicative of MMEJ, rather than NHEJ, and results through fork stalling and template switching during DNA
replication or through microhomology-mediated break induced repair.\textsuperscript{43, 44} The proteins involved in MMEJ include PARP1, Rad50, and Ercc1 whereas MMEJ is inhibited by functional ATM, H2AX, 53BP1 and BRCA1.\textsuperscript{42} We have previously shown that mutation of ATM, BRCA1 and other genes involved in DNA homologous recombination are associated with in increased levels of loss of heterozygosity in MM patients.\textsuperscript{45} It is likely that disruption of this pathway is key in genomic instability and progression of disease.

The non-Ig chromosomal partners of \textit{MYC} are not random and are known to contain super-enhancer elements.\textsuperscript{5, 6} From our analysis of the breakpoints at the most frequent non-Ig locations (6p24.3 (TXNDC5/BMP6), 1p12 (FAM46C), 6q21 (FOXO3)) we show that the breakpoints at these genes are also clustered. The breakpoints are, in general, contained within TADs which are more likely to interact with one another.\textsuperscript{46, 47} Each TAD at the partner chromosome contains a super-enhancer and breakpoints rarely fall outside of the TAD. The rearrangements are predicted to result in a changed TAD structure that places \textit{MYC} in the same domain as the super-enhancer from the partner locus. If breakpoints were to occur outside of the TAD with the super-enhancer there would be a lower likelihood of it interacting with \textit{MYC} and expression would not be enhanced.

We identified 149 partner loci for \textit{MYC} rearrangements, but 67.2\% of the samples with translocations involve one of the six main partners. The Ig partners have strong super-enhancers in MM, but there are many other active super-enhancers and so it is likely that these six main partners are constrained by chromatin structure. The breakpoints at 8q24 surround an epigenetically active region, defined by the active chromatin marks H3K27Ac, H3K36me3 and,
H3K4me1 as well as DNaseI hypersensitivity sites. It may be that epigenetically active, and therefore accessible, loci are preferred translocation partners\textsuperscript{48, 49}, and the nuclear localization of chromosomes may play a part too.\textsuperscript{50}

Each of these different rearrangements results in over-expression of \textit{MYC}. \textit{MYC} is not the only gene at 8q24, and indeed \textit{PVT1} is significantly over-expressed in our dataset. \textit{PVT1} is a long non-coding RNA and is associated with inhibition of apoptosis and increased proliferation.\textsuperscript{51} It has also been shown that \textit{PVT1} interacts with \textit{MYC}, resulting in a stable protein, and that ablation of \textit{PVT1} results in diminished tumorigenicity.\textsuperscript{52} It may be that the gene complex encompassing \textit{MYC} and \textit{PVT1} is required for oncogenesis and merits further study.

Other than \textit{PVT1}, we also identified other genes that are direct targets of c-Myc and are over-expressed in 8q24-rearranged samples. These included \textit{HK2}, a key enzyme involved in glucose metabolism. It has previously been shown that silencing of \textit{HK2} sensitizes cancer cells to other drugs, and so over-expression of \textit{HK2} in \textit{MYC}-rearranged myeloma may be a key drug resistance mechanism.\textsuperscript{53} Additional genes involved in important cellular functions that increase the oncogenic potential of myeloma cells were also identified, such as ribosome biosynthesis and translation initiation are likely to contribute to the poor prognosis seen in \textit{MYC}-rearranged myeloma.\textsuperscript{5, 14} Targeting \textit{MYC} could therefore be an effective way to disrupt many essential tumor features in one hit.
This study provides evidence of complex chromosomal rearrangements at 8q24 as a key cause of MYC oncogenic up-regulation. Although we found that several MYC abnormalities are associated with prognosis in this dataset, including MYC-IGL and complex translocations, we have previously shown that the association is not independent of other genomic and clinical markers. However, it may be possible that with longer follow-up MYC abnormalities may be independently associated with overall survival and be a marker of poor outcome. We also show a specific pattern of chromosomal breakpoints suggesting the role of the chromatin landscape in tumorigenesis. The mechanism of DNA breaks is clearly different between MYC rearrangements, resulting from MMEJ rather than NHEJ, and differs in myeloma compared to primary MYC translocations in lymphoma.
Author Contributions

AM, CA, FED, GJM and BAW conceived and designed the study; AM, CA PQ, AR, AH and AT collected clinical data, sequencing data and performed statistical testing; JK, DA and AT were responsible for collection and availability of CoMMpass study related data; JAD, KRR, SY and FED developed and maintained mouse models; RGT, SD and ET processed the samples and performed laboratory protocols; AM, CA, ET, JK, DA, GHJ, GJM and BAW analyzed and interpreted data; CA, MAB, CPW, KM and MT performed the bioinformatics of the sequencing data; All authors discussed the results, wrote, reviewed and approved the manuscript.

Conflict of Interest

Celgene Corporation: Employment, Equity Ownership: KM, MT, AT. Funding for data processing and storage was provided by Celgene Corporation. Other authors declare no competing interests.
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Figures

Figure 1: Effect of 8q24 abnormalities on patients’ outcome. (A) 8q24 abnormalities and hyperdiploidy. (B) Translocation complexity. (C) Translocations involving specific types of immunoglobulin locus. Statistically significant levels are as follows: ***P<0.001, **P<0.01 and *P<0.05.
Figure 2: Circos plots of MM samples showing various MYC rearrangements. 
(A) MYC translocations partners in the dataset of the 1253 non-complex cases; loci present in 5–9 cases (orange lines) and ≥10 cases (red lines) are highlighted. 
(B) Complex chromoplexy involving seven chromosomes, including the MYC locus. 
(C) Simple IGH-MYC t(8;14). 
(D) t(14;16) with a secondary translocation to MYC. 
(E) Non-Ig MYC translocation involving TXNDC5/BMP6 on chromosome 6. 
(F) Non-Ig MYC translocation involving FAM46C on chromosome 1. 
(G) Inversion on chromosome 8. 

Annotated genes in uncertain loci were chosen as the closest highly-expressed gene(s) (within 1 Mb maximum distance) defined as being present in >95% of patients with log$_2$ normalized counts >10 in the dataset of 571 cases tested by RNA-sequencing.
Figure 3: RNA-sequencing expression analysis of MYC and PVT1 in relation to chromosomal abnormalities at 8q24. Effect of abnormality type [(A) and (D)], translocation category [(B) and (E)] and translocation breakpoint position [(C) and (F)] are shown for MYC and PVT1, respectively. Statistically significant levels are as follows: ***P<0.001, **P<0.01 and *P<0.05.
Figure 4: Primary IGH rearrangements and MYC rearrangements occur through different mechanisms. (A) The locations of classical IGH (green dots) and IGH-MYC (red dots) translocation breakpoints on 14q32.33. Yellow bars show super enhancers identified in MM.1S cell line; Purple bars show AID motif clusters (>200 RGYW motifs per 2.5 kb) indicating switch (S-) regions. IGH constant regions are indicated as red blocks. (B) IGH-MYC breakpoints on 8q24.21 (red dots). Blue bars show the two breakpoint hotspots identified in Figure 5. The location of MYC (red) and other genes (gray) are indicated. (C) Primary IGH translocations, MYC translocations and other translocations were compared for microhomology between chromosomes surrounding the breakpoints. Primary translocations have significantly more blunt-ended rearrangements compared to MYC rearrangements (P<0.001), consistent with MMEJ.
Figure 5: Distribution of chromosomal breakpoints and minimally altered regions detected at the MYC region. Percent values show proportion of breakpoints in the defined hotspot for a specific category of abnormalities. (A) Three breakpoints hotspots. (B) Minimal tandem-duplicated region. (C) Two minimal copy number gained regions (excluding tandem-duplications). (D) Two minimally deleted regions. (E) Minimal copy-number lost region (excluding deletions). Details of copy-number abnormalities analysis are given in Supplementary Figure 2–3. Upper dotted line shows GC content, ENCODE open chromatin markers identified by a combination of DNase-seq and FAIRE-seq in cell line K562, BLUEPRINT DNase-seq analysis of U266 cell line and BLUEPRINT ChIP-seq analysis in U266 cell line and four myeloma patients’ samples.
Figure 6: Chromosomal breakpoints in MYC translocation partners’ regions. (A) IGH locus at 14q32.33. (B) IGL locus on 22q11.22–22q11.23. (C) IGK locus on 2p11.2. (D) TXNDC5/BMP6 locus on 6p24.3. (E) FAM46C locus on 1p12. (F) FOXO3 locus on 6q21. Yellow bars show super-enhancers identified in the MM.1S cell line; Green bars show topologically associated domain (TAD) boundaries identified in RPMI-8226 and U266 cell lines. Ig genes are separated into constant (C, red), joining (J, blue), diversity (D, green) and variable (V, purple) regions; non-Ig highly-expressed genes (present in >95% of patients with log_2 normalized counts >10 in the dataset of 571 cases tested by RNA-sequencing) are in red and other genes in gray color.
Figure 7: TAD reorganization through rearrangements places a super-enhancer next to MYC. The TAD architecture (colored triangles) surrounding MYC is indicated in the central panel (red box) as defined in U266 cells. (A) A patient sample with a t(4;8) involves the insertion of a super-enhancer from PCDH10 (chr4) into chr8, creating a neo-TAD containing MYC and the super-enhancer. (B) A translocation from a key MYC partner introduces a super-enhancer into the MYC TAD. (C) Deletions centromeric of MYC result in fusion of TADs containing MYC and the super-enhancer next to NSMCE2.
Figure 8: Integration of ChIP-seq for c-Myc and gene expression data identifies direct targets of MYC rearrangements. (A) 121 genes that were significantly changed in expression between samples with or without a MYC abnormality (FDR<0.05, fold-change ≥1.8) in the dataset of 526 MM patients with RNA-sequencing. (B) All c-Myc ChIP-seq peaks detected in MM.1S and KMS11 cell lines and ordered by -log10 P value. (C) Significant c-Myc ChIP-seq peaks (-log10 P value > 100) with highlighted PVT1 gene and genes that overlap with 121 genes (A).