Translocations at 8q24 juxtaspese MYC with genes that harbor superenhancers resulting in overexpression and poor prognosis in myeloma patients

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Secondary MYC translocations in myeloma have been shown to be important in the pathogenesis and progression of disease. Here, we have used a DNA capture and massively parallel sequencing approach to identify the partner chromosomes in 104 presentation myeloma samples. 8q24 breakpoints were identified in 21 (20%) samples with partner loci including IGH, IGL and IGI, which juxtaspose the immunoglobulin (Ig) enhancers next to MYC in 8/23 samples. The remaining samples had partner loci including XBP1, FAM46C, CCND1 and KRAS, which are important in B-cell maturation or myeloma pathogenesis. Analysis of the region surrounding the breakpoints indicated the presence of superenhancers on the partner chromosomes and gene expression analysis showed increased expression of MYC in these samples. Patients with MYC translocations had a decreased progression-free and overall survival. We postulate that translocation breakpoints near MYC result in colocalization of the gene with superenhancers from loci, which are important in the development of the cell type in which they occur. In the case of myeloma these are the Ig loci and those important for plasma cell development and myeloma pathogenesis, resulting in increased expression of MYC and an aggressive disease phenotype.

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INTRODUCTION

Rearrangements at 8q24 have been reported in up to 47% of myeloma patients by a combination of fluorescence in situ hybridization (FISH), spectral karyotyping and classical cytogenetics. In presenting myeloma this frequency is lower, with abnormalities of 8q reported in 15% of cases using mapping arrays and FISH. The gene of interest in this region is MYC, an oncogene that has a pivotal role in cell growth, proliferation, tumorigenesis and stem cells.

The importance of MYC activation in myeloma has been shown through the use of the VkMYC transgenic mouse model, where activation of MYC arises through AID-dependent somatic hypermutation during B-cell development, resulting in the onset of myeloma in these mice. MYC has also been shown to be activated in the transition from monoclonal gammapathy of undetermined significance to myeloma, implicating it in disease progression. Myeloma cells have been shown to have a dependency on MYC for survival, where inhibition of MYC by small hairpin RNA or small-molecule inhibitors results in cell death indicating that MYC is a promising therapeutic target.

The mechanism of MYC activation is mainly through secondary translocations involving the immunoglobulin (Ig) loci (IGH > IGL > IGI), which juxtaspose the strong B-cell enhancers present at these loci and MYC, resulting in overexpression of the oncogene. Unlike primary translocations in myeloma, which are often simple reciprocal exchanges of chromosomal material, the rearrangements that result from MYC translocations are often complex, involving many partner chromosomes. Interestingly, it has been reported that up to 74% of MYC rearrangements do not involve an Ig locus leading to the conclusion that other mechanisms of activation may also be important in myeloma. Using FISH, partner chromosomes at 1p13, 1p21–22, 6p21, 6q12–15, 13q14 and 16q22 have been identified, but the specific loci involved have remained elusive. The breakpoints on 8q24 have been mapped in a large number of myeloma cell lines and the majority are found within 1 Mb of MYC, but some can be greater than 3 Mb either telomeric or centromeric of the locus. The loci surrounding MYC are POUSF18 (centromeric) and PVT1 (telomeric). PVT1 is a non-coding RNA that has been shown to be the location of variant t(8;22) breakpoints in Burkitt’s lymphoma, as well as generating fusion genes with WWOX and NBEA in myeloma patients with an 8q24 rearrangement.

Here, we have used targeted capture followed by massively parallel sequencing to pull down the region surrounding MYC in a series of presenting myeloma cases in order to identify any translocations in this area and the mechanism of action involved.

MATERIALS AND METHODS

Cell selection

CD138-positive bone marrow plasma cells were selected to a purity >95% using magnetic assisted cell sorting (Miltenyi Biotech, Bisley, UK). Tumor DNA and RNA were extracted using the AllPrep kit (Qiagen, Manchester, UK). All patients were at presentation and had not received any treatment when the sample was taken.

FISH

Probes have been previously published with the addition of the LPL (8q22), CEP 8 and MYC (8q24.1-24.21) probes (Abbott, Maidenhead, UK).
MYC abnormalities were defined using the t(8;14) fusion probe, LPL/CEP 8/ MYC probes, IGH@ translocations (IGH@ break-apart probe followed by fusion probes for the common partner chromosomes). FISH results were interpreted alongside karyotype data, where available.

Targeted capture of the MYC locus
A targeted capture system was designed using the SureSelect system (Agilent, Santa Clara, CA, USA) that was based on tiling RNA baits across the MYC, IGH, IGK and IGL loci as previously described. The region captured surrounding MYC spanned from 127.5–129.8 Mb on chromosome 8, roughly 1 Mb on either side of MYC, which is located at 128.75 Mb. This region includes POU5F1B and PVT1, which are common sites of 8q24 translocations in myeloma.

DNA from 104 samples were assayed using 150 ng of DNA and a modified capture protocol with eight cycles of prehybridization PCR and 11 cycles of posthybridization PCR. Samples were barcoded using Illumina (San Diego, CA, USA) indexes and up to 27 samples were sequenced per lane on a HiSeq2000 generating 76-bp paired-end reads. After base calling and quality control metrics, the raw fastq reads were aligned to the reference human genome (build GRCh37) resulting in a median depth of 289 × per sample after de-duplication for the captured region.

Translocation breakpoints were identified in the sequencing data using DELLY. Breakpoints called using the bioinformatic approach were further filtered based on depth, unique mappability for 76 bp reads, number of supporting reads and whether or not they were detected in non-tumor samples. The coordinates of the breakpoints and superenhancers were compared with randomized data produced using Monte Carlo sampling using the Genomic Hyper Browser. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Discrete data were expressed as frequencies and percentages and were assessed by Fisher’s exact test or the (2-test, as appropriate. Survival curves were plotted using the Kaplan–Meier method.

RESULTS
MYC rearrangements detected by FISH
MYC rearrangements were tested for using interphase FISH on CD138– selected plasma cells on patient samples from the UK MRC Myeloma IX trial using a combination of probes for MYC (8q24), LPL (8p22) and CEP 8. Interphase FISH was successful on 751 patients and was interpreted with data from FISH probes for the IGH locus and karyotyping. Table 1 shows the frequency of MYC abnormalities, split by translocation group in these samples. MYC abnormalities were detected in 26% of samples with the most frequent abnormality being gain of the locus, followed by a split probe signal. In 50% (32/63) of those with a split probe signal the IGH/K/L locus was identified as the partner. Of the 32 samples with an Ig partner 28 were with the IGH locus. In the remaining 50% the partner remained undetermined, but may involve the light chain loci in samples where cytogenetics or karyotyping was unsuccessful or uninformative. MYC rearrangements were not associated with any particular primary translocation, but were associated with ISS II (P = 0.022) or ISS III (P = 0.0027). There was no negative impact on PFS or OS in patients with a MYC abnormality.

| Translocation (Tx) group | t(4;14) | t(11;14) | t(6;14) | t(14;16) | t(14;20) | HRD | HRD + Tx | None |
|-------------------------|---------|---------|---------|---------|---------|-----|---------|------|
| Normal                  | 49      | 74      | 4       | 17      | 6       | 294 | 20      | 90   |
| Gain                    | 7       | 17      | 2       | 8       | 4       | 46  | 10      | 17   |
| Split                   | 3       | 10      | 0       | 2       | 2       | 40  | 1       | 5    |
| DEL                     | 5       | 0       | 0       | 0       | 0       | 4   | 1       | 14   |
| Total abnormal (%)      | 23.4    | 26.7    | 33.3    | 37.0    | 50.0    | 23.2| 37.5    | 28.5 |

Abbreviation: FISH, fluorescence in situ hybridization.

Table 2. Incidence of 8q24 breakpoints in sequence-capture samples

| Translocation | Assayed (%) | With 8q24 breakpoint (%) of group | P-value |
|---------------|-------------|-----------------------------------|---------|
| t(4;14)       | 13(12.5)    | 0(0)                             | 0.031   |
| t(6;14)       | 8(7.7)      | 1(12.5)                           | NS      |
| t(11;14)      | 28(26.9)    | 5(17.8)                           | NS      |
| t(14;16)      | 17(16.3)    | 11(64.7)                          | <0.001  |
| t(14;20)      | 6(5.8)      | 1(16.6)                           | NS      |
| HRD           | 31(29.8)    | 3(9.6)                            | 0.037   |
| Othera        | 1(0.9)      | 1(100)                            | NS      |
| Total         | 104         | 21                                |         |

Abbreviations: HRD, hyperdiploidy; NS, not significant. aThis sample has a secondary t(7;14).
there was an enrichment for t(14;16) and a depletion of t(4;14) and hyperdiploidy associated with an 8q24 breakpoint, Table 2, which was not seen with FISH alone.

The positions of the breakpoints on 8q24 were spread out over 1.2 Mb. The majority of the breaks were situated telomeric of PVT1 (51.5%) with 12% within PVT1, 15% between MYC and PVT1 and 15% between POU5F1B and MYC. Of the three samples with breakpoints within PVT1 two were found to have potential fusion genes, one with FOXO3 and the other with LINCO0309.

Nine samples had two breakpoints on 8q24, often with two different chromosome partners. In samples with two breakpoints, the sequencing reads indicate that MYC is only involved in one of the breakpoints (that is, only one side of the translocation was captured). The reason for this is not clear and will require long reads to clarify the final genomic rearrangements. Several samples had complex rearrangements resulting in multiple chromosomal segments being joined together, often involving MYC, an Ig enhancer and the primary Ig partner oncogene (for example, sample 11/625 that has a t(6;14) and a t(6;8) or sample 12/0404 that has a t(11;14) and a t(8;11)). Details of breakpoints are shown in Table 3. It is possible that the Ig enhancer is influencing both the target oncogene of the primary translocation (for example, CCND1 or CCND3) and MYC through the assembly of a complex t(8;11;14) or t(6;8;14) derivative chromosome.

Superenhancer colocalization with MYC

A total of 8/21 samples (38%) with MYC abnormalities had rearrangements with an Ig locus on the partner chromosome. Ig partner loci are recognized to upregulate expression of the target oncogene and have been shown to have clinical relevance as prognostic markers. Given that the mechanism of upregulation of

Table 3. Genomic locations of MYC breakpoints

| Sample | IGH translocation | 1st Chr | 1st breakpoint | 1st Gene* | Distance (bp) to MM1.1 enhancer from breakpoint | 2nd Chr | 2nd breakpoint | 2nd Gene* |
|--------|------------------|---------|----------------|-----------|-----------------------------------------------|---------|---------------|-----------|
| 29     | t(7;14)          | chr6    | 7966298        | Telomeric | MYH9                                          | chr8    | 129251475     | POUS1-CCDC26 |
| 29     | t(7;14)          | chr6    | 7966298        | Telomeric | TXNDC5                                         | chr8    | 129375213     | POUS1-CCDC26 |
| 176    | None             | chr1    | 118246012      | Telomeric | FAM66-GDA2                                     | chr8    | 129266432     | POUS1-CCDC26 |
| 222    | t(14;16)         | chr2    | 64412927       | Centromeric | LINC00309                                     | chr8    | 129064694     | PVT1 |
| 222    | t(14;16)         | chr2    | 64468628       | Telomeric | LINC00309-LGALSL                               | chr8    | 129104714     | PVT1 |
| 471    | None             | chr22   | 24071965       | Telomeric | IGL                                            | chr8    | 128737937     | POUS1-CCDC26 |
| 475    | t(14;16)         | chr12   | 25507375       | Telomeric | IGL-LITLD1                                      | chr8    | 129368991     | PVT1-CCDC26 |
| 478    | t(11;14)         | chr2    | 77937177       | Telomeric | LRTIM4-SNAR-H                                   | chr8    | 128896717b    | MYC-PVT1 |
| 592    | t(14;16)         | chr11   | 11119351       | Telomeric | C11ORF93-MIR4491                               | chr8    | 129247671     | POUS1-CCDC26 |
| 673    | t(14;16)         | chr14   | 106119946      | Telomeric | IGK                                            | chr8    | 129290655     | POUS1-CCDC26 |
| 730    | None             | chr22   | 41824373       | Centromeric | TEF-TOB2                                        | chr8    | 129345574     | PVT1-CCDC26 |
| 730    | None             | chr22   | 23280899       | Centromeric | IGK                                            | chr8    | 129228289     | PVT1-CCDC26 |
| 984    | t(14;16)         | chr2    | 89151255       | Centromeric | IGK                                            | chr8    | 129228289     | PVT1-CCDC26 |
| 984    | t(14;16)         | chr2    | 89151255       | Centromeric | IGK                                            | chr8    | 129228289     | PVT1-CCDC26 |
| 1112   | t(11;14)         | chr11   | 69283791       | Centromeric | CCND1                                          | chr8    | 128427581     | POUS1-CCDC26 |
| 1112   | t(11;14)         | chr11   | 146700223      | Centromeric | MIR501-FMRI-AS1                                 | chr8    | 129075004     | PVT1-CCDC26 |
| 1310   | t(11;14)         | chr11   | 108911604      | Telomeric | FOXO3                                          | chr8    | 129254453     | PVT1-CCDC26 |
| 1310   | t(11;14)         | chr11   | 108908007      | Centromeric | FOXO3                                          | chr8    | 129000293     | PVT1 |
| 1108   | t(14;16)         | chr10   | 125858805      | Telomeric | CHST15-OAT                                      | chr8    | 129235977     | PVT1-CCDC26 |
| 1138   | t(14;16)         | chr11   | 23307780       | Telomeric | IGK                                            | chr8    | 128727226     | MYC-PVT1 |
| 1138   | t(14;16)         | chr11   | 23307780       | Telomeric | USP9 (near CCND1)                              | chr8    | 128244760     | PCAT1-POUS1F |
| 1174   | t(14;16)         | chr2    | 29210349       | Telomeric | XBP1-ZNBF3                                     | chr8    | 128772206     | MYC-PVT1 |
| 1174   | t(14;16)         | chr2    | 134989574      | Telomeric | MIR3679-MGATS                                   | chr8    | 128772258     | MYC-PVT1 |
| 214    | t(14;16)         | chr22   | 23285257       | Telomeric | IGK                                            | chr8    | 129214619     | PVT1-CCDC26 |
| 11212  | t(14;16)         | chr22   | 23258401       | Centromeric | IGK                                            | chr8    | 129301246     | PVT1-CCDC26 |
| 111227 | t(11;14)         | chr14   | 105969653      | Telomeric | MIR548AS-DAOA-AS1                               | chr8    | 128504864     | POUS1-CCDC26 |
| 120213 | t(11;14)         | chr1    | 11830287       | Telomeric | FAM66-GDA2                                     | chr8    | 128869631     | MYC-PVT1 |
| 120365 | t(14;16)         | chr10   | 125880805      | Telomeric | CHST15-OAT                                      | chr8    | 129235977     | PVT1-CCDC26 |
| 120365 | t(14;16)         | chr17   | 74521561       | Telomeric | RHDD2-CYGB                                      | chr8    | 129262577     | PVT1-CCDC26 |
| 120404 | t(11;14)         | chr11   | 69425933       | Centromeric | MYEOV-CCND1                                     | chr8    | 128719531     | POUS1-CCDC26 |

*Where >1 gene is named, the breakpoint is in the intergenic space between genes. *bBreakpoint within inversion. *cConfirmed by FISH. *dComplex rearrangement containing 400 bp of chrX with translocations leading to chromosomes 14 (IGH@) and 8 (PVT1) as well as a t(8;11) and t(11;14). *eConfirmed by karyotype underlined = derivative does not contain MYC.
Ig loci partner oncogenes is through colocalization of active enhancer elements with the oncogene, we examined the remaining samples with rearrangements for the presence of enhancer elements. We used data from two papers in which binding sites of BRD4 and MED1, which occur at transcriptionally active sites, enhancers and superenhancers in MM1.s cells had been annotated using only enhancer sites on the assembled derivative chromosome.\(^{23,24}\) As expected, the Ig loci breakpoints were typically close to or within an enhancer/superenhancer (minimum distance = 0, maximum distance = 26 kb) with the exception of IgKC breakpoints, which were ~292 kb from the nearest superenhancer (Table 3). This discrepancy is likely owing to MM1.s cells being a lambda light chain expresser that do not express a kappa light chain. If a cell line expressing a kappa light chain had been used in the analysis an enhancer may have been detected much closer.

In order to determine whether there was an enrichment of superenhancer sites in the vicinity of MYC breakpoints, we used the genomic locations of superenhancers from a variety of the cell types determined in Hnisz et al.\(^{23}\) There was a statistically significant enrichment for breakpoints within 1 Mb of a superenhancer in the MM1.s myeloma cell line and in CD19\(^+\) B cells (\(P = 0.0049\) and 0.041, respectively). No enrichment was seen with enhancer locations in K562 cells, CD3\(^+\) T cells or skeletal muscle myoblasts indicating a cell-specific enrichment for superenhancers in the B-cell lineage.

The partner loci to MYC are also mostly related to myeloma pathogenesis or B-cell biology (Table 3). In addition to the Ig loci, partner genes of MYC rearrangements included FAM46C, KRAS and CCND1. All of these genes are candidates in many genetic studies of myeloma. FAM46C is deleted in ~20% of samples and mutated in ~3% of samples.\(^{2,25}\) KRAS is mutated in 31% of samples and CCND1 is overexpressed in t(11;14) accounting for ~15% of samples.\(^{26,27}\) In addition, XBP1 is involved in plasma cell differentiation, the unfolded protein response and is mutated in a low percentage of relapsed refractory myeloma.\(^{26,28}\) The genes located next to breakpoints in the remaining samples may also be active in B-cell biology or myeloma pathogenesis as recurrent translocations are found near the CHST15 locus, which is known to be involved in B-cell signaling,\(^{29,30}\) and FOXO3, which is involved in B-cell development.\(^{31}\) Some rearrangements from these samples are shown in Figure 2 to illustrate the colocalization of superenhancers to MYC.

**Impact on MYC expression**

Previously published gene expression array data were available on 33 of the samples (Supplementary Table 2).\(^2\) We categorized the

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![Figure 2](image-url)

**Figure 2.** MYC rearrangements result in superenhancer colocalization on the derivative chromosome. Superenhancer elements are colocated near to MYC from a t(1;8) (top) or a t(8;22) (middle and bottom) where the partner chromosome gene (FAM46C, XBP1 and IGL, respectively) has a known function in myeloma or B-cell biology.
samples according to the presence (n = 9) or absence (n = 24) of a MYC translocation, as detected by the capture assay, and used the MYC probe set (202431_s_at) to examine any difference in expression between the groups. Those samples with any MYC translocation had higher expression of MYC compared with those without a translocation (median 703.2 vs 2313), but this did not reach significance with this number of samples (P = 0.065). Figure 3a. This was extended in the full Myeloma IX data set for which any translocation was detected by FISH or capture and for which expression array data were available. In this larger data set, samples with any translocation (n = 27) had a significantly higher expression of MYC (2003 vs 945.8, P = 0.009) compared with those with no translocation (n = 142), even allowing for those samples on which no capture had been performed (and may contain translocations not detected by FISH) (Figure 3b). The increase in expression of MYC is consistent with its deregulation being the central mechanism.

Impact on clinical outcome
A subset of patients (n = 55) from the capture panel had taken part in the UK MRC Myeloma IX trial, so we performed an analysis of the impact of MYC translocations on survival. MYC translocations had a significant impact on OS and PFS in univariate analysis (Table 3), which carried over into multivariate analysis, resulting in a significant decrease in OS and PFS for patients with a MYC translocation (Figure 4).

DISCUSSION
Here, we have investigated the partner loci involved in rearrangements with 8q24, namely MYC. We analyzed presenting cases of myeloma characterized by a range of different primary IGH translocation events and found evidence of a MYC translocation in 21% of samples, making it the most frequent translocation in presenting myeloma cases.

Although FISH analysis of 8q24 rearrangements found no significant association with primary translocation groups, we did find an enrichment for samples with a MYC breakpoint in samples that had a t(14;16) and a depletion of samples with a t(4;14) or hyperdiploidy when screened by the sequence capture technique. Other studies using FISH agree with our FISH-based results in that it has been reported that rearrangements at the MYC locus show a similar prevalence in hyperdiploid and non-hyperdiploid tumors\(^1\) or show some trend to being associated with t(4;14).\(^2\) The discrepancy between our capture results and those determined by FISH may lie in the technique used to study the abnormality. One study used both FISH and mapping arrays to examine MYC breakpoints and found that 33% of breakpoints detected by array were not detected by routine FISH analysis.\(^3\) Therefore, a higher resolution assay, such as genome sequencing, may identify more breakpoints and explain some of the discrepant results. However, care must be taken regarding the frequency of sequence capture translocations within the primary translocation groups owing to the bias in sample selection and the relatively small number of samples studied.

It is notable that all groups find MYC rearrangements to be complex, often involving many chromosomes. We only capture the region surrounding MYC and are not able to detect any rearrangements downstream on the partner chromosome, which could result in inversions, insertions or duplications of DNA segments. Whole-genome sequencing of samples will provide more complex and detailed information regarding the final composition of the genome in these samples.

The translocations at 8q24 results in overexpression of MYC due to the colocalization of active superenhancers in the partner loci. The obvious examples of this belong to the known active enhancers in the B-cell lineage, the Ig loci, but in addition to these a series of previously unknown partners have also been identified. A similar study in B-cell lymphomas identified non-immunoglobulin partners to MYC rearrangements and concluded that these are non-random processes that juxtapose MYC with genes involved in lymphomagenesis (namely BCL6, PAX5 and IKAROS).\(^32\) We have identified a different set of genes in this study and interestingly these genes are related to B-cell biology and myeloma pathogenesis. We hypothesize that in different diseases with MYC rearrangements there is a selection process, which results in active superenhancers from genes that are expressed in that cell type being placed near MYC. In all B-cell neoplasias these include the Ig loci, but in different B-cell subtypes the non-Ig loci differ to include those that are expressed in that cell type. In myeloma these non-Ig partner genes include FAM46C, KRAS, XBP1.

Figure 3. Expression of MYC in samples with a breakpoint is higher than in those without a breakpoint. (a) Expression data from 33 samples with sequence capture-determined translocations for those samples with no breakpoint at 8q24 (normal) and for those with an identified breakpoint (split MYC). (b) Expression data from 169 samples with any translocation detected by capture or FISH. Whisker plots show the 10–90 percentiles.
and CCND1. The other partner genes identified here, which have no known function in myeloma or plasma cells, are likely also to be important in plasma cell development or myeloma disease as they have active superenhancers that are sequestered by MYC.

Some of the loci identified have active superenhancers up to 22 Mb away from the breakpoint. These distal superenhancers may be responsible for MYC overexpression, but as these results are based solely on data from the MM1.s cell line it may be that in different myeloma cell types an enhancer closer to the breakpoint is active, and it is this as yet unidentified superenhancer that is located near MYC in this patient. This argument is exemplified by the lack of a superenhancer at the IGK locus in MM1.s and can be explained as this cell line expresses the lambda light chain and not the kappa light chain. Similar studies involving a large cohort of myeloma cell lines would give further insight into the complex nature of superenhancers in this disease.

As the mechanism of action for MYC overexpression is through the juxtaposition of superenhancers specific to each disease, it is possible that this unifying mechanism can be therapeutically targeted. Given that it has been shown that superenhancers can be disrupted using BET-bromodomain inhibitors, such as JQ1, it makes patients with MYC rearrangements good candidates for treatment with this class of drugs. We show here that patients identified with a MYC translocation have a poor PFS and OS compared with those with no rearrangement. If these patients could be identified in advance they may benefit from treatment with this class of targeted drugs.

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