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Identification of a Novel t(7;14) Translocation in Multiple Myeloma Resulting in Over-expression of EGFR

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ABSTRACT

IGH translocations in myeloma are a primary event and determine the prognostic outcome of a patient. These events are characterized by FISH and classical cytogenetics, but in a small proportion of samples a translocation involving the IGH locus can be detected but the partner chromosome cannot be identified. These cases are usually genetically complex and are the result of cryptic events which cannot be discerned at the resolution of FISH. Here we analyzed a sample with an unidentified translocation partner using a targeted capture and massively parallel sequencing. We identified the partner chromosome as a t(7;14) with the breakpoint upstream of EGFR. This sample over-expresses the target oncogene, EGFR. This case represents a rare and novel translocation in myeloma, from which a targeted personalized treatment, in the form of EGFR inhibitors which are commonly used in other cancer types, could be used.
INTRODUCTION

Multiple myeloma is a complex disease comprising distinct genetic subgroups (Morgan et al., 2012). The two main sub-classifications delineate samples into those with trisomies of chromosomes, resulting in hyperdiploidy, and those with translocations involving the IGH locus on 14q32.33. This latter group comprises about 45% of presentation myeloma patients and arises through aberrant events during immunoglobulin gene heavy chain (IGH) rearrangement. These aberrant events usually occur in the germinal centre during class switch recombination (CSR), where the switch regions upstream of the constant IGH regions recombine with a partner chromosome (Gonzalez et al., 2007). However, we have recently suggested that a proportion of these translocations can occur through non-CSR mechanisms involving V(D)J recombination and somatic hypermutation (Walker et al., 2013).

There are five main translocations which account for the vast majority of primary IGH translocations in myeloma (Bergsagel et al., 1996; Chesi et al., 1996; Boersma-Vreugdenhil et al., 2004). These are t(4;14), t(11;14), t(6;14), t(14;16) and t(14;20) which result in juxtaposition of an IGH enhancer to an oncogene resulting in over-expression of the partner gene. The respective main partner genes are FGFR3 and MMSET, CCND1, CCND3, MAF and MAFB and the over-expression of these genes drives clustering of samples in expression microarray analyses. These primary translocations are usually detected in all myeloma cells and are thought to be initiating events. Secondary translocations involving the IGH locus are also detected the most frequent involving MYC on chromosome 8 (Gabrea et al., 2006). These can either be simple reciprocal t(8;14) translocations or complex non-reciprocal translocations or insertions juxtaposing MYC with an IGH enhancer, often involving three or more chromosomes.

IGH translocations are usually detected using fluorescence in situ hybridization (FISH) with a breakapart probe for the IGH locus to determine if a translocation has occurred. If positive, fusion probes for the five main partner chromosomes are tested to determine the translocation. In a small proportion of samples an IGH translocation that appears to be primary is detected but the partner chromosome cannot be determined. Here we used a custom capture pull-down panel of RNA
baits, complementary to the $IGH$ locus, and paired-end massively parallel sequencing to determine the unknown partner chromosome.

**MATERIALS AND METHODS**

Conventional Cytogenetics and FISH

CD138-positive bone marrow plasma cells were selected to a purity >95% using magnetic assisted cell sorting (Miltenyi Biotech, Bisley, UK). G-banded chromosomal analysis was carried out as previously described (Chiecchio et al., 2006) on 1-200 metaphases depending on availability. Interphase FISH was performed on plasma cells using a panel of commercial and in-house probes, as previously described (Ross et al., 2005; Chiecchio et al., 2006). Results were available for del(13q), $IGH$ break-apart, t(4;14)(p16;q32), $CCND3$ break-apart (6p21), t(11;14)(q13;q32), t(14;16)(q32;q23), $MAFB$ break-apart (20q11), $TP53$ (17p13), $CDKN2C$ (1p32), and $CKS1B$ (1q21) together with 17 and 22 centromere, and ploidy status. The interphase FISH method used to estimate ploidy and classify patients into groups with and without hyperdiploidy had been previously designed and assessed in myeloma patients (Chiecchio et al., 2006). The cut-off levels for interphase FISH scoring recommended by the European Myeloma Network (EMN) FISH workshop (10% for fusion/break-apart probes and 20% for numerical abnormalities) were followed.

Capture Design and Sequencing

We developed a targeted capture using the SureSelect (Agilent, Stockport, UK) system by tiling RNA baits across the $IGH$, $IGK$ and $IGL$ loci. Baits covered the V, D and J segments as well as being tiled across the entire constant region, including the switch regions in the $IGH$ locus.

DNA from four samples where an unknown $IGH$ translocation partner had been identified were assayed using 150 ng of DNA and a modified capture protocol (Kozarewa et al., 2012) with eight cycles of pre-hybridization PCR and 11 cycles of post-hybridization PCR. Samples were barcoded using Illumina indexes and were sequenced 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 289X per sample after deduplication for the captured region.

Translocation breakpoints were identified in the sequencing data using both a visual scanning of the alignments in IGV and using SVDetect (Zeitouni et al., 2010). Derivative chromosome breakpoints were reconstructed using Velvet or by manually aligning reads. Once derivative chromosome breakpoints were reconstructed they were validated by PCR. PCR primers used were 5’-GGGGAGCATGGAGTTTATTCAG -3’ and 5’-AGCATACTGCAGTTTGGCC-3’.

**RESULTS**

Myeloma samples routinely underwent G-banding analysis and FISH in order to determine key prognostic genetic markers, including IGH translocation partners. One historical sample for which an IGH translocation was detected, but the partner unidentified, was investigated further using a targeted capture pull-down followed by massively parallel sequencing. Using the IGH breakapart probe this sample showed signals on chromosome 8 (red, IGH 3’ flanking probe) and chromosome 14 (fusion). 18% of the interphase nuclei showed a single fusion with the IGH/MYC probe but there were no dual fusions. Karyotype and FISH data for this sample are shown in Tables 1 and 2.

In this sample, 90029, 273 paired reads identified a translocation between chromosomes 7 and 14. On chromosome 14 the paired reads mapped to the IGHA constant regions. Due to the homology between constant regions of the same isotype we found paired-end reads which mapped to chromosome 7 in both the IGHA1 and IGHA2 constant regions. This is most likely due to random assignment of reads to either IGHA1 or IGHA2 by the alignment algorithms and it is bioinformatically impossible to identify in which of these constant regions the breakpoint actually resides. Although the breakpoint lies within the constant region of the IGHA segments it is likely that this translocation was mediated by class switch recombination. On chromosome 7 the breakpoint was located at position 55,056,006 in the intergenic space between the genes SEC61G and EGFR. A schematic of the rearrangement is shown in Figure 1A. The breakpoint is 30,719 bp
upstream of \textit{EGFR} and 229,066 bp upstream of \textit{SEC61G}. The breakpoint was realigned and mapped against the original chromosome sequences, as shown in Figure 1B. Primers were designed either side of the breakpoint and a PCR performed to confirm the sequence (Figure 1C). The paired reads on both chromosomes are centromeric of the breakpoints, indicating the resulting derivative chromosome would be dicentric. However, the karyotype for this sample is complex and does not indicate a dicentric chromosome. Based on the karyotype and metaphase FISH it is more likely that a complex rearrangement involving chromosomes 7, 14 and 8 occurred. FISH results indicate that this translocation may be a secondary event as other chromosomal abnormalities (del(13q), 1q+, 11q+, gain 5/9/15) in this sample were detected in 97-99% of cells, whereas the split \textit{IGH} was detected in 85% of cells.

We used expression microarray data using a published dataset (GSE15695 (Dickens et al., 2010)) containing expression values for this sample to look for over-expression of both \textit{SEC61G} and \textit{EGFR}. We plotted the expression values for the probes for both \textit{SEC61G} and \textit{EGFR} (Figure 2) and it can clearly be seen that the expression of \textit{SEC61G} in this sample is no different to the other samples, whereas the expression of \textit{EGFR} is markedly higher in the sample with the t(7;14) and is the target oncogene. It is interesting to note that in this dataset of 258 presentation myeloma samples this is the only sample which expresses \textit{EGFR} to any level, indicating that this is a rare translocation event. We also analyzed a gene expression dataset (Mulligan et al., 2007) consisting of 264 relapsed myeloma patients (GSE9782) using the same probeset and saw no expression of \textit{EGFR}.

This patient was enrolled as part of the UK MRC Myeloma IX trial, which has been reported previously (Morgan et al., 2010). The patient received zoledronic acid and cyclophosphamide, thalidomide and dexamethasone (CTD) as part of the intensive pathway. The overall survival for this patient was 25.3 months compared to the median for this treatment arm which was 61.0 months (Morgan et al., 2010).

**DISCUSSION**

Certain \textit{IGH} translocations in myeloma are considered primary events and are present in up to 45% of presenting myeloma cases (Morgan et al., 2012). The
determination of these primary translocations is important for determining the prognosis of the patient (Walker et al., 2010; Boyd et al., 2012). Translocations can be divided into those with neutral or poor prognosis where the t(11;14) and t(6;14) are considered neutral and the t(4;14), t(14;16) and t(14;20) are considered poor (Boyd et al., 2012). Interestingly, the poor prognostic translocations result in over-expression of \textit{CCND2} whereas the neutral prognostic translocations over-express \textit{CCND1} or \textit{CCND3} (Bergsagel et al., 2005). More recently, the development of new drug treatments has indicated that the poor prognostic impact of the t(4;14) can, in part, be negated by the use of bortezomib (Avet-Loiseau et al., 2010). It is therefore important, both biologically and clinically, to be able to determine primary translocations in myeloma patients.

Both FISH and chromosomal banding analysis have been used to identify the translocations in myeloma and, in this respect, FISH is considered the gold standard technique. However, subtle or complex rearrangements between chromosomes can make the interpretation of FISH results difficult. As new technologies have been developed they can be used in conjunction with the existing techniques to more accurately determine the chromosomal structure in these difficult to analyze cases. We therefore set out to use a targeted capture and next generation sequencing to assist in the elucidation of the partner chromosomes.

Here we report a case where an \textit{IGH} translocation was detected by FISH but the partner chromosome could not be identified. This sample represents a tiny proportion of total FISH tests carried out, but nevertheless this minority of samples could be interesting both clinically and biologically. We therefore set out to determine the partner chromosome in this case.

The sample in which the unidentified translocation partner was elucidated identified a t(7;14) with the breakpoint 5’ of \textit{EGFR}. This sample over-expresses \textit{EGFR} due to its juxtaposition with the 3’ \textit{IGH} enhancer. The sample has a complex karyotype and combined with FISH, a targeted capture and expression array data it is still not possible to fully understand the chromosomal structure. However, it is clear that the positioning of the \textit{IGH} enhancer results in over-expression of \textit{EGFR}. It is the only
sample in our dataset of 258 presentation myeloma samples to express EGFR and represents a rare event.

EGFR is a member of the HER family of tyrosine kinase receptors whose principal ligands are EGF, TGF-α and amphiregulin (Okines et al., 2011). Stimulation of the receptor induces homo- or heterodimerization, stimulating the intracellular signaling cascade through Ras, Raf and MEK resulting in cell-cycle progression, cell division, survival, invasion and adhesion. EGFR over-expression is not common in myeloma but is a common finding in advanced-stage oesophagogastric cancer and correlates with poor prognosis. Although EGFR activation is rare in myeloma, activation of the Ras pathway is frequent through activating mutations in KRAS, NRAS and BRAF which account for ~50% of presentation myeloma samples (Chapman et al., 2011; Walker et al., 2012). Ras pathway mutations are not regarded as primary events in myeloma and can be found in presentation myeloma as a sub-clone. This EGFR translocation was also found at a lower frequency compared to some other genetic abnormalities, such as del(13q), and is also probably a secondary event in disease progression.

The identification of a t(7;14) indicates an alternative mechanism for activation of the pathway and that this patient could have benefited from a targeted treatment. EGFR can be targeted using HER-inhibiting antibodies such as cetuximab, panitumumab and matuzumab which have been trialed successfully in oesophagogastric cancer, adenocarcinoma and squamous-cell carcinoma (Okines et al., 2011). Tyrosine kinase inhibitors, such as erlotinib or gefitinib, also exist to act against EGFR activation. This targeted treatment aligns well with current clinical trials in myeloma using BRAF inhibitors for the 4% of patients with a BRAF^{V600} mutation.

Continued molecular analysis of complex genetic myeloma cases can result in the identification of novel oncogenes relevant to the disease. In some cases, such as the t(7;14), molecular characterization of rare events could be therapeutically beneficial to the patient if activated oncogenes are identified in which a targeted therapy exists in another cancer, including EGFR.
Acknowledgments
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REFERENCES


Age has a profound effect on the incidence and significance of chromosome abnormalities in myeloma. Leukemia 19:1634-1642.


FIGURE LEGENDS

Figure 1: Detection of a t(7;14) in multiple myeloma. 
A, Schematic of the t(7;14) juxtaposing the 3’ Enhancer next to EGFR. 
B, Breakpoint sequence of the t(7;14) showing the base pair locations on chromosomes 7 and 14. 
The derivative chromosome sequence is shown in bold. 
C, Sanger sequencing trace of the amplified translocation indicating the boundary of chromosome 7 and 14 sequences.

Figure 2: Expression of EGFR and SEC61G in 258 presentation myeloma samples. 
The open box data points show the value for sample 90029 with the t(7;14).
Figure 1

A

B

Chr.14 GCCGTCCACCCTCGCCATGACACACACATTGAGTGGTGGTTTACCGCCACTGCAGGTCGATGGTC 106173586
Der GCCGTCCACCTCGCCATGACACACACATTGAGTGGTGGTTTACCGCCACTGCAGGTCGATGGTC 106173586
Chr.7 TAAATAGAAAGCTTGATTTCAAGGCCAAAGAAAGTGGGCTTTTGTGTCTAAAAATACAGGGTTCTATAA 55056010
Chr.14 TTCTGTGAGGACGGGAGGGCCGGCGCTCGTGGGCCCACCGATGACGAGGTTGTCCTCCTCTCCAGT 106173656
Der GCCAGAGCTTTACTTGATAAGACTTTTTCCAAAAATTCATTTCTTTAATTTCAACTATTCAATTTTAATTGT 55056080
Chr.7 GCCAGAGCTTTACTTGATAAGACTTTTTCCAAAAATTCATTTCTTTAATTTCAACTATTCAATTTTAATTGT 55056080

C

Chap. 14  

Chap. 7  

Figure 1
Figure 2