Genetics in myeloma: genetic technologies and their application to screening approaches in myeloma

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Abstract

Background: Despite advances in the treatment of multiple myeloma (MM), it remains an incurable malignant disease. Myeloma genetics is intrinsically complex, but it offers an opportunity to categorize the disease and apply a personalized medicine approach.

Areas of agreement: Research into the genetics of myeloma is moving at a fast pace and is highlighting areas and patient cohorts likely to benefit from specific treatment. Targeting residual disease is likely to be crucial to improved clinical outcome.

Areas of controversy: Patients in clinical trials are more likely to receive genetic diagnosis than non-trial patients, for whom access is ad hoc and dependent upon regional commissioning arrangements.

Areas timely for developing research: Relating genetics to potential treatment pathways will become crucial for improved myeloma outcomes. Universal access to standardized genetic testing will facilitate modern personalized treatments.

Key words: myeloma, diagnosis, personalized medicine, genetic technologies

Introduction

Multiple myeloma (also known as plasma cell myeloma) is a neoplastic disorder characterized by an abnormal monoclonal proliferation of plasma cells in the bone marrow (BM) and overproduction of circulating monoclonal immunoglobulin (paraprotein). The paraprotein product circulates in blood and is deposited in various tissues including the renal
tubules, causing substantial renal impairment, and in other organs including the heart and the gut. At these sites, the paraprotein is prone to transformation into a degradation-resistant protein known as amyloid, which significantly impairs organ function. Malignant plasma cells secrete osteoclast-activating factors and osteoblast-deactivating factors leading to destructive, osteolytic bone disease. Malignant plasma cells accumulate within the BM effectively crowding out normal haemopoietic tissue leading to BM failure manifesting as anaemia, thrombocytopenia and leukopenia as well as impaired cellular and antibody-mediated immunity.

Plasma cell neoplasms progress through distinct clinical phases: monoclonal gammopathy of unknown significance (MGUS), asymptomatic myeloma or smouldering myeloma (SM), plasma cell myeloma, progressing to plasma cell leukaemia. Other clinical phases following treatment can include myeloma in remission (termed 'plateau phase'), relapsed myeloma and refractory myeloma (where disease is non-responsive to treatment). Plasma cell myeloma is defined by the presence of >10% clonal plasma cells in the BM, a paraprotein and the presence of end-organ damage, which can be summarized by the acronym, CRAB.

- HyperCalcaemia
- Renal insufficiency
- Anaemia
- Bone lesions

Hypercalcaemia results from bone destruction and is seen in 20% of patients at diagnosis. Renal insufficiency and ultimate failure is due to tubular damage resulting from proteinuria. Anaemia is seen in ~67% of patients, and results from effacement of BM by plasma cells and renal damage resulting in loss of erythropoietin. Osteolytic disease is seen in 70% of patients with MM.

Myeloma is the 17th most common cancer in the UK accounting for 1.5% of all new cases reported (source: Cancer Research UK, 2009). This equates to ~5000 new patients being diagnosed with myeloma in the UK per year. MM is a cancer of older adults: the majority of cases occur in patients over the age of 65. Twenty-six per cent of patients are aged between 65 and 74 at diagnosis, and 37% are over 75. MM is diagnosed in younger people, with 37% of patients aged 65 or under, and 2% below 40 years of age. MM is not a disease that is seen in children. Myeloma is likely to increase in incidence in the UK in line with the ageing population. Data suggest that MM is more common in men than in women (ratio 7.1:4.3), and more common in Black people than in people of Asian or Caucasian descent (ratio ~2:1).

There is a 3.7-fold increased risk to first-degree family members of patients with MM, suggesting some inherited component. Fifteen per cent of MM patients have no symptoms at presentation, and a high paraprotein may be discovered following routine screening. Approximately 40% present with more substantial morbidity including anaemia, renal failure and skeletal disease including pathological fractures, hypercalcaemia, spinal cord compression or generalized bone loss (osteoporosis). Less critical symptoms can include backache, bone pain, anaemia and tiredness. Diagnosis is dependant on results from a number of clinical tests, including full blood count and chemical analysis, serum and urine electrophoresis, BM morphology, radiography and genetic analysis.

Treatment strategies are conventionally divided into intensive and non-intensive regimens. The former feature chemotherapy using a combination of steroids (e.g. dexamethasone), alkylating agents (e.g. cyclophosphamide) and immunomodulatory agents (e.g. thalidomide or lenalidomide) plus or minus a proteasome inhibitor (e.g. bortezomib or carfilzomib) for younger (<60 years), fitter patients, followed by autologous stem cell transplantation. High-dose melphalan (i.e. 200 mg/m²) is used as a consolidation therapy prior to autologous stem cell transplantation, further reducing plasma cell load within the BM. Non-intensive regimens comprise similar combinations used at attenuated doses and without autologous stem cell transplantation.

Immunomodulatory drugs, such as lenalidomide (and other thalidomide analogues), have been shown to have clinical efficacy in the treatment of myeloma. Recent work has shown that these immunomodulatory drugs can bind to and inhibit the cereblon ubiquitin ligase. Furthermore, lenalidomide-bound
cereblon acquires the ability to target two specific lymphoid transcription factors, \textit{IKZF1} and \textit{IZKF3}, both known to play a central role in B and T cell biology, for selective ubiquitination and degradation.\textsuperscript{7,8} Proteasome inhibitors are also extremely effective in myeloma treatment. The proteasome is an intracellular enzyme complex that breaks down damaged proteins. The exquisite sensitivity of myeloma to proteasome inhibitors remains largely unexplained, although the drugs are thought to act in a multifaceted and extensive mechanistic fashion. They have been thought to stimulate apoptotic pathways, inhibit the NF-\textit{kB} pathway, down-regulate expression of genes associated with DNA repair and induce an endoplasmic reticulum stress response.\textsuperscript{3–11} Bisphosphonates are an important class of drugs used to treat bone manifestations in MM. They are potent inducers of osteoclast apoptosis, thereby reducing elevated bone resorption associated with MM.

Shorter survival times correlate with higher clinical stage at diagnosis, renal insufficiency, degree of marrow replacement, increased proliferative activity and certain karyotypic abnormalities.\textsuperscript{12} Although significant advances have been made in MM treatment over the past two decades, and it is now regarded as highly treatable, myeloma remains almost always incurable. The only wholly curative option available for the treatment of myeloma is allogeneic stem cell transplantation. However, although this intensive procedure can achieve long-term remission, it is associated with a high-treatment related mortality and risk of relapse and tends to be considered only in a small minority of patients.\textsuperscript{13} Eighty-eight per cent of myeloma patients will survive 1 year, median survival is 3 years and 10-year survival is only achieved in 10\% of patients.\textsuperscript{12} Importantly, there is a subset of patients for whom current treatment modalities are not effective, with \~20\% of clinical responses sub-optimal and 5\% non-responsive.

**Plasma cell biology**

The human immune system has evolved to confer resistance to infection. Plasma cells are part of the ‘adaptive’ component and are required to produce antibodies in response to antigenic insult.\textsuperscript{14–16} The immunoglobulin (Ig) antibody molecule is composed of two heavy chain and two light chain proteins. These are encoded by the \textit{IGH} gene for the heavy chain, located on chromosome 14, and the \textit{IGK} and \textit{IGL} genes for the light chain, located on chromosomes 2 and 22, respectively.\textsuperscript{15} Variable gene segments at these loci undergo irreversible rearrangement, at the DNA level, and this creates individual B cells with specificity for a single antigen. In summary, the several stages of B-cell development each represent a change to the genomic DNA involving the variable (V), diversity (D) and junctional (J) gene segments of the immunoglobulin genes. These stages can be divided into three processes, all of which generate double-stranded DNA breaks: VDJ recombination, somatic hypermutation and IgH-switch recombination.\textsuperscript{17,18}

Following maturation, plasma cells have undergone the final stages of development and home to the BM.\textsuperscript{17} These cells are long-lived, terminally differentiated and non-dividing. They are highly dependent on the BM microenvironment where their survival is favoured by survival factors found in permissive niches.\textsuperscript{19,20}

Plasma cells interact with the BM microenvironment via a number of complex interactions, which are crucial to tumour survival and disease progression.\textsuperscript{19} The BM microenvironment is made up of extracellular matrix and five types of BM stromal cells: fibroblastic stromal cells, osteoblasts, osteoclasts, vascular endothelial cells and lymphocytes.\textsuperscript{21,22} The crosstalk between myeloma cells and the microenvironment can occur either directly or indirectly, via local secretion of adhesion molecules, cytokines and growth factors. The strong association between these cell types is thought to be pivotal in the pathogenesis of osteolytic lesions and bone loss associated with MM.\textsuperscript{17} In both MGUS and early MM, plasma cells are highly stromal dependant; this dependence decreases as the disease progresses. Many of the cellular interactions involved are governed by genetic changes affecting their normal regulation.\textsuperscript{19}

While key to the creation of a diverse adaptive immune repertoire, these complex developmental processes imply an inherent genome instability, and it is this required instability that may facilitate oncogenic transformation.
Genetics of plasma cell neoplasms

Myeloma is a genetically complex disorder characterized by multiple genetic changes, affecting different pathways, that have the ability to deregulate plasma cell biology leading to a broadly similar phenotypic manifestation of disease. This genetic heterogeneity, in part, is likely to have hindered the development of effective treatments. A deeper and clearer understanding of the genetic abnormalities, and their role in specific pathways, may offer new routes for drug development. This could enhance the clinicians’ ability to offer a personalized medicine approach when treating patients with MM.

Hyperdiploidy vs. hypodiploidy

From a genetic perspective, myeloma can be divided into those with and without a hyperdiploid karyotype. Hyperdiploidy is seen in ~30–40% of patients. Chromosome number ranges from 48 to 75, median 53, and the chromosome gains are non-random, often involving the odd numbered chromosomes, 3, 5, 7, 9, 11, 15, 19 and 21. These changes are not seen as serial gains, but rather as the result of a single catastrophic mitotic event. Hyperdiploidy in myeloma has been associated with a better overall survival when compared with the non-hyperdiploid group.

The non-hyperdiploid group includes karyotypes with hypodiploid, pseudodiploid or near tetraploid chromosome number. The near tetraploid groups appear to be a doubling (i.e. 4n) of the hypodiploid and pseudodiploid cell lines. The non-hyperdiploid groups are typically associated with IGH translocations, although IGH rearrangements are also present in ~10% of the hyperdiploid group.

Translocations

Non-hyperdiploid patients are frequently (55–70%) associated with rearrangements of IGH on chromosome 14. IGH rearrangements are considered promiscuous as they have many partner genes. They are usually simple reciprocal translocations juxtaposing the IGH enhancers to an oncogene. This gives rise to abnormal expression of the oncogene and contributes to the myelomagenic effect. Detailed in Table 1, the five main translocation partners are the following: t(4;14), t(11;14), t(6;14), t(14;16) and t(14;20); together these are seen in ~40% of patients. These rearrangements are thought to be associated with up-regulation of one of the cyclin D genes. t(11;14) and t(6;14) directly deregulate CCND1 and CCND3, respectively; t(14;16) up-regulates CCND2 as MAF directly binds to the CCND3 promoter and t(4; 4) also up-regulates CCND2 via FGFR3 and MMSET, although the exact mechanism is unknown. Such translocations are mediated by errors in DNA modification associated with B-cell maturation, following plasma cell–antigen interactions. IGH switching is considered the most common, while somatic hypermutation and VDJ recombination are likely to be less frequent events.

The t(4;14)(p16.3;q32) translocation is cytogenetically cryptic. As a result, fluorescence in situ hybridization (FISH) is required to determine the presence of this rearrangement. The t(4;14) rearrangement involves the IGH gene and two protein-coding genes located at 4p16.3, multiple myeloma SET domain (MMSET) and the fibroblast growth factor receptor 3 (FGFR3), an oncogenic receptor tyrosine kinase. In the balanced translocation, both FGFR3 and MMSET are juxtaposed to two IGH enhancers. FGFR3 is overexpressed from the derivative chromosome 14, and MMSET is overexpressed from the derivative chromosome 4. Twenty-five percent of cases are seen in an unbalanced form, with loss of the derivative chromosome 14, which is associated with the loss of the aberrant FGFR3 expression. This translocation is universally associated with a poor prognosis. It is seen in MGUS (~3% of cases), but more highly associated with SM and MM. Data suggest that these patients benefit from bortezomib therapy, and in addition, TKI-258, an FGFR3 inhibitor, is also undergoing clinical evaluation.

t(11;14)(q13;q32) involves translocation of the CCND1 gene located at 11q13, where it comes under the regulatory influence of IGH at 14q32. This translocation is balanced in the majority of cases, although additional copies of the derivative...
chromosome 14 have been seen and are thought to be associated with progressive disease.

The t(6;14)(p21;q32) involves the gene CCND3 at 6p21, resulting in its up-regulation. It is often seen on a backdrop of a complex karyotype, and the derivative chromosome 14 can be present in multiple copies. This is a rare event and has only been associated with ∼3% of MM patients.19

t(14;16)(q32;q23) and t(14;20)(q32;q11) both show juxtaposition of the IGH gene to a MAF family gene, MAF and MAFB, respectively.34 MAF rearrangements have been described in 5–7% of MM and considered to be mediated by a fragile site in the WWOX gene on the long arm of chromosome 16.17,23 This results in MAF coming under the regulatory influence of IGH, which in turn has the effect of up-regulating CCND2.19 MAF rearrangements have been associated with a more aggressive clinical course.23 Data on MAFB rearrangements associated with t(14;20) (Fig. 1A and B) are not robust due to their rarity, although a similar clinical course would be predicted.23

Translocations not involving IGH do occur, but they are considered unusual and are most likely to be seen in progressive disease. CCND3-MAF, MAF-FGFR3/MMSET, CCND3-FGFR3/MMSET rearrangements have all been described.19

MYC translocations are seen in ∼15% of patients with myeloma at presentation21,35,36 and up to 45% of patients with advanced disease.21,23 MYC translocations are not considered to be initiating events, but rather late events associated with increased proliferation and stromal independent plasma cells.21 They are frequently seen as non-reciprocal translocation events involving more than one chromosome and associated regions of amplification and duplication (Fig. 1C).21 The t(8;14)(q24;q32) accounts for only 25% of MYC rearrangement,17,36 and recent studies have demonstrated that MYC is able to recruit active super enhancers from highly expressed genes associated with B cell, plasma cell or myeloma development.37 Examples include enhancers associated with CCND1, XBP1, KRAS, FAM46C and CHST15.37

Deletions and duplications

MM genetics includes copy number variation as a common and recurrent finding.19,23 Theoretically, and simplistically, deletions are likely to involve loss of tumour suppressor genes at those sites, and duplications are likely to be associated with overexpression of genes within the region. Regions of recurrent deletion and/or duplication include 1p, 1q, 9q, 11q, 12p, 13q, 15q, 16q, 17p, 19q and 22q,17,19,23,38 and a number of these are described further.

Deletion of chromosome 13 was the first chromosome abnormality to be associated with a poor prognosis in myeloma.39 Chromosome 13 abnormalities are seen in 50% of myeloma patients;23 of these, 85% show chromosome 13 monosomy, and the remaining 15% involve an interstitial deletion of 13q14 which includes the RB1 gene.36,40 Recently, the poor prognosis associated with deletion/monosomy of chromosome 13 has been contested in the literature. Its close association with the presence of t(4;14) suggests that the statistically poor prognosis may be a result of association alone.23,41

Table 1 Detailing the five main translocations associated with IGH in myeloma and the normal gene role or function

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Gene(s)</th>
<th>Frequency</th>
<th>Prognosis</th>
<th>Gene role/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(4;14)(p16.3;q32)</td>
<td>FGFR3 MMSET</td>
<td>15%</td>
<td>Poor</td>
<td>Bone development and maintenance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oncogene, overexpression results in proliferation and anti-apoptotic effects</td>
</tr>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>CCND1</td>
<td>15%</td>
<td>Good</td>
<td>Cell cycle G1/S transition</td>
</tr>
<tr>
<td>t(14;16)(q32;q23)</td>
<td>MAF</td>
<td>5%</td>
<td>Poor</td>
<td>Oncogene, enhances tumour stroma interactions</td>
</tr>
<tr>
<td>t(6;14)(p21;q32)</td>
<td>CCND3</td>
<td>3%</td>
<td>Good</td>
<td>Cell cycle G1/S transition</td>
</tr>
<tr>
<td>t(14;20)(q32;q11)</td>
<td>MAFB</td>
<td>2%</td>
<td>Poor</td>
<td>Regulation of lineage-specific haematopoiesis</td>
</tr>
</tbody>
</table>
Deletion of the short arm of chromosome 17, the site of the TP53 tumour suppressor gene, remains the most important prognostic factor in myeloma genetics conferring an extremely poor prognosis. Deletion of 17p has been associated with shorter survival, aggressive disease, increased hypercalcaemia and the presence of extramedullary disease. Chromosome 1 rearrangements are the most common aberrations in myeloma. These usually result in deletions of the short arm and duplications of the long arm. Short arm deletions have been shown to span a region from 1p13 to 1p31 and have been associated with a poor prognosis. Genes that have been associated with this deletion include CDKN2C, FAF1 and FAM46C. Rearrangements of the long arm of chromosome 1 are often complex and have been associated with pericentromeric instability. They are seen in 40% of myeloma patients at diagnosis and 70% of cases at relapse. ‘Jumping’ translocations are frequently associated with chromosome 1q duplication and this term describes rare chromosomal events in which the same donor chromosome segment is translocated onto two or more recipient chromosome sites, such that it can be present in different guises in different cells. Implicated genes include CKS1B and...
ANP32E. Duplication of 1q is also associated with poor prognosis, although the intrinsic relationship with deletion 1p creates difficulties in assessing these abnormalities separately. A common manifestation of del1p/dup1q is the isochromosome 1q, (Fig. 1D and E), where the short arm is lost and the long arm is duplicated and mirrored around the centromere.

Uniparental disomy (UPD) represents a further mechanism by which alleles can be lost. UPD is a term used to describe loss of a chromosome, or chromosome part, which is then duplicated from the second allele. This results in loss of heterozygosity (LOH) without the associated loss of copy number. UPD can be seen associated with cancer genotypes and is termed ‘acquired UPD’. Walker et al. have demonstrated that acquired UPD/LOH is prevalent in MM with a median number of three UPD regions per sample. The size ranged from 677 kb to whole chromosomes, but tended to implicate relatively small regions at 1p, 1q, 6q, 8p, 13q and 16q. These areas of UPD may highlight areas containing genes important in myeloma pathology.

Mutations and implicated pathways
Recent studies involving whole exome sequencing (WES) have suggested that an average of 35 genetic mutations is present per MM patient sample. This figure sits part way between the suggested mutation level of 8 in the more genetically simple haematological malignancies and ~540 mutations detected in genetically complex epithelial tumours.

Determination of these multiple mutations, and the general genetic heterogeneity, supports the hypothesis that a pathological requirement of MM is pathway deregulation, rather than specific gene rearrangement or modification. Do the multiple genetic abnormalities converge to result in a more simplified effect, of targeting a smaller number of specific functional pathways?

Keats et al. have demonstrated constitutive activation of the NF-kB pathway in 50% of MM cases using gene expression profiling. This is not caused by a single mutation, but rather a collection of gene mutations and deletions. Genes implicated include BIRC2 and BIRC3 at 11q, CYLD at 16q and TRAF3 at 14q32. Increased NF-kB nuclear activity is thought to have an anti-apoptotic effect. In addition, data suggest that low-level TRAF3 expression is associated with a better response to bortezomib.

Other affected pathways include the Wnt signalling pathway, RANK, PI3K, JAK and the mitogen-activated protein kinase (MAPK) pathways. Bone damage associated with MM results from an imbalance in osteoblast and osteoclast function. The Wnt signalling pathway supports osteoblast function; therefore, pathway inhibition results in osteoblast suppression and reduced bone formation. The RANK pathway is often enhanced in myeloma, and the resulting MIP1-α activation stimulates osteoclasts, resulting in increased bone resorption. The MAPK pathway is dysregulated in ~55% of MM patients. Genes that have been associated with MAPK signalling include KRAS, NRAS, BRAF and more recently NFI and RASA2.

Epigenetic changes
Epigenetic factors are also involved in the aetiology of MM, with changes to both DNA glycosylation/acetylation and histone modification playing a part in modulating gene expression. Global DNA hypomethylation and specific gene hypermethylation have been reported in association with the transformation of MGUS to MM. Fifteen per cent of t(4;14) patients show a gene-specific hypermethylation pattern. Overexpression of MMSET leads to histone modification which in turn promotes cell survival and cell cycle progression. Dysregulation of miRNA has been associated with a particular gene cluster on chromosome 13 implicated in the MGUS to MM transition.

Genetic predisposition
An inherited genetic variation at 2p23.3, 3p22.1 and 7p15.3 has been associated with a genetic predisposition to MGUS. More recently, genome-wide association studies (GWAS) have shown further regions of common variation at 3q26.2, 6p21.33, 17p11.2 and 22q13.1. Chubb et al. suggest that the seven loci identified are likely to account for ~13% of the familial risk of myeloma, and this highlights the likelihood
that many more variants associated with a genetic susceptibility await discovery.

Intraclonal heterogeneity

Recently, WES, cytogenetics and copy number analysis have been used to demonstrate that the majority of myeloma cases have a complex subclonal structure. Bolli et al.\textsuperscript{50} have demonstrated, using serial sampling, that clonal evolution is diverse and includes both linear and branching evolution. This suggests that myeloma populations are not homogeneous, but made up of different populations with different clonally related and unrelated changes. These clones can demonstrate a differential response to therapy. This raises treatment issues; reducing the level of one clone may result in expansion of a more prognostically detrimental clone. The process of branched evolution and the existence of intra-tumour heterogeneity provide further ambiguity, since a single genetic picture at a single time point is likely to under-represent the complexity of the underlying disease.\textsuperscript{55}

Genetic analysis in myeloma (advantages and disadvantages)

Genetic analysis of BM samples in myeloma patients has proved to be useful, offering both diagnostic and prognostic information to the clinician and the patient.\textsuperscript{4} Since myeloma is a genetically heterogeneous disease, it is likely that genetic abnormalities will increasingly be used to inform treatment decisions.\textsuperscript{13,56} Patients exhibiting a t(4;14) are more likely to be offered more aggressive therapy, including bortezomib, first line, and \(BRAF\) inhibitors incorporated where \(BRAF\) mutations exist.

Cytogenetic analysis

Cytogenetic analysis in MM relies on the ability to capture plasma cells during the metaphase period of cell division. Robust techniques are employed in many laboratories to manipulate the cell cycle. These often utilize colchicine, or one of its analogues, to disrupt formation of the spindle apparatus and thereby induce cell-cycle arrest at the metaphase stage of mitosis. At metaphase, the chromosomes have condensed and are preparing to arrange themselves on the equator of the spindle apparatus, before anaphase proceeds to split the two chromatids into separate daughter cells. Harvesting samples at this stage and treating chromosomes with trypsin and stain (e.g. Leishmans or Giemsa) produces a characteristic banding pattern, allowing chromosomes to be karyotyped.

Although conventional G-banded cytogenetic analysis offers the benefit of a whole genome analysis, albeit at low resolution, the requirement for cells in metaphase can be extremely problematic due to the low proliferative rate of terminally differentiated mature plasma cells. Even introducing adaptations to the culturing process, allowing for longer term culturing (4–6 days), cytogenetic abnormalities are only reported in \(\sim 30\%\) of myeloma patients.\textsuperscript{32} This low-level detection is likely to reflect failure to capture the plasma cells in division, rather than a reflection of the true abnormality rate. This is borne out by the much higher abnormality rate reported by FISH techniques.

Cytogenetic analysis requires the individual karyotyping of a number of cells, this time consuming, skilled analytical step contributes to the slow and expensive nature of this methodology.

FISH

FISH involves hybridizing fluorescently labelled target DNA, from a region of interest, to metaphase or interphase cells to allow enumeration or rearrangement detection (Fig. 2). The ability to utilize interphase cells eliminates the problems associated with metaphase capture for cytogenetic analysis.

FISH allows analysis of increased cell numbers (50–200 cells). However, this remains problematic since plasma cell populations can be as low as 10% in MM patients and lower still in MGUS. Methods of plasma cell enrichment are usually employed to overcome this issue. Plasma cells can be sorted using magnetic-activated cell sorting (MACS\textsuperscript{TM}), which
entails collection of CD138-positive cells or coFISHed using fluorescent cytoplasmic immunoglobulin (cIg) stain, to highlight plasma cells. An abnormality rate of 90% is reported in MM samples when FISH techniques are utilized.

The resolution of FISH is limited by probe size, which ranges from 150 kb to 1 Mb. Small deletions or mutations within genes or within the probe target cannot be detected. Specific probes have an associated false-positive rate, dependant on the probe set design. This rate is typically quoted at 1–4% by manufacturers.

FISH can be an expensive technique when multiple probes are used, but small panels offer an efficient and accurate way to detect rearrangements and copy number changes. Hyperdiploidy can be confidently assessed using a 3-chromosome combination of probes (chromosomes 9, 11, 15). The presence of two of the three chromosomes is a highly specific indicator of hyperdiploidy.57

DNA microarray/comparative genome hybridization

DNA microarrays employ similar technology to comparative genome hybridization (CGH). Both DNA of interest and control DNA are prepared and fluorescently labelled: control DNA in red and DNA of interest in green. The two DNA samples competitively hybridize to an array chip displaying complementary DNA oligonucleotides. In the case of equal copy numbers in the two DNA samples, the competitive hybridization will be equal. Increased copy number in the DNA of interest will result in a stronger green fluorescence signal. Conversely, when deletions are present in the DNA of interest, reduced competition will result in dominant red fluorescence. The level of fluorescence is measured across the chip for each oligonucleotide to identify regions of loss and gain across the genome.

DNA microarrays are designed to provide a full genome screen with a backbone level of resolution.

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**Fig. 2** Schematic diagram to demonstrate the process of FISH. Probe DNA of interest, usually relating to a specific gene rearrangement or deletion, is labelled with a fluorescent dye and co-denatured alongside the target DNA to create single-stranded DNA. The single-stranded DNA is hybridized together allowing competitive reannealing to occur. A series of post-hybridization washes remove any excess probe and the target and probe can be visualised using a fluorescence microscope.
across the genome, and areas of specific interest (for example, cancer-related probe sets) can have enhanced resolution. Current arrays permit interrogation of the entire genome using >2.6 million markers for copy number analysis and ∼750 000 single nucleotide polymorphisms (SNPs). The higher probe density areas include >500 cancer genes, all of which are covered with 25 markers per 100 kb region. Although SNP incorporation allows for extended analysis of LOH, DNA array technology remains predominantly a high-resolution method for detecting copy number changes since balanced translocations and mutations at a base pair level are not detectable.

**Gene expression analysis**

Gene expression profiling (GEP) analysis takes the analysis of genetic change one step further and allows the impact of these changes on the messenger RNA (mRNA) to be measured by assessing the activity of the cells at a point in time. This is usually done by simultaneous comparison between patient sample time points or against a normal control. DNA modifications in myeloma have been shown to be accurately assessed by GEP, offering a comprehensive and sensitive approach for myeloma classification and risk stratification. Issues relating to the complexity of the analysis, interpretation and maintenance of good quality control make this a less robust technique in a routine clinical setting. The high risk groups, specifically, have a broad range of clinical outcomes, suggesting that further work is required to create robust analytical methodologies before GEP can be utilized routinely.

**Multiplex ligation-dependant probe amplification**

Multiplex ligation-dependant probe amplification (MLPA) is a multiplex polymerase chain reaction (PCR) methodology, which allows simultaneous detection of deletions or duplications of up to 50 targeted gene regions. As the probe sequence is small (~60 nucleotides), it can detect deletions of an exon, smaller than conventional FISH techniques. MLPA probes are designed in pairs with the same two primer sequences present in each of the pairs. One member of the pair incorporates a stuffer sequence, thus creating a known total probe length. The DNA is denatured and hybridized with the MLPA probes that attach to the region of interest. When the DNA target is present with no alterations, the probe pairs lie immediately adjacent to each other and can ligate. When there is a deletion, or in some cases a mutation, the probes cannot hybridize to the DNA correctly, and ligation does not occur. PCR is used to directly amplify the ligated probe pairs and not the target sequence. As only ligated probes will be exponentially amplified, the number of products is a direct measure of the number of target sequences, compared with control. Separation of the products is carried out using electrophoresis, and the known probe lengths allow identification of the specific target region; the steps are depicted in Figure 3.

MLPA offers a cost-effective, multiplex technique to assess loses and gains in up to 50 regions across the genome. The limitations of MLPA are that, used in isolation, only copy number changes can be detected (and only in the regions that have been targeted by the probe set). MLPA cannot distinguish between a mutation and a deletion, as both scenarios result in non-amplification. The technique is highly sensitive to contamination, and furthermore, low-level abnormal populations can be problematic. In the case of MM, plasma cell purification to a minimum 50% purity would be a prerequisite. MLPA has proved its worth in studies of MM; however, as its power is restricted to detection of gains and losses, it should be used in conjunction with FISH for detection of *IGH* rearrangements.

**Next-generation sequencing**

The emerging technologies of high-throughput or next-generation sequencing (NGS), including whole genome sequencing (WGS), WES and targeted NGS panels, offer the highest level of resolution for genetic screening. NGS has evolved dramatically over the last few years; it offers the power of simultaneous sequencing of multiple DNA templates, without the requirement of the same number of target-specific DNA primers. Commercial platforms have also evolved...
providing confidence in the robustness of this approach to provide accurate and comprehensive means of mutational analysis.

There are a range of NGS platforms currently on the market, each of which employs different sequencing chemistries but with similar data output. All begin with the production of short DNA fragments with the addition of non-specific adaptor DNA sequences. This can be achieved either by chemical binding or ligation. Adaptor sequences allow DNA fragments to be captured onto minute bead structures or tethered to a solid surface (microchip). Subsequent PCR is then carried out in emulsion within tiny wells (big enough to hold one bead) or by implementing a bridging PCR reaction. In all approaches, this step allows single DNA fragments to be amplified non-specifically from the same clone, making the signals in the later stages more easily readable.

Emulsion PCR is based on the technique of pyrosequencing in which the reaction is flooded sequentially and repeatedly with each of the four deoxyribonucleotides, G, C, A and T. Incorporation of a nucleotide results in emitted light, or a change in pH, which is captured, allowing the sequence to be accumulated over hundreds of flow cycles. The Illumina™ chemistry utilizes sequencing primers that are complementary to the ligated adapter sequences. The PCR reaction mix is made up of chain terminating deoxyribonucleotides, labelled with a specific fluorescent tag, which corresponds to the base identity. During the extension phase, termed sequencing by synthesis (SBS), each individual base is recorded by its fluorescence. These

Fig. 3 Schematic diagram to demonstrate the process of MLPA. Paired probes around an area of interest are labelled with primer sequence. A stuffer sequence of known length is also included to allow for probe identification. The probes are annealed to the DNA of interest. When no DNA alteration is present, the probes lie adjacent to each other allowing probe ligation to occur. Ligation is not possible where deletions or mutations are present. Ligated probes are amplified and represent a measure of the number of copies. The products are separated by electrophoresis based on their size.
fluorescent tags are then cleaved, removing the chain terminating effect and allowing further extension to occur and the sequence to build up sequentially. Once the sequence fragments are created, they are aligned to the known reference sequence using bioinformatic algorithms. The computational technology is able to measure the number of fragments over a given region (read depth) and highlight (call) sites of discordance to the reference sequence (variance).

There are pros and cons relating to each chemistry type, but modifications and updates are fast coming and many of these issues are being overcome with time.

NGS technology has superseded Sanger sequencing, which has been the primary sequencing method for ~30 years. Sequencing the human genome took years to complete using Sanger technology. NGS, at its current best, reports production of 25 gigabases per day—the equivalent of eight human genomes.

What NGS can offer in the research setting, by way of novel findings and identification of mutations, could be considered problematic when transitioning this technology into the diagnostic forum. The expense, the magnitude of data, time-consuming analysis and interpretation, limited analytical software currently available, limited theoretical understanding and issues of consent and ethics relating to incidental findings could all remain cause for concern. Nonetheless, implementation of NGS technology in diagnostic laboratories is moving forward apace.

There are number of ways of applying NGS technology in a more manageable way, from a technological, analytical and financial point of view. This often involves targeting smaller regions of interest—WES. In this scenario, only the exonic, protein-coding gene regions are sequenced. This amounts to ~1.5% of the whole genome, 30 megabases of DNA or 180 000 exons, but is believed to contain 85% of disease-causing mutations.

NGS panels offer a chance to harness the accuracy and depth of NGS analysis in an even more targeted fashion. This method has proved a popular way to introduce NGS sequencing to the diagnostic setting. A set of genes is selected to create a study panel. Sequencing can be aimed at a particular chromosomal region, genes implicated in a specific disease or a specific function. Analysis is limited to the pre-selected genes, and therefore, the functionality of novel gene discovery is lost. Despite this, panels can be designed with specific objectives in mind, for example tailored to the cohort of patients/samples to be analysed. In the case of MM, we may choose to target the ‘osteome’—genes implicated in the normal or pathological biology of bone.

To date, NGS has been applied successfully by the haematology research community, but it is slower to translate to diagnostic services. Myeloma is included in this flurry of research activity. In 2011, Chapman et al. applied both WGS and WES technologies to a cohort of 38 myeloma patients, and their data present novel mutations in genes associated with histone methylation and protein translation, and in addition, they provided additional evidence of the importance of the NF-KB pathway, demonstrating mutations in 11 relevant genes. The study also reported BRAF involvement in 4% of MM patients, raising the possibility of incorporating BRAF inhibition in treatment for these patients. WGS has also been assessed at four time points across a single patient’s disease course. The patient was shown to have t(4;14), seen at all time points, as well as 10 single-nucleotide variants. Other genetic changes appeared and disappeared throughout the disease. Five new events were seen at the final time point of plasma cell leukaemia, and these changes have been postulated as leukaemic transformation events.

NGS remains relatively expensive, time consuming and has the potential to generate excessively large data sets. More targeted approaches offer realistic aims in terms of the diagnostic management of MM, since they retain the ability to determine the mutation status over a large number of relevant genes. Although adaptations are fast being included in the NGS repertoire, it is not designed to assess large regions of duplication or deletion, or balanced rearrangements.

Conclusions

Despite advances in the treatment of MM, it remains an almost invariably incurable malignant disease. Myeloma genetics is intrinsically complex and heterogeneous, but it offers an opportunity to categorize the
disease into genetic subtypes. Myeloma genetic research is highlighting areas and patient cohorts where targeted treatments would be appropriate. As a result of this genetic, as well as clinical, complexity, it is becoming clear that there is unlikely to be a single approach to treatment of myeloma patients, but rather a series of genetically targeted and systems targeted treatments that are likely to be used in combination to create an orthogonal cancer therapy approach based on the genetic subtypes of MM.66

The complex genetic findings in MM mean that a single genetic technology cannot currently provide information across the breadth of genetic diversity encountered. Cytogenetic analysis is expensive and only offers clinically significant information in a fraction of patients; FISH is an accurate and highly applicable test, but it becomes costly and time consuming as increased numbers of probes are utilized; MLPA and array technology offer the higher resolution and the ability to target multiple lesions, but it cannot detect balanced translocations or rearrangements; GEP has the ability to risk stratify patients, but it is not currently able to determine all areas of prognostically useful information. NGS is a novel technology, and although well used in the research setting, it is not yet incorporated into all NHS diagnostic centres. Importantly, this ‘high-throughput’ approach has the potential flexibility to create a testing strategy aimed to cover all known prognostically relevant MM aberrations.

Targeting residual disease is likely to be crucial to improved clinical outcome. Current treatments offer effective means to target bulk disease, but they are unable to eradicate residual disease, resulting in eventual disease relapse in the majority of patients. Understanding the genetics related to this residual disease population may allow more targeted treatment approaches aimed at total eradication.

Current genetic testing strategies employed for MM, or MM trials, across the UK are ad hoc and dependent upon regional commissioning arrangements. There is a requirement for the production of best practice guidelines for the genetic analysis of patients with myeloma. Initially, it is likely that genetic testing will be restricted to those patients with confirmed plasma cell myeloma, by the presence of >10% plasma cells in the BM aspirate or trephine. An age limit or fragility limit may be appropriate, meaning that patients not likely to be considered for treatment may not benefit from genetic testing, although there is a strong argument that the prognostic information is useful in patient management even if hard line treatment is not an option. It is generally accepted that genetic testing should be carried out on selected plasma cell populations. The abnormalities targeted are likely to include those with a prognostic impact; IGH rearrangement (and its partners), TP53 deletion, 1p and 1q abnormality, but should perhaps be extended to include hyperdiploidy, or those abnormalities that already have inhibitors that are potentially available, for example BRAF rearrangement. Then the question of technology arises; it may be that this is not the most important factor. While potentially NGS could offer the flexibility of analysis of a whole range of abnormalities, its expense precludes it currently. A single test does not yet cover the range of appropriate testing, but a comprehensive FISH/MLPA regime or FISH/array regime could be envisaged at a cost that is likely to be considered appropriate within the context of myeloma diagnosis and treatment. Best practice guidelines would not only provide uniformity, but also offer the most comprehensive information possible to clinicians and therefore the best treatment options for patients with MM.

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**Conflict of Interest statement**
The authors have no potential conflicts of interest.

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