Review Article

Routine laboratory diagnosis of chromosome aberrations in multiple myeloma

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ABSTRACT

Multiple Myeloma (MM) is a Plasma Cell (PC) malignancy characterized by proliferation of differentiated B cells mainly in the bone marrow. Genetic abnormalities are powerful prognostic factors in MM for risk stratification and therapeutic strategies. The standard diagnostic tests to detect genetic abnormalities in MM include Conventional Cytogenetic Analysis (CCA) and Interphase Fluorescence In Situ Hybridization (FISH). Due to the low proliferative activity of the abnormal clone, only 30-50% of newly diagnosed MM demonstrate an abnormal karyotype by CCA. CCA is a biological test which requires dividing cells for analysis. The t(4;14) translocation which carries a poor prognosis is cryptic and cannot be detected by CCA. These limitations were overcome partly by the incorporation of interphase FISH as a routine diagnostic test in MM. There is an international consensus that FISH should be performed in all newly diagnosed MM to detect high-risk genetic abnormalities. FISH testing must be done on purified PCs or by simultaneous labeling of cytoplasmic immunoglobulin light chain to allow identification of PCs. The minimum essential abnormalities to test for are t(4;14), t(14;16) and del(17)(p13). However, there is no consensus on the optimal protocol for CCA and interphase FISH. We review here the types of chromosomal aberrations found in MM, the prognostic significance of these abnormalities, methodologies in CCA to improve on the low yield of abnormal karyotypes, and protocols in interphase FISH.

Keywords: Multiple myeloma, Chromosome abnormalities, Prognosis, Conventional cytogenetic analysis, FISH analysis

INTRODUCTION

Multiple Myeloma (MM) is a B cell neoplasia, characterized by proliferation of malignant plasma cells in the bone marrow. It is a heterogeneous disease at the genetic level and in terms of clinical outcome. The incidence of MM varies with ethnicity, with Asians having a lower incidence than Caucasians. However, there were reports that its incidence is increasing in some Asian countries such as Taiwan and Korea probably due to industrialization and aging. MM is usually preceded by an asymptomatic premalignant condition called Monoclonal Gammopathy of Undetermined Significance (MGUS) or Smoldering Myeloma (SM). The diagnosis of MM include clonal plasma cells of more than 10% in the bone marrow, presence of monoclonal (M) protein in either serum or urine, and evidence of end organ damage ['CRAB' criteria: calcium (elevated), renal failure, anemia, and bone lesions]. Patients diagnosed with MM are staged according to the International Staging System (ISS), which divides myeloma into 3 stages (Stages: I, II, and III) based on serum beta 2-microglobulin and serum albumin levels. Some of the prognostic factors in MM include serum beta 2-microglobulin level, bone marrow Plasma Cell (PC) labeling index, genetic abnormalities and age.
The standard diagnostic test to detect genetic abnormalities for newly diagnosed MM includes both Conventional Cytogenetic Analysis (CCA) and Interphase Fluorescence In Situ Hybridization (FISH). In newly diagnosed MM, the abnormal PCs have a low proliferative activity, and the analyzable metaphase spreads from CCA are derived from normal hematopoietic cells, thus resulting in a normal karyotype. About 30-50% of newly diagnosed MM patients have an abnormal karyotype, while the karyotype of the remaining 50-70% of patients would be normal.6,10 The low mitotic activity of the abnormal PCs is a limitation of CCA which requires dividing cells for analysis. Some aberrations are cryptic, for example t(4;14) translocation is submicroscopic and cannot be detected by CCA. These limitations were overcome partly by the incorporation of FISH analysis of interphase nuclei11,12 as a routine diagnostic test. FISH can be done on non-dividing cells and detect cryptic translocations. It is also used for targeted detection of specific aberrations with known prognostic significance. Chromosomal aberrations identified by CCA and FISH are important for risk stratification and help to determine therapeutic strategies.13,14 Both CCA and FISH also provide complementary information, and can be used to monitor the response to therapy.

In this paper we review the types of chromosomal aberrations found in MM, the prognostic significance of these abnormalities, methodologies in CCA to improve on the low yield of abnormal karyotypes, and methodologies in interphase FISH.

CHROMOSOME ABERRATIONS IN MM

Primary genetic events

Chromosomal aberrations in MM are usually complex with multiple numerical and structural abnormalities. Patients may have 8 or more karyotypic abnormalities at diagnosis, and some of these clones have generated heterogeneous subclones with many secondary aberrations that indicate disease progression.15,16 At the genetics level, MM comprises of two broad subtypes of disease that may reflect different oncogenic pathway: hyperdiploid and nonhyperdiploid disease. About 50-60% of MM patients belong to the hyperdiploid group which is characterised by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21; and fewer structural aberrations. Patients will usually remain hyperdiploid throughout the course of the disease. The nonhyperdiploid group usually has structural aberrations, mainly translocations of the immunoglobulin heavy chain (IgH) gene at chromosome 14q32, and frequent loss of chromosomes 13, 14, 16 and 22. The prevalence of IgH translocations in the nonhyperdiploid and hyperdiploid group are >85% and <30% respectively.17,18 The hypodiploid group is associated with a more aggressive disease and shorter survival compared to the hyperdiploid group.19 IgH translocations are considered as early genetic lesions in the pathogenesis of MM because of their similar prevalence in MGUS and SM (about 35-50% of patients). However, some variants of IgH translocations may be secondary translocations and are probably involved in tumor progression. Primary immunoglobulin translocation are probably due to errors in B-cell specific DNA modification processes: mostly IgH switch recombination, somatic hypermutation, and rarely VDJ translocations.20 The IgH translocations in MM are promiscuous and involve nonrandom partners, mainly 11q13 (CCND1), 4p16 (FGFR3 and MMSET), 16q23 (MAF), 20q12 (MAFB) and 6p21 (CCND3).21 The two most frequent translocation in MM are t(11;14)(q13;q23) and t(4;14)(p16;q32). The t(11;14) translocation which is found in about 15-17% of MM patients, results in upregulation of cyclin D1 oncogene. This translocation has a favorable outcome and is regarded as neutral to prognosis. The t(11;14) translocation is found at a high frequency in light chain amyloidosis (35-50%) and in IgM MM (>90%).15 The t(4;14)(p16;q32) translocation which is present in about 15-20% of MM patients, involves two genes on 4p16: the Multiple Myeloma SET domain gene (MMSET) and fibroblast growth factor receptor 3 gene (FGFR3), which translates a methyltransferase protein and a transmembrane tyrosine kinase receptor respectively. As a result of the t(4;14) translocation, two genes with oncogenic potential are dysregulated simultaneously, MMSET on der(4) [derivative chromosome 4] and FGFR3 on der(14).22 About 25% of the cases lack FGFR3 expression, due to loss of FGFR3 on der(14).23 The t(4;14) is cryptic to metaphase analysis, and only detectable by FISH or reverse-transcriptase PCR. Patients with t(4;14) translocation have a poor prognosis with short remission durations and aggressive relapses.24 Two clinically important translocations t(14;16)(q32;q23) and t(14;20)(q32;q12), are found in 6-7% and 2% of MM patients respectively.18 Both translocations are associated with an unfavorable outcome and usually detected by FISH. The t(6;14)(p21;q32) translocation which is found in about 4-6% of MM patients has a favorable outcome. The frequency, upregulated oncogenes, and prognosis of the 5 common IgH translocations in MM are shown in Table 1.21

Table 1: IgH translocations in multiple myeloma.21

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Frequency (%)</th>
<th>Upregulated Oncogenes</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(4;14)(p16;q32)</td>
<td>15-20</td>
<td>MMSET, FGFR3</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>t(14;16)(q32;q23)</td>
<td>5-9</td>
<td>MAF</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>t(14;20)(q32;q12)</td>
<td>1-2</td>
<td>MAFB</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>15-17</td>
<td>CCND1</td>
<td>Favorable / neutral</td>
</tr>
<tr>
<td>t(6;14)(p21;q32)</td>
<td>4</td>
<td>CCND3</td>
<td>Favorable / neutral</td>
</tr>
</tbody>
</table>
Figure 1 shows the hyperdiploid karyotype of a male multiple myeloma patient with structural abnormalities including IgH translocation t(11;14)(q13;q23).

Figure 1: Karyotype of male patient with multiple myeloma:
48,XY,der(6)t(1;6)(q21;q16),+7,+8,t(11;14)(q13;q23),15ps+. Hyperdiploidy with loss of chromosome segment 6q12qter & gain of segment 1q21qter on derivative chromosome 6, trisomies of chromosomes 7 & 8, and IgH translocation t(11;14). The increase in size of satellites on the short arm of one chromosome 15 is a normal chromosomal variation. Arrows indicate chromosomal abnormalities.

Secondary genetic events

Multiple secondary chromosomal aberrations such as reciprocal and non-reciprocal rearrangements, duplications, amplifications, deletions, etc. are found during the progression of MM. The common abnormalities include translocations of MYC, chromosome 13 aberrations (monosomy or deletion of chromosome 13), deletions of 17p13 and/or chromosome 1 aberrations (deletions in 1p and amplifications in 1q).16 Translocations and amplifications of MYC at 8q24 are present in 45% of patients with advanced MM. MYC rearrangements that do not involve the IgH, as well as rearrangements involving both IgH and MYC which are often very complex, show similar prevalences in hyperdiploid and nonhyperdiploid myeloma.25 Clg-FISH analysis detected rearrangements involving MYC with immunoglobulin light chain kappa (IgK) or lambda (IgL) in about 3% of MM patients.26

Chromosome 13 aberrations (detected by FISH rather than metaphase cytogenetics) are present in 50% of MM patients, of which 85% are monosomy 13 and the remaining 15% are deletions of chromosome 13.27 Chromosome 13 aberrations used to be associated with a poor prognosis, but however it was shown later that this was related to its coexistence with high risk genetic abnormalities, such as t(4;14).15

Deletion of 17p13 [del(17)(p13)] which leads to inactivation of a tumor suppressor gene, p53 is detected in 10% of MM patients by interphase FISH. The deletion is associated with an extremely poor prognosis in MM: shorter survival, more aggressive disease, higher prevalence of extramedullary disease and hypercalcemia.28 There is no clear evidence till now that maintenance therapy, with any agent, benefit patients with 17p13 deletions.29

Chromosome 1 aberrations are highly prevalent in MM and mostly involve deletions in 1p and amplifications in 1q. Chromosome 1 abnormalities are associated with a poor prognosis.16,30 The deletions in 1p are mainly interstitial deletion. Chromosome 1q abnormalities are usually complex and unstable. Chromosome 1q aberrations include amplifications, duplications (direct and indirect), isochromosomes of 1q, and jumping translocations. Amplification of 1q21 is absent in MGUS, but is observed in 43% of newly diagnosed MM and 72% of relapsed MM.31

Identification of high-risk genetic aberrations such as t(4;14), t(14;16), t(14;20), del(17)(p13), abnormalities of 1p and 1q in all newly diagnosed MM patients are important for risk adaptive treatment strategies. MM is incurable. Current therapy for high-risk MM patients are inadequate and they should be considered for clinical trials using novel combinations; including chemotherapeutic, novel or investigational agents.32 Studies have shown that the adverse effect of t(4;14) may be overcome partially by newer agents such as bortezomib-based therapy.32

LABORATORY METHODS FOR CONVENTIONAL CYTOGENETICS ANALYSIS

The reluctance of abnormal PCs to divide in culture has resulted in the analysis of normal metaphases and normal karyotype being obtained. Dewald et al. (1985),33 found that the frequency of chromosomal abnormalities in MM was 27% when the BM cells were harvested directly or after short-term culture in mitogen-free medium. In our cytogenetic laboratory, we perform CCA as a routine diagnostic test for all patients with hematological malignancies. In our overnight culture (about 15-16 hours) of bone marrow cells without the addition of any mitogen, chromosomal aberrations were detected in less than 10% of newly diagnosed MM patients. These culture conditions used probably enabled the identification of abnormal karyotypes from the most aggressive and rapidly dividing myeloma cells. CCA is a biological test. In early MM, the myeloma cells are stroma-dependent; taking them out of their supportive microenvironment will result in apoptosis, and therefore no mitosis. If the myeloma cells have become stroma-independent in the advanced stages of MM, the myeloma cells can survive outside their microenvironment and will proliferate to give rise to abnormal metaphases.34 The presence of abnormal metaphase is usually correlated with an
increase in plasma cell labeling index and tumor burden, thus indicating a higher mitotic rate and bone marrow plasmacytosis. Finding abnormal mitosis may be an indication of stroma-independent cells and advanced myeloma. Identifying cytogenetic abnormalities by CCA depend on a number of factors. The percentage of plasma cells in the bone marrow aspirate must be more than 10%. Gole et al., (2014) reported that abnormal karyotypes are detected in 50% of newly diagnosed MM patients with more than 10% plasma cells, but when PC content was lower the cytogenetics showed a normal karyotype. Campbell (2005) reported that the presence of 30% of PCs or more in the bone marrow aspirate will increase the probability of finding a cytogenetic abnormality significantly: the median percentage of PCs in abnormal vs. normal karyotype was 48% vs. 25% respectively. Out of 200 cases of MM, cytogenetic abnormalities was detected in 45.5% of patients. Studies using different combination of cytokines and culture conditions were carried out to induce the myeloma cells to divide. This include cultures from one to seven days, and stimulation with Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), interleukin-2 (IL-2), IL-3, IL-4, IL-6, and/or tumor necrosis factor-alpha. There is evidence that by setting up more cultures from a sample, there is a higher probability of finding a cytogenetic abnormality. Different cytogenetic laboratories follow different cell culture protocols. For example, one cytogenetic laboratory would set up a minimum of two cultures routinely: a 24-hour unstimulated culture, and a 72-hour culture stimulated with IL-4. If there is sufficient sample, a 72-hour unstimulated culture would be established. Another laboratory would set up only one 24-hour unstimulated culture, and a 72-hour culture stimulated with IL-6 if there were sufficient cells. Although there is no consensus on which is the optimal culture technique, cytogenetic laboratories must try to increase the rate of detecting an abnormal karyotype in MM if the yield is low (less than 30%). Factors to be taken into account include PC concentration of more than 10% (some laboratories >30%), and the establishment of different culture conditions from a sample.

**LABORATORY METHODS FOR INTERPHASE FISH**

The International Myeloma Working Group (IMWG) 2009 recommended that FISH testing in MM must be performed on nuclei from purified PCs or by simultaneous labeling of cytoplasmic immunoglobulin light chain (for identification of PCs) to improve on the rate of detecting cytogenetic abnormalities. FISH studies in unsorted samples should not be reported because of its low sensitivity for detection of abnormalities. PCs can be purified by using flow cytometry or immunomagnetic beads. The most practical and widely used technique of sorting PCs is by CD138 positive magnetic microbeads. Although this method does not distinguish between normal and tumor PC, it was accepted that the PCs sorted are suitable for most genetic studies. The more commonly used technique are the slide-based assays; cytoplasmic immunoglobulin FISH (clg-FISH or FICTION) which involves staining the PCs with lambda/kappa antibodies followed by conventional FISH. Using clg-FISH, tumor and normal PCs can be distinguished from other cells. There are many variations and modifications of clg-FISH protocols. In one clg-FISH protocol, the red cells are removed by lysis, the white cells are spun, dropped onto slides, stained with lambda/kappa antibodies, followed by conventional FISH. However, preparation of cytopsin slides is tedious, requires additional equipment and more bone marrow aspirate. Another clg-FISH protocol uses fixed cell pellets. The advantage of this technique is that cell pellets from conventional cytogenetics can be used for clg-FISH as well. To prevent the cells from aggregating and clumping, the cells were washed in 100% methanol (instead of 96% ethanol), dropped onto slides, followed by adding gently 96% ethanol. The modified clg-FISH technique of Gole et al., (2014) does not require additional chemicals, reagents and equipment; did not miss any chromosomal abnormalities; and is easy to perform.

Translocations may be detected by using a ‘break apart’ strategy that detects any IgH translocation using probes that localize to the variable and constant IgH region. Translocations detected by a ‘fusion’ strategy is very specific; fusion of probes for regions of interest indicates a translocation. Due to the unbalanced nature of IgH translocations in MM, FISH probes that enable a double fusion strategy should be used. Some of the technical recommendations by European Myeloma Network (EMN) 2012 for interphase FISH in MM were as follows: the sample should be part of the first draw of the aspirate, PCs must be purified or specifically-identified, conservative cut-off levels of 10% for fusion or breakapart probes, and 20% for numerical abnormalities, at least 100 PC should be scored; and the clinical report should be clear and should include the method used for PC identification, the probes used, the total number of PCs scored and the percentage of abnormality. The method of choice to use is left to individual laboratories.

The IMWG 2009 recommended that the minimum FISH panel for risk stratification should include testing for t(4;14), t(14;16) and 17p13 deletions. A more comprehensive panel should include testing for t(11;14), chromosome 13 deletion, ploidy status, chromosome 1p and 1q abnormalities. The EMN 2012 recommended that the essential abnormalities to be tested are t(4;14), t(14;16) and 17p13 deletions, and where possible 1p and 1q abnormalities. Chromosome 13 deletion is not an independent prognostic marker and its adverse effect is due to its close association with high risk abnormalities.
The t(4;14), t(14;16), t(14;20), del(17)(p13), and 1q amplifications, identified by FISH, confer an adverse outcome, and these abnormalities should be specifically sought at diagnosis to enable the appropriate management of these MM patients.48

DISCUSSION

Nearly all MM patients have cytogenetic abnormalities if tested for hyperdiploidy and the common translocations involving 14q32 using FISH,49 as compared to 30-50% for CCA. The low proliferating malignant PCs in MM which makes it different from other hematological malignancies (such as leukemias), has been a challenge for CCA. Early in the disease, the tumor cells are stroma dependent and taking them out of their supportive microenvironment will result in cell death, and thus no mitosis. Therefore, research on novel culture media to mimic the supportive microenvironment and novel mitogens to stimulate the malignant PCs in MM to proliferate is much needed for cytogenetic studies. Finding abnormal karyotype in newly diagnosed MM is an indication of stroma-independent tumor cells, and therefore advanced myeloma.46 Just like in acute leukemia, CCA should be performed on all new MM patients to assess risk and predict outcome.8

There is an international consensus that FISH analysis should be done in all newly diagnosed MM for the identification of high-risk genetic abnormalities.15,47,48,50 The minimum abnormalities to test for are t(4;14), t(14;16) and del(17)(p13). An extended panel may include testing for abnormalities of 1p and 1q, t(11;14), t(14;20), and ploidy status. This is to provide further assessment of the disease biology, clinical features and likely outcome. Besides chromosome aberrations, other important prognostic factors such as tumor characteristics, tumor stage (ISS), and age of patients are included in risk stratification in the clinical management of MM patients.29 Due to advancement in technology, next generation sequencing which can identify copy number alterations, translocations and somatic mutation may succeed FISH testing in the future.48

CONCLUSION

Interphase FISH is currently the most useful molecular cytogenetic tool for the identification of recurrent genetic abnormalities with major prognostic impact and predictive outcome in MM.

There is no consensus on the optimal protocol for CCA and interphase FISH to detect genetic abnormalities in MM. To improve the yield of getting abnormal metaphases, the concentration of PCs and the setting up of several culture conditions are important factors to be considered. The method of choice for purification or identification of plasma cells for interphase FISH analysis should be decided by each laboratory based on their own expertise, facilities and requirements.

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