Detection of 14q32 rearrangements in multiple myeloma, using simultaneous FISH analysis combined with immunofluorescence

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BACKGROUND: 14q32 rearrangement has been identified as a recurrent hotspot of translocations in multiple myeloma (MM). The Fluorescence Immunophenotyping and Interphase Cytogenetics as a tool for the Investigation of Neoplasms (known as FICTION technique) for evaluation of chromosomal changes in MM. The aim of this work is to detect 14q32 rearrangement, using FICTION technique, on archival bone marrow (BM) slides of MM patients, and to study its prognostic value.

METHOD: This study was conducted at Ain Shams University Hospital. The FICTION technique, which uses CD138 and dual color, and the break-apart 14q32 rearrangement probe, was performed on archived smears of BM slides for 50 MM patients at the time of diagnosis.

RESULTS: A significantly higher percentage of cases were positive for 14q32 rearrangement by FICTION (32%) compared to fluorescence in situ hybridization (FISH) (12%) (p = 0.04). Cases positive by FICTION for the rearrangement were designated as Group A, while negative cases were designated as Group B. Significantly lower Hb and CRP levels were found among Group B when compared to Group A patients (p = 0.001 and 0.01, respectively). Serum albumin level and Bence Jones protein (BJP) significantly affect overall survival (OS) (p = 0.01, 0.007, respectively). However, a statistically non-significant shorter mean survival time was found in positive cases through FICTION versus negative cases.

CONCLUSION: FICTION technique provides a sensitive tool for establishing clonal plasma cells (PC) infiltration of BM aspirates, and is amenable for use on archived as well as fresh smears.

KEYWORDS: Multiple myeloma; Plasma cells; 14q32 rearrangement; FICTION; Archived smears

Multiple myeloma (MM) is characterized by a clonal proliferation of malignant, immunoglobulin (Ig) producing plasma cells (PCs) in the bone marrow (BM).1

It has been reported that all MM patients harbor cytogenetic abnormalities, sometimes during the course of the disease.1 The chromosomal 14q32 region has been identified as a recurrent hotspot of translocations in myeloma.2 However, this is not specific to MM as the chromosomal 14q32 region abnormalities are also detected in mantle cell lymphoma and chronic T-cell leukemia.3 These abnormalities are involving the immunoglobulin heavy chain (IgH) locus at chromosome 14q32, and one of several partner genes.7 14q32 rearrangements can be detected by conventional as well as molecular cytogenetic methods, such as FISH. Conventional cytogenetic analysis of the BM is an important tool in evaluating karyotype abnormalities in MM. However, due to the in vitro hypoproliferative nature of the myeloma, it is not always
possible to obtain good metaphases for analysis. For example, t(4;14) and t(14;16) are cryptic by classical cytogenetics. However, FISH analysis performed on BM aspirates of MM patients are technically challenging because the neoplastic PCs may represent only a small proportion of the total nuclei present. To overcome this difficulty, several techniques have been advocated. Some have used antibody-based sorting techniques to purify PCs from the BM aspirate before FISH analysis. Another proposed technique is to use an initial May-Grünwald Giemsa stain with mapping of PCs by image analysis software, followed by subsequent FISH analysis of the previously mapped PCs. However, both cell sorting and image analysis techniques involve protocols not routinely performed in most pathology laboratories. Importantly, none of the previously published techniques have been applicable to routinely archived BM smears.

FISH analysis combined with simultaneous immunofluorescence (IF) using CD138 is an attractive alternative approach for the evaluation of chromosomal changes in MM. The use of combined IF and FISH was first reported by Weber-Matthiesen et al. as a procedure termed FICTION. So far, FICTION has been applied to BM smears, peripheral blood (PB) and cytospin samples with good results. This technique is potentially very useful due to its easy applicability, rapidity, and amenability to automation. Owing to its capacity to improve the efficacy of result interpretation (due to the combination of more than one technique on the same sample), FICTION could be extremely useful in tumor diagnosis, especially in the detection of low-level disease, minimal residual disease (MRD) or diagnosis of composite tumors.

FISH can be performed on de-waxed tissue sections, but this technique remains potentially challenging: the scoring of individual nuclei is difficult due to cellular overlap and nuclear truncation. Moreover, fixation and embedding procedures may produce artifacts in tissue, thereby interfering with DNA hybridization. Alternatively, Buño et al. clearly showed that FISH using tissue imprints, cytopreps, and BM smears, including those stored for long periods of time (up to 12 years) is a reliable method to detect abnormalities with high sensitivity and specificity.

The aim of this work is to detect the 14q32 rearrangement using FISH analysis combined with simultaneous IF on archival BM slides of MM patients and to study its prognostic value.

SUBJECTS AND METHODS

This study was conducted at Ain Shams University Hospital, in the period from June 2012 to February 2014, after procuring the approval of the Scientific and Ethical Committee of Ain Shams University.

Subjects

The subjects of the study comprised 50 patients initially diagnosed with MM according to World Health Organization (WHO) diagnostic criteria for plasma cell myeloma.

The archived smears of the BM of 50 MM patients at the time of first diagnosis were collected for processing.

The clinical, laboratory, and radiological data of the patients were obtained from patients’ records. Laboratory data included complete blood count (CBC), serum protein electrophoresis (SPEP), Bence Jones protein (BJP) in urine, immuno-fixation (IFX), serum calcium level, chemistry profile (LDH, BUN and serum creatinine), serum beta-2-microglobulin (β2m) and C-reactive protein (CRP).

All patients received bortezomib (VELCADE)-based combination chemotherapy. This treatment was composed of bortezomib (1.3 mg/m²; days 1, 4, 8 and 11) plus dexamethasone (40 mg/m²; days 1–4 and 9–12). The cycle was repeated every 3 weeks for four cycles.

Methods

The following was performed for all patients:

- Morphological re-evaluation of at least two Leishman-stained smears per patient was performed by two experienced hematologists.
- FICTION technique was used to further analyze the collected smears and involved a two-step procedure, as follows:

STEP 1: immunofluorescence staining for CD138

(a) FITC-labelled anti-CD138 was diluted in phosphate buffer saline (PBS) in a ratio of 1:40 and added to each slide so that it covered the tissue in the marked area.
(b) The slides were then incubated for 30 min at 37 °C in a dark chamber.
(c) Before performing FISH analysis, the slides were covered with a cover slip and revised by fluorescence microscopy for the presence of good IF staining. If not used immediately, slides were kept at 4 °C for a maximum of 1 week.
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14Q32 REARRANGEMENT IN MM BY FICTION TECHNIQUE

(d) Differential count of CD138 positive cells was done before performing FISH analysis on the inverted fluorescence microscope using the low power magnification lens (40×).

**STEP II: fluorescence in situ hybridization (FISH) for 14q32 rearrangement**

FISH analysis was performed using Vysis LSI (locus specific identifier) IgH dual color, break-apart 14q32 rearrangement probe (Abbott, Germany). 10 μl probe mixture (7 μl buffer, 1 μl probe 2 μl sterile water) was added to the marked area on each slide followed by denaturation (for 5 min at 73 °C) using HYBrite (Vysis) followed by overnight incubation at 37 °C. Post hybridization washes of the slides were performed first for 2 min at 73 °C in R1 solution (0.4 × SSC/0.3% NP-40 at pH 6.8–7.2), then another 2 min at 37 °C in R2 solution (2 × SSC/0.1% NP-40 at pH 7.0–7.5). Finally, slides were blotted and left to dry in the dark. DAPI II (4',6-Diamidino-2-Phenylindole) was then applied to each slide as counter stain. The slides were scanned using fluorescent microscope (CytoVision image analysis system) and signals were viewed in at least 200 interphase cells.

**Probe interpretation**

CD138-FITC allowed the identification and enumeration of cell populations expressing the CD138 antigen, with cells positive for CD138 antigen showing green rim.

The LSI IgH dual color, break-apart probe was designed as a dual-color split probe to detect translocations at 14q32. A break was defined when a red/green or yellow fusion signal (F) split into separate red and green signals. Only red and green signals, which were more than one signal diameter apart from each other, were counted as a break. Normally, cells showed two fusion signals. Cells positive for 14q32 rearrangement showed single fusion signal, one red signal, and one green signal. To establish the cutoff value for positive result, 20 archived BM slides of non-malignant cases and 10 BM slides with reactive increase in the plasma cells (mean = 6.7 ± 0.7) were examined using the FISH technique, and the positive result was determined as the mean ± 4SD as described by Cook et al. Cutoff value was set at more than 4% of the cells.

**Statistical methods**

Collected data were analyzed using SPSS (version 12) statistical software package under Windows XP operating system. Data were expressed as mean ± SD for quantitative measures, and both number and percentage for categorical data. Test for data normality was done using Shapiro–Wilk test. Unpaired t-test and Mann Whitney test were used to compare parametric and non-parametric quantitative variables, respectively. Chi-Square and Fisher’s exact tests were used for inter-group comparisons. Overall survival (OS) was estimated from the time of diagnosis to the date of death or last visit. The OS was determined using Kaplan–Meier curves; log-rank test was used to calculate p value. The probability of error at <0.05 was considered significant.

**RESULTS**

The results of this study are shown in Tables 1 and 2, Fig. 1, and Photo 1.

This study included 50 MM patients, admitted to the Hematology Unit at Ain Shams University Hospital. They comprised 12 (24%) males and 38 (76%) females, with a male to female ratio of 1:3.3. Their ages ranged from 28 to 74 years (with a mean of 57.3 ± 11.6 years). Clinical follow-up revealed that 14 of the 50 patients died (28% mortality rate). The OS of patients ranged from 5 months to 30 months.

**Correlation between BM PCs% morphologically and by CD138 staining (Fig. 1)**

A highly significant positive correlation was found between morphologically assessed BM PCs percentage and by IF staining for CD138 (r = 0.67, p = 0.003).

**Diagnostic validity of 14q32 rearrangement detection by FISH and FICTION**

Patients were considered positive for presence of 14q32 rearrangement when >4% of cells exhibited it (out of all cells in case of FISH and out of CD138 positive cells in case of FICTION). A significantly higher percentage of cases were positive for 14q32 rearrangement by FICTION (16/50, 32%) compared to FISH (6/50, 12%) (χ² = 3.14, p = 0.04). FICTION was therefore more sensitive than FISH. Cases positive by FICTION for the rearrangement (16/50) were designated Group A, while negative cases were designated Group B.

**Comparison between Group A and Group B**

(Table 1)

Significantly lower Hb and CRP levels were found among Group B when compared to Group A patients (p = 0.001 and 0.01, respectively). Although median level of serum β2m and monoclonal band were higher in Group B than in Group A, the p value was borderline (0.06).
Survival studies (Table 2)
The mean survival for all patients was 13.3 ± 6.6 months. The impact of selected poor prognostic factors, according to Dmoszynska, on the OS of the presently studied MM patients revealed that serum albumin level and BJP significantly affected OS ($p = 0.01, 0.007$, respectively). Regarding the effect of 14q32 rearrangement using FICTION technique, shorter mean survival time was found in positive cases; however, the $p$ value was not statistically significant. The effect of $\beta_2m$ and LDH levels on OS could not be studied because all MM patients expressed high levels that exceeded the suggested levels for discriminating poor versus good prognosis.
DISCUSSION

In the present study, the percentage of PCs was estimated using CD138 immuno-stained BM slides of MM patients as compared to their traditional differential count of BM PCs. This IF slide technique gave a higher percentage of positive cases, compared to that estimated by morphological assessment of Leishman-stained smears. Smith and Elnawawi\(^\text{11}\) reported that CD138 immuno-staining achieves...
superior results because, unlike traditional methods, it makes it possible to recognize PCs under low-magnification microscopy when their numbers can be better judged in relation to other cells of the marrow. Thus, CD138 estimates improved the diagnostic accuracy of counting PCs in MM. The same authors also reported a more accurate estimate of the PC population within the entire BM core biopsy using IF staining for CD138 than the Giemsa-stained aspirate smears and Hematoxylin-Eosin (H&E) sections. Stifter and colleagues\(^\text{12}\) recommended the combined analysis of BM aspirates and core biopsies, using the IF staining for CD138 of BM PCs to achieve more accurate and informative data on the diagnosis of PC infiltrates of BM. PC quantification is also used in the evaluation of morphological remission and minimal residual disease (MRD) in MM patients. A high percentage of PC infiltration in BM has been recognized as a reliable predictor of relapse in cases of treated MM. The PC fraction in the BM is therefore critical for the classification and optimal clinical management of patients with MM.\(^\text{12}\)

In the present study, FISH analysis was used with simultaneous CD138 IF on archived BM smears. The CD138 IF allows the identification and enumeration of PCs and improves the sensitivity of the FISH method, in accordance with the reports of previous studies.\(^\text{1,5,13,14}\)

To establish the cutoff value for positive results, 20 archived BM slides of non-malignant cases and 10 BM slides with reactive increase in the plasma cells were examined using the FISH technique. Our cutoff value was set at more than 4% of the cells. This is in accordance with Cook et al.\(^\text{5}\) who examined paraffin sections of formalin-fixed and bone marrow clot preparations by FICTION technique in MM cases using CD138 and break-apart probe for IGH translocations. They used intact paraffin sections of only five cases of reactive lymphoid hyperplasia as negative controls. They established cutoff thresholds for interpretation at four standard deviations above the mean of the negative controls, yielding cutoffs of >5%.

FICTION technique was found in the present study to be more sensitive than FISH analysis in detecting 14q32 rearrangements in archived BM aspirate smears (32% versus 12%, respectively). In 2006, Cook et al.\(^\text{5}\) reported that scoring of only CD138-positive PCs allow efficient detection of myeloma-associated chromosomal abnormalities, which may be demonstrated in a very high proportion of plasma cells counted, even when the overall percentage of plasma cells in the marrow is low. This technique offers several advantages over other proposed methods. Unlike techniques using cytoplasmic immunoglobulin light chain staining, prior knowledge of the restricted light chain involved is not required. Specialized procedures such as cell sorting or computerized image analysis are unnecessary. Most importantly, this technique is applicable to routinely archived, formalin-fixed, paraffin-embedded and BM clot preparations. Therefore, this

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**Photo 1.** Positive FICTION technique: CD138 positive cells show green rim; cells positive for 14q32 rearrangement show single fusion signal, one red signal, and one green signal.
method allows for both prospective analysis and retrospective studies of previously archived and even paraffin-embedded material. The present study, a significant association was found between the presence of 14q32 rearrangements and an IgG isotype. Interestingly, in 2002, Moreau et al. investigated 168 MM patients and found a strong positive correlation between t(4;14) and IgG isotype, with no correlation observed in patients with translocations involving another partner, or those lacking any 14q32 rearrangement. Additionally, Chen et al. studied 48 newly diagnosed untreated MM patients, using interphase FISH and reported no correlation between 14q32 rearrangements and the type of para-protein.

In 2002, Avet-Loiseau and colleagues reported a strong association of β2m to t(4;14) and t(14;16), but not to t(11;14), or to patients lacking 14q32 rearrangements. In the present study, the association between β2m and 14q32 rearrangements was of borderline significance. This could be attributed to the conglomerate approach of FICTION to 14q32 rearrangements in the present study versus studying each translocation partner separately.

The survival of myeloma patients is highly variable, ranging from few months to more than 10 years. This heterogeneity is related mainly to prognostic factors associated with specific characteristics of both the tumor itself and the host.

In the present work, there was a non-significant association between 14q32 rearrangements and shorter OS. Moreau et al., Dewald et al., and Gertz et al. reported that patients with t(4;14) and t(14;16) had shorter OS, while patients with t(11;14) had exceptionally long OS survival. The inability to establish a significant association between OS and the presence of 14q32 rearrangements in the present work may be in part due to the heterogeneous impact of different partner chromosomes on survival, or due to the short duration of follow up in relation to the median survival of patients in records.

An ideal prognostic model should associate cytogenetics to other prognostic parameters. This justifies the routine application of FICTION in the workup of all newly diagnosed and relapsed myeloma patients, where a more precise prognostic classification and therapeutic management focuses on the molecular genetics of every patient, as the patient-dependent toxicity of most drugs available for myeloma treatment is related to constitutional genetic variability.

The detection of t(4;14), t(14;16), deletion 13 and deletion of p53 by FICTION will define high-risk prognostic groups that are not generally controlled with high-dose melphalan and autologous stem cell transplantation (ASCT), and should therefore be treated with investigational therapies. On the other hand, patients who do not have these poor risk factors are more likely to benefit from a high-dose melphalan-based regimen followed by ASCT. More intensive chemotherapy accompanied by transplantation, tandem ASCT, as well as targeted therapies (e.g., bortezomib and thalidomide), should be carefully evaluated in an effort to distinguish those patient subgroups more likely to respond, and to gain maximum profit from the appropriate therapeutic agent.

Finally, the present work concluded that FICTION technique provides a more sensitive tool for establishing clonal PC infiltration of BM aspirates, and is amenable for use on archived as well as fresh smears. The use of the FICTION technique is recommended for the simultaneous detection of other clinically relevant chromosomal abnormalities in MM, lymphoma, and other hematological malignancies.

**CONFLICT OF INTEREST**

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