GENES, CHROMOSOMES & CANCER 00:000–000 (2010)

RESEARCH ARTICLE

Interphase Fluorescence In Situ Hybridization on Selected Plasma Cells is Superior in the Detection of Cytogenetic Aberrations in Plasma Cell Dyscrasia

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Interphase fluorescence in situ hybridization (FISH) detects nonrandom cytogenetic abnormalities in plasma cell (PC) dyscrasia according to PC burden. However, when performed on cultured whole bone marrow (BM), it often fails to detect these aberrations. We have compared this interphase FISH technique with FISH after PC purification or identification to detect recurrent aberrations. In this study, 235 BM samples were collected from patients with multiple myeloma (MM) or related PC disorders regardless of disease status. All samples were analyzed in parallel. Clonal abnormalities were detected in 34.9% of cultured samples compared with 71.0% PC selected samples ($P < 0.001$). Moreover, FISH on PCs allowed to detect more abnormalities per case ($P < 0.001$) and identified higher percentages of abnormal nuclei ($P < 0.001$). This study indicates that FISH on PCs is the preferred technique for routine cytogenetic investigation of MM. © 2010 Wiley-Liss, Inc.

INTRODUCTION

Multiple myeloma (MM) is a clonal plasma cell (PC) disorder characterized by the early presence of cytogenetic aberrations. Over the past 15 years, insights in genetic abnormalities in this disease have grown significantly. First, conventional cytogenetic studies identified recurrent numerical as well as structural aberrations (Dewald et al., 1985; Weh et al., 1993; Lai et al., 1995; Sawyer et al., 1995; Calasanz et al., 1997). Because of the extremely low proliferation rate of PC dyscrasias, typically with less than 1% of cells synthesizing DNA, until late stages of MM (Debes-Marun et al., 2003), and limited bone marrow (BM) infiltration, abnormal karyotypes are reported in only 30–40% of newly diagnosed cases (Dewald et al., 1985; Weh et al., 1993; Sawyer et al., 1995; Calasanz et al., 1997). The advent of interphase fluorescence in situ hybridization (FISH) allows for an increased rate of detection of aberrations and for identification of some recurrent cryptic changes involving immunoglobulin loci, which are prognostically significant (Liebisch and Döhner, 2006; Avet-Loiseau, 2007). The aim of this study was to assess the impact of different interphase FISH procedures on the detection of chromosomal abnormalities in MM and related PC disorders.

MATERIALS AND METHODS

Patients

Consecutive BM samples from 1923 patients diagnosed with MM/PC dyscrasia were referred between June 2001 and October 2008. Samples from different hospitals were collected and analyzed in one single cytogenetic center. Of those, only samples analyzed in parallel, by FISH on cultured BM cells and FISH on PCs, were included in the study, regardless of disease status. Two hundred and thirty-five BM samples fulfilled these criteria.

The proportion of PCs was determined by morphological analysis in the referring center. In addition, sample cellularity was determined before cultures were set up (Supplementary Table 1).

Additional Supporting Information may be found in the online version of this article.

Supported by: Fonds voor Wetenschappelijk Onderzoek Vlaanderen, Research Foundation Flanders (FWO), Grant number: G.0610.07, KUL GOA MaNet, KUL CoE EF/05/007, BelSPO IUAP P6/25, IWT SBO-MoKa, IWT TBM-IOTA3, FOD Cancer.

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Received 21 May 2010; Accepted 28 June 2010

DOI 10.1002/gcc.20809

Published online in Wiley InterScience (www.interscience.wiley.com).

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TABLE I. Information on Patients' Characteristics, Samples, and Applied FISH Techniques

Age	
Median (years)	66.8
Range (years)	35.3–89.4
Gender	
Male (*n*)	128
Female (*n*)	107
PCs[a]	
Median (%)	32.5
Range (%)	1.0–100.0
FISH technique[b]	
PC selection (*n*)	235
MACS (*n*)	8
Easy Sep (*n*)	11
Clg (*n*)	216
Cultured BM (*n*)	235
Number of probes per case	
1 probe (*n*)	129
2 probe (*n*)	40
3 probe (*n*)	41
4 probe (*n*)	25
Total probes in 235 cases (*n*)	432

n, number; PC, plasma cell; BM, bone marrow.
[a]Percentage BM PCs by morphological assessment.
[b]FISH was performed on whole cultured BM in all cases. In parallel, FISH was performed on either purified CD138-positive PCs obtained with the AutoMACS system or Easy Sep, or on PCs identified by cytoplasmic light chain immunostaining (Clg).

Interphase FISH Analysis

FISH on cultured whole bone marrow cells

FISH was performed on whole BM cells after a 4-day culture without stimulation and followed standard protocols and manufacturer's procedures.

FISH on plasma cells

FISH was performed on uncultured BM, either on immunologically identified κ/λ positive (cIg-FISH) or on purified CD138-positive PCs.

Clg-FISH

Cytospin slides of fresh whole BM were made and stored at −20°C. Subsequently, the slides were thawed and fixed with Carnoy. Further processing of slides was identical as for FISH on cultured whole BM.

Finally, immunostaining was performed with FITC-labeled commercially available antibodies for κ and λ (Dako, Enschede, The Netherlands) to identify cells with positive cytoplasmic staining. Based on our own experience, slides labeled with FITC were found more stable than those labeled with AMCA, enabling visualization of the signal for several months. In addition, the cytoplasmic green fluorescence is usually weak and does not interfere with the green signals generated by FITC in the nucleus. Therefore, FITC was preferred over AMCA.

FISH on CD138-purified plasma cells

Mononuclear cells from fresh whole BM were enriched by Ficoll-gradient centrifugation. An immunomagnetic cell selection procedure with anti-CD138 specific antibodies was applied, using either the AutoMACS system (Miltenyi Biotec, Utrecht, The Netherlands) or CD138-coated magnetic beads (Easy Sep, Stem Cell Technologies, Grenoble, France), according to the manufacturer's instructions. Cytospin slides were made and fixed with Carnoy. Further processing of slides was identical as for FISH on cultured whole BM.

FISH probes

All probes used in the routine panel are commercially available (Vysis/Abbott, Downers Grove, IL). The classical panel included the following probes: LSI 13 (*RB1*, 13q14) and LSI p53 (*TP53*, 17p13) to assess gains and losses, CEP9 (centromere 9) or LSI BCL6 (*BCL6*, 3q27) for ploidy, and LSI IGH Dual Color Break Apart (*IGH*, 14q32) for translocations. In case of translocation involving the *IGH* gene, additional applied probes obtained from Abbott included LSI IGH/CCND1 dual color dual fusion, LSI IGH/CCNDI-XT dual color dual fusion, LSI MYC dual color break apart (*MYC*, 8q24), LSI IGH/FGFR3 dual color dual fusion. When needed, the following noncommercial BACs and fosmids were used: for *CCND3* (6p21) RP11-533O20 and RP11-7K24 labeled with spectrum orange-dUTP (SO)/RP11-696P19 and RP11-298J23 labeled with spectrum green-dUTP (SG), for *TCRad/TRAF3* (14q11/14q32) RP11-447G18 (SG)/G248P83103H7, G248P8205B4, and G248P89287F6 (SO) and for *PBX1* (1q23) 1127J20 (SO).

Correct hybridization of probes was previously assessed on normal metaphases. Specimens known to display either t(6;14)(p21.1;q32), t(11;14)(q13;q32), or t(4;14)(p16.3;q32) (clinical samples) were used to confirm the ability of specific probes to detect each translocation.

FISH scoring

FISH signals were evaluated by the same scorer during the whole period of the study. Only

nuclei with one or more FISH signals of each probe were scored. Between 20 and 200 cultured BM cells and 20–100 PCs were counted. Less than 20 evaluable cells per probe was considered as a failure.

In cIg FISH, the scoring process included a cytomorphologic assessment to exclude non-PCs with false positive cIg staining, and we attempted to score only the cIg-positive PCs. The European Myeloma Network (EMN) recommendations (http://www.cytogenetics.org.uk/prof_standards/myeloma.htm) were used for cut-offs, i.e., 20% for losses (deletions and monosomies) and 10% for gains and structural aberrations.

Rearrangements of the 14q32/*IGH* region were determined by means of a dual-color probe (Abbott). Nuclei with a clear split signal were considered to harbor a translocation involving the immunoglobulin heavy chain gene (*IGH*) locus. Physiological rearrangements of *IGH* may lead to signal loss of the green-labeled probe covering the *IGH* variable (V) genes. Therefore, loss of the green signal was considered aberrant, only in case of an identified translocation partner. In contrast, loss of the orange-labeled LSI IGH 3′ flanking probe (constant region of *IGH*) was always considered abnormal. Cases with 14q32 rearrangements were subsequently analyzed with translocation-specific probes. The latter are reported here only in case of parallel assessment by both FISH techniques.

Statistical Analysis

Differences between proportions of detected abnormalities were assessed using the McNemar test with continuity correction (McNemar, 1947; Agresti, 2002), or an exact version, as appropriate (Fay, 2010). Differences between detection rates were assessed with a Wilcoxon signed rank test. The relationship between sample cellularity, percentage of PCs and the detection of aberrations was modeled using a logistic regression model accounting for overdispersion (Agresti, 2002).

The effect of the applied FISH technique on the probability of detecting an abnormality was assessed with a subject-specific mixed effect logistic regression model with a random intercept for each patient. Estimates were adjusted for number of investigated PCs per probe and probe type using interaction terms and/or main effects (Agresti, 2002). Adjusted analysis is indicated in text with *. Backward model selection was performed. Results were considered significant at the 0.05 level.

Ethics

Approval was obtained from the local Institutional Review Board and the experiments were performed according to the International Conference on harmonization on good clinical practice (www.ich.org).

RESULTS

Clinical Findings

Two hundred and thirty-five BM samples were analyzed. Of those, 144 were diagnostic and 91 were follow-up samples. Ten patients were evaluated twice during disease evolution (Supplementary Table 1). Detailed information on patients and sample characteristics is shown in Table 1 and Supplementary Table 1. One hundred and twenty-two patients were male and 103 female. The median age was 66.8 years (range 35.3–89.4 years). The median percentage of PCs assessed by morphology on the BM aspirate was 32.5% (range 1–100%) and the median sample cellularity was 9.0 × 10^6/ml (range 0.7–110.0 × 10^6/ml) (Table 1).

FISH Analysis

The results of the FISH studies are summarized in Table 2. Detailed information is shown in Supplementary Tables 2 and 3. FISH was performed on purified PCs, obtained from fresh BM samples by immunomagnetic selection using anti-CD138 (AutoMACS, $n = 8$ or Easy Sep, $n = 11$) or on BM smears in which PCs were identified by κ/λ cytoplasmic immunostaining ($n = 216$).

An average of four probes was applied on PCs, but in this study we considered only the probes that were used in parallel on cultured BM. This explains the high number of cases analyzed with LSI IGH (Supplementary Tables 1–3). When an abnormal signal distribution was observed on PCs with the latter probe, further investigation was preferentially performed on cultured BM, so as to find the partner of translocation in metaphases.

The threshold of 20 evaluable PCs per probe was not reached in 111/432 tests and therefore, these tests were excluded for further analysis (Supplementary Table 3). These are 34, 34, 25, 16, 1, and 1 tests with probe LSI 13, LSI p53, LSI IGH, LSI BCL6, CEP9, and LSI IGH/

TABLE 2. Detailed Information on FISH on PCs vs. FISH on Whole Cultured BM[a]

	PCs	Cultured BM	*P*-value	*P*-value (odds ratio)[b]
Total				
Abnormal tests	228/321 (71.0%)	112/321 (34.9%)	<0.001	<0.001 (9.6)
Abnormal cases	176/205 (85.9%)	95/205 (46.3%)		
Aberrant nuclei[c]				
Higher percentage	91/110 (82.7%)	6/110 (5.5%)	<0.001	NA
Similar percentage	13/110 (11.8%)	13/110 (11.8%)		
Abnormalities per patient			<0.001	NA
0 abnormalities (*n*)	29	110		
1 abnormality (*n*)	135	83		
2 abnormalities (*n*)	31	8		
3 abnormalities (*n*)	9	3		
4 abnormalities (*n*)	1	1		
LSI IGH				
Abnormal (all patterns)	142/172 (82.6%)	73/172 (42.4%)	<0.001	<0.001 (9.6)
Abnormal (split pattern)	103/172 (59.9%)	55/172 (32.0%)	<0.001	<0.001 (11.0)
LSI P53				
Abnormal (loss or gain)	20/45 (44.4%)	8/45 (17.8%)	<0.001	<0.001 (9.6)
Abnormal (loss of 1 signal)	8/45 (17.8%)	3/45 (6.7%)	=0.48	NA
LSI 13				
Abnormal	36/51 (70.6%)	10/51 (19.6%)	=0.001	<0.001 (9.6)
LSI BCL6				
Abnormal	10/25 (40.0%)	7/25 (28.0%)	=0.45	<0.001 (9.6)
LSI IGH/CCND1				
Abnormal	12/15 (80.0%)	7/15 (46.7%)	=0.07	<0.001 (9.6)
LSI IGH/FGFR3				
Abnormal	1/4 (25.0%)	1/4 (25.0%)	NA[d]	<0.001 (9.6)
LSI MYC				
Abnormal	1/3 (33.3%)	1/3 (33.3%)	NA[d]	<0.001 (9.6)
LSI IGH/CCND1-XT				
Abnormal	3/3 (100.0%)	3/3 (100.0%)	NA[d]	<0.001 (9.6)
TCRad/TRAF3 (home-made probe)				
Abnormal	1/1 (100.0%)	1/1 (100.0%)	NA[d]	<0.001 (9.6)
CCND3 (home-made probe)				
Abnormal	1/1 (100.0%)	1/1 (100.0%)	NA[d]	<0.001 (9.6)
PBX1 (home-made probe)				
Abnormal	1/1 (100.0%)	0/1 (0.0%)	NA[d]	<0.001 (9.6)

FISH, fluorescent in situ hybridization; PC, plasma cell; BM, bone marrow; *n*, number; NA, not available.

[a]Excluded cases, i.e., cases with <20 evaluable PCs per probe, are not shown. In addition, only probes tested by both FISH techniques, i.e., on cultured whole BM and on selected PCs, are considered.

[b]Adjustment for number of investigated PCs per probe and probe type was done using interaction terms and/or main effects, as described in the Materials and Methods section.

[c]When the abnormality was detected by both FISH techniques ($n = 110$), the percentage of aberrant nuclei was compared. Differences ≥10% were considered to be significant, differences <10% were considered similar.

[d]Probes were applied in small numbers of cases, therefore no reliable *P*-value could be calculated for these cases.

CCND1, respectively. In 30/235 cases, all FISH tests failed (i.e., less than 20 PCs were assessable for each probe). Of note, an abnormality was detected on whole cultured BM in 8/111 and on PCs in 50/111 excluded tests, respectively, despite the low numbers of scored PCs.

In the remaining valid tests (Table 2, Supplementary Table 2), an abnormality was detected in 228/321 experiments on PCs versus 112/321 on cultured whole BM (71.0 versus 34.9%, respectively) ($P < 0.001$, odds ratio 9.6*). Results for the individual probes are shown in Table 2. Only in two tests with LSI BCL6, FISH on PCs failed to detect the abnormality, whereas FISH on cultured whole BM cells showed gains of 3q27. This can be explained by the presence of abundant background signals hampering the scoring process of PCs in case 190329 and by the low ratio (i.e., near the EMN cutoff) of aberrant BM nuclei and PCs in case 193418. In both cases, presence of gains of 3q27 was demonstrated on previous or following samples.

At least one abnormality was detected in 176 and 95 (85.9 and 46.3%) of the 205 eligible cases on PCs and cultured whole BM, respectively. Moreover, FISH on PCs was able to detect more

abnormalities per case (Table 2) ($P < 0.001$). When an abnormality was detected by both techniques ($n = 110$), the percentage of aberrant nuclei was compared. Differences >10% were considered to be significant. FISH on PCs detected a higher percentage of aberrant nuclei in 92 cases, while the percentage was higher on cultured whole BM in six cases ($P < 0.001$). A similar percentage of aberrant nuclei, this is a difference of ≤10%, was observed in 12 cases.

Adjustment for number of investigated PCs per probe and different probes was done using interaction terms and/or main effects. Probe type was not associated with the probability of detecting an abnormality*. In addition, there was no interaction effect between the number of investigated PCs per probe and the applied FISH technique*, i.e., the effect of number PCs is identical in both techniques. In contrast, main effects for the number of PCs and for technique were strongly significant*, indicating that abnormalities are more likely to be detected on selected PCs, and when the number of investigated PCs increases. This holds true when the number of investigated PCs per probe was included as a continuous variable and as a dichotomized variable (i.e., threshold of ≥100 PCs)*.

We examined the association between the reported percentage of BM PCs (assessed by morphology), the measured sample cellularity and the detection of chromosomal aberrations. A higher sample cellularity was associated with a higher probability of detection of at least one abnormality using at least one FISH technique ($P = 0.019$). There was also a strong association between the percentage of PCs (assessed by morphology) and the detection of at least one aberration on whole BM ($P < 0.001$) and on PCs ($P < 0.001$). However, the applied statistical models did not allow for definition of a threshold level of PC infiltration that would favor either FISH on PCs or FISH after cytogenetic culture. However, as illustrated in Figure 1 and Supplementary Table 4, moderate (≥50%) and high (≥80%) aberration detection rates were observed on cultured whole BM in cases with a BM PC infiltration >30% and >70%, respectively, whereas an overall high detection rate ≥70% was found on selected PCs, independent of the BM PC infiltration.

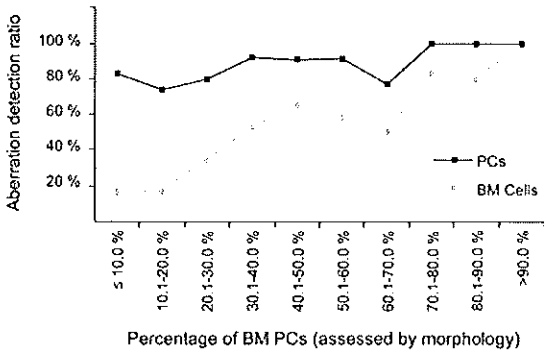

Figure 1. Aberration detection ratio versus percentage of BM PCs (assessed by morphology) in eligible cases (less than 20 evaluable PCs per probe was considered insufficient for inclusion). BM, bone marrow; PC, plasma cell.

DISCUSSION

The clinical course of MM is heterogeneous, with survival ranging from a few months to more than 10 years (Sirohi and Powles, 2006; Rajkumar and Buadi, 2007). Genetic alterations in MM are correlated with prognosis (Avet-Loiseau, 2007). However, karyotyping fails to detect aberrations in 50–70% of the cases, probably because metaphases do not properly represent the malignant hematopoietic component (Fonseca et al., 2004). In addition, prognostically unfavorable aberrations, e.g., t(4;14)(p16.3;q32) and t(14;16)(q32;q23), can be cryptic (Chesi et al., 1998; Avet-Loiseau et al., 1999). In contrast, interphase FISH can circumvent the problem, and has a resolution of at least one Megabase, allowing to reveal cytogenetically cryptic aberrations.

Based on practical experience, FISH after PC selection has been preferred over FISH on cultured BM. The superiority of PC selection was suggested in a small comparative series of 13 patients, in which 13q14 deletions were observed in 9/13 and 5/13 cases, using PCs and BM cells, respectively (Fiserova et al., 2002). In another study, two separate cohorts of 132 and 50 MM cases and eight and two cases with monoclonal gammopathy of unknown significance (MGUS) were analyzed without and with PC identification, respectively. In both cohorts, aberrations were detected in 46 and 92% of MM cases, respectively (Christensen et al., 2007). These results support the recommendation, initially based on a consensus agreement, that FISH should be performed after PC sorting or identification in the routine investigation of MM (Fonseca et al., 2004).

In this study, we have performed a direct comparison of interphase FISH on PCs versus cultured whole BM, in one single cohort of 235 cases. Three hundred and twenty-one parallel

tests performed on 205 samples were considered eligible. Abnormalities were detected in 34.9% versus 71.0% of the tests on whole cultured BM and selected PCs, respectively ($P < 0.001$, odds ratio 9.6*). At least one clonal abnormality was detected in 46.3 and 85.9% of the eligible cases on cultured whole BM and PCs, respectively. The latter percentages are similar compared to earlier observations (Christensen et al., 2007).

The prevalence of loss of *TP53* was 17.8% after PC selection, consistent with data reported in the literature (Fonseca et al., 2003, 2004; Avet-Loiseau, 2007). The higher prevalences of 14q32/IGH aberrations and loss of 13q, i.e., 82.6 and 70.6%, respectively (Fonseca et al., 2003, 2004; Avet-Loiseau, 2007), are possibly related to selection bias, as in cases with aberrant IGH patterns on PCs, special efforts were made to identify a translocation partner. Therefore, these cases were almost systematically analyzed by FISH on metaphases. For LSI 13, higher detection rates, namely in 11/15 (73%), have previously been described in MM patients with relapse (Zojer et al., 2000), and are particularly frequent in cases with 14q32/IGH translocations (Fonseca et al., 2004).

Moreover, FISH on PCs detected more abnormalities per case ($P < 0.001$) and identified higher percentages of abnormal nuclei ($P < 0.001$). Even in non eligible tests, the detection rate of abnormalities in PCs was higher compared to whole cultured BM (50/111 versus 8/111) cases. In this study, 25.7% ($n = 111$) tests were considered non eligible, that is, less than 20 PCs per probe were assessable. Christensen et al., (2007) reported 10% ($n = 5$) of non- (i.e., < 20 PCs) or only partially (i.e., 20–50 PCs) evaluable cases. The higher percentage of nonvalid cases in our study can be explained by several factors: (i) some samples were of suboptimal quality due to delay in transport and differences in manipulation in the referring centers, (ii) there was a selection bias, namely when only very few PCs were sorted or identified, additional FISH experiments were preferentially performed on whole cultured BM, and (iii) the analysis was not restricted to MM, as other PC dyscrasias were included.

As observed by Avet-Loiseau et al., (2007) and Fonseca et al., (2009), the portion of a BM aspirate dedicated to cytogenetic analysis is commonly the last tube collected. Consequently, dilution in peripheral blood will result in a lower concentration of PCs in the cytogenetic than in the morphological sample. This might jeopardize the detection rate of abnormalities by FISH on whole cultured BM. As there is a strong association between the detection rate of aberrations and the morphologically assessed PC infiltration ($P < 0.001$), FISH on selected PCs should be the standard technique, especially in cases with low PC counts. After adjustment for number of investigated PCs per probe and probe type*, the effect of the investigated number of PCs per probe was found to be identical in both techniques. In addition, abnormalities were more likely to be detected on selected PCs, and when the number of investigated PCs increases.

In summary, the detection rate of chromosomal abnormalities and the number of abnormalities per case in MM and PC dyscrasia significantly improves when PCs are selectively analyzed. This study confirms FISH on PCs to be the preferred technique for routine cytogenetic investigation of PC dyscrasia.

ACKNOWLEDGMENTS

We thank all referring clinicians and laboratory technicians. We are also indebted to A. Ferrant and K. Rack for critical reading of this manuscript.

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