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REVIEW ARTICLE

FISH diagnostics in plasma cell myeloma: recommendations and our own experience

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Abstract

Plasma cell myeloma (PCM) is disease with heterogeneous clinical outcomes. It is increasingly evident that the genetic features of the tumor cells largely dictate the clinical heterogeneity of PCM. Primary chromosomal alterations in myeloma can be divided into hyperdiploid and non-hyperdiploid subtypes. Secondary chromosomal changes occur during progression of disease. Cytogenetic abnormalities are important prognostic markers in PCM and some of them were incorporated into the current prognostic staging system of PCM. The presence of t(4;14), t(14;16), t(14;20), gain of 1q or *TP53* deletion is considered to be high-risk myeloma. Detection of these alterations can be performed by interphase fluorescence *in situ* hybridization (FISH) after separation or identification of the plasma cells. The proper FISH examination in myeloma has to meet further requirements regarding aspirating and timing of samples, probe selection and their cut-off levels, the criteria of accurate analysis and reporting. Based on the literature, we here present technical recommendations regarding FISH in PCM. Furthermore, we share our own experience in FISH diagnostics acquired over 12 years. In this period, we have performed nearly 2,050 FISH tests in 603 myeloma patients and used two different methods of myeloma FISH: FISH on immunolabeled plasma cells, and target FISH with the BioView system.

Key words: myeloma, plasmacytoma, FISH, c-IG, target FISH, BioView

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Introduction

Plasma cell myeloma (PCM) is the most common malignant gammopathy and accounts for 10% of all hematological neoplasms [1]. The disease virtually always starts with a premalignant monoclonal gammopathy of undetermined significance (MGUS) that variably progresses to symptomatic PCM within months or years [2]. PCM is a heterogeneous disease with variable courses, responses to therapy, and survival outcomes that range from less than one year to more than 10 years. This clinical variety reflects the biological diversity driven by genetic abnormalities. Much has been learned regarding these genetic abnormalities. For instance, the translocations affecting immunoglobulin heavy chain (*IGH*) locus are essential in the pathogenesis of PCM in nearly 50% of patients. Most of the remaining patients have hyperdiploidy (trisomies of odd-numbered chromosomes) as the hallmark of the disease [1, 3]. In addition to these primary genetic events, presentation of myeloma is frequently accompanied by secondary chromosome abnormalities including deletion of chromosome 13q, gain of chromosome 1q [gain(1q)], and deletion of chromosome 1p [4]. Cytogenetic diagnosis constitutes an important part of the risk stratification of PCM and genetic diagnostic recommendations are constantly being updated. It must be underscored that the cytogenetic analysis of PCM can be challenging. Due to low proliferating features of malignant plasma cells and multiple marrow infiltrates, karyotyping is not recommended. Moreover, some chromosome aberrations such as t(4;14)(p16;q32)

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Table I. Cytogenetic abnormalities in newly diagnosed plasma cell myeloma (modified from [1, 2, 4, 6–9])

Type of genetic event	Cytogenetic abnormalities	Gene affected	Frequency [%]
Primary	Trisomies of odd-numbered chromosomes with the exception of chromosomes 1, 13		42–60
	IGH translocations:		30
	• t(11;14)(q13;q32)	*CCND1*	15–20
	• t(4;14)(p16;q32)	*MMSET, FGFR3*	6–15
	• t(14;16)(q32;q23)	*MAF*	2–7
	• t(14;20)(q32;q11)	*MAFB*	1
	• t(6;14)(p21;q32)	*CCND3*	1–4
	• t(12;14)(p13;q32)	*CCND2*	~1
	IGH translocations and trisomies of odd-numbered chromosomes		15
	Deletion of whole *IGH*	*?TRAF3*	4,5–45
Secondary	17p deletion	*TP53*	5–15
	13q deletion/monosomy of chromosome 13	*RB1, DIS3,DLEU2*, miR-15a, miR-16-1	50
	1q21 gain	*CKS1B*	34–40
	1p32 deletion, 1p22 deletion	*CDKN2C*	7–17
	Translocation of 8q24/other aberration of 8q24	*MYC*	15–35

are karyotypically cryptic. For these reasons, interphase fluorescence *in situ* hybridization (FISH) is the most useful cytogenetic method. Unlike with other hematological malignancies, in PCM, FISH should not be performed directly on bone marrow. Due to frequent low plasma cell percentage, plasma cell selection must be carried out. There are two popular methods of plasma cell enrichment: labeling of the cytoplasmic immunoglobulin light chains (c-IG), and plasma cell sorting.

The alternative method of plasma cell identification is target FISH. In this method, automated image analysis system combines the images of May-Grünwald-Giemsa (MGG) staining and FISH study on the same plasma cell for analysis [5].

Herein, we present international recommendations for FISH in PCM, together with own experience of c-IG FISH and target FISH.

Chromosomal abnormalities in PCM

Current understanding regarding the chromosomal abnormalities in PCM and the association of these genetic events with clinico-pathological features has enabled the creation of a biological genetic classification of PCM [3, 4, 6]. This classification denotes primary and secondary abnormalities (Table I) [1, 2, 4, 6–9]. Primary alterations divide myeloma into hyperdiploid and non-hyperdiploid subtypes. The first is characterized by trisomies of odd-numbered chromosomes: 3, 5, 7, 9, 11, 15, 19, 21 and is associated with a more indolent form of the disease. However, not all trisomies have the same prognostic impact: trisomy 21 impairs, while trisomies 3 and 5 actually improve, survival [7]. The non-hyperdiploid subtype is characterized by the *IGH* translocations and is associated with a more aggressive course. Primary *IGH* translocations with oncogenes include (in descending order of frequency): t(11;14)(q13;q32) (*CCND1*), t(4;14)(p16;q32) (*MMSET, FGFR3*), t(14;16)(q32;q23) (*MAF*), t(14;20)(q32;q11) (*MAFB*), t(6;14)(p21;q32) (*CCND3*), t(12;14)(p13;q32) (*CCND2*). Hyperdiploidy is almost mutually exclusive with *IGH* translocations, but in very rare cases, both trisomies and *IGH* translocations can be present. Hyperdiploidy and *IGH* translocations are present in all stages of gammopathy, suggesting that primary alterations initiate preneoplastic MGUS, but are not sufficient to cause the progression to PCM [4].

Besides trisomies and *IGH* translocations, the monosomy of *IGH* is considered as another primary chromosomal aberration [1]. Recent studies suggest that deletion of the whole *IGH* is an early event in the pathogenesis of myeloma [8, 9]. In addition to early (primary) chromosomal events, the presentation of myeloma is accompanied by acquisition of secondary chromosomal alterations. These secondary abnormalities are generally associated with a poor prognosis. The major secondary changes are: chromosome 13 deletion/monosomy, which co-occurs with t(4;14) and t(4;16), 1q21 gain and 1p32 deletion, which are closely related and chromosome 17p (*TP53*) deletion [del(17p)]. Other frequent secondary genetic events are alterations of the *MYC* involving not only translocations

Table II. Cytogenetic risk stratification of newly diagnosed plasma cell myeloma patients (acc. to International Myeloma Working Group classification [6] and Mayo Clinic classification [1])

High risk factors	Standard risk factors
t(4;14)	All other including:
t(14;16)	• t(11;14)
t(14;20)	• t(6;14)
del(17p)	• trisomies of odd-numbered chromosomes
gain(1q)	
'Double hit': two high risk factors	
'Triple hit': three or more high risk factors	

but also amplifications, duplications and inversions [3]. Deletion of *TP53* is a particularly poor prognostic factor, and is unresolved even by modern therapies or allogeneic stem cell transplantation [4, 6].

Cytogenetic risk stratification

Identifying high-risk patients and treating them properly is essential to improve outcomes in PCM [2]. Chromosomal abnormalities have important prognostic value for PCM, especially in identifying high-risk patients (Table II). According to classifications of the International Myeloma Working Group and Mayo Clinic, the high risk cytogenetic abnormalities are: t(4;14), t(14;16), t(14;20), del(17p), gain(1q). All other changes including: t(11;14), t(6;14), trisomies of odd-numbered chromosomes (hyperdiploidy) are considered as standard risk factors [1, 6, 10]. Compared to the previous risk classification, this stratification has incorporated new risk factors, 'double hit' and 'triple hit'. Recently it has been reported that patients with 'double hit' defined by the co-occurrence of at least two high risk alterations have an especially poor prognosis [11].

The cytogenetic risk stratification may change with treatment modalities. At present, the improved current prognostic staging system of PCM (The Revised International Staging System for Myeloma) incorporates the presence of three high risk abnormalities: t(14;16), t(14;20), del(17p) for the better stratifications of PCM patients [12].

Recommendations for FISH in PCM

Compared to other hematological neoplasms, an accurate FISH analysis in PCM is more complicated and more time-consuming. Practical guidelines for FISH testing have been developed by the European Myeloma Network and the European Cytogeneticists Association [3, 13, 14]:

- Morphological assessment of bone marrow cannot be used to decide whether or not to carry out FISH.
- Material should be a part of the first draw of aspirate, and the needle must be repositioned for further aspiration.
- The aspirate should be sent at a suitable time, because laboratory PCM processing is time-consuming.
- It is very important to purify or to identify the plasma cells (PC), but the method used should be chosen by the laboratory.
- It is strongly advised that cut-off levels for a positive result should be relatively conservative: 10% for dual fusion or break-apart probes, and 20% for single fusion probes and numerical abnormalities. These recommendations are subject to controversy and some laboratories may want to use their own threshold. Therefore, it should be pointed out that in purified or identified PCs, the vast majority of I PCs are expected to have primary changes. However, secondary abnormalities can only exist in a part of the PC population.
- Minimal panel of probes at the time of diagnosis should detect *FGFR3/IGH*, *MAF/IGH* and deletion of *TP53*.
- It is recommended that 100 cells be scored wherever possible. However, if high purity/identified PC samples were being analyzed, 50 cells should be sufficient for a normal result of primary abnormality. In exceptional circumstances, an abnormal result in as few as 10–20 PCs can be reported, but all doubts should be stated in the report.
- It is considered that a single experienced analyst is sufficient to examine the FISH specimens. However, cases with a low proportion of cells with alteration or a low level of plasma cells have to be analyzed by a second diagnostician.
- The report should be stated clearly for clinicians. It should include the method of PC identification, the probes used, the number of scored cells, and the percentage of cells with alterations. The European Myeloma Network [13] does not recommend International System for Human Cytogenomic Nomenclature (ISCN), but according to the guidelines of the European Cytogeneticists Association [14], a full ISCN should be stated on the report.
- The frequency of FISH testing is not well defined. It is accepted that primary abnormalities will not change over time. However, disease progression can be accompanied by genetic evolution. In the case of disease relapse, it is now recommended to test del(17p) and gain(1q) [6].

The extension of probes panel at the time of diagnosis may be necessary as it can yield more information regarding disease biology, clinical features and outcome. The extended, more comprehensive, panel may include testing for chromosome 1 abnormalities, t(11;14), t(14;20), chromosome 13 deletion, and ploidy status (to establish aneuploidy for any two chromosomes out of 5, 9, 11 and 15) [6, 7, 13, 14].

FISH diagnostics in myeloma — laboratory experience

FISH analysis of identified plasma cells in PCM became part of our laboratory practice in 2009. Between September 2009 and March 2021, bone marrow samples (or other extramedullary tissues) of 603 patients with suspected plasma cell myeloma or extramedullary plasmacytoma were investigated. Over that period, we performed nearly 2,050 FISH tests in myeloma patients (Table III).

For PC identification, we applied immunostaining of cytoplasmic immunoglobulin chains. In this method, AMCA anti-human kappa, anti-human lambda and anti-human IGG chains antibodies are used for staining of PC cytoplasm (Vector Laboratories, Burlingame, CA, USA). For this purpose, we use cultured cells fixed in 3:1 methanol:acetic acid. Excitation and emission parameters of our fluorescent microscopes (triple Filter set 25HE, Carl Zeiss Jena, Germany) enables us to see AMCA stained cytoplasm of plasma cells as brown/yellow (Figure 1).

In March 2018, we introduced target FISH as the second method of PC identification. In this method, bone marrow cells are separated by density gradient centrifugation to prepare cytospins. Cytospin slides are stained with MGG and scanned by multiparametric BioView system (Abbott Molecular, Abbott Park, IL, USA). The system automatically selects plasma cells, but review by a diagnostician is necessary to classify PCs for further FISH analysis. According to our experience, the optimal number of classified PCs is 200–250. This is many more than should be analyzed, but it is necessary because of hybridization failure or detaching of cells. The next steps are destaining of the slides, FISH procedure, and repeated scanning. The system automatically finds previously selected PCs and enables simultaneous observation of FISH results and MGG morphology of cells (Figure 2).

Currently we use both methods of PC identification (Figure 3) and we apply the FISH algorithm presented in Figure 4. If the amount of bone morrow (BM) is adequate, every sample is *in vitro* cultured for c-IG FISH (and karyotyping, if necessary) and prepared for target FISH. In the cases of extramedullary plasmacytoma, biopsies of other tissues are *in vitro* cultured for c-IG FISH and karyotyping. Employing two methods is more time-consuming, but provides advantages. The identification of PCs is crucial for proper FISH analysis. c-IG FISH is the established method of PC identification, but in some cases the labeling of cytoplasm is weak and the selection of plasma cells is very difficult. MGG morphology as the first step of PC identification minimizes the troubles with PC selection. On the other hand, the procedure of cytospin preparation can lead sometimes to destruction of PCs. Plasma cells are sensitive for centrifugations, because of the abundance of cytoplasm. In this case, c-IG of cultured cells facilitates PC selection.

Table III. Data regarding myeloma fluorescence *in situ* hybridization (FISH) tests performed in Cytogenetic Laboratory, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland

Year	Number of PCM patients	Number of EMP patients	Number of patients	Number of tests
2009	15	2	17	111
2010	44	5	49	310
2011	34	4	38	170
2012	42	2	44	179
2013	28	1	29	150
2014	48	0	48	214
2015	52	3	55	190
2016	44	3	47	145
2017*	45	2	47	97
2018#	82	6	88	185
2019	69	2	71	141
2020	53	2	55	120
2021 (January–-March)	15	0	15	39
Total: September 2009 to March 2021			603	2051

*Introduction of basic panel of probes: IGH breakapart, *TP53*/centromere 17; #introduction of target FISH; PCM — plasma cell myeloma; EMP — extramedullary plasmacytoma

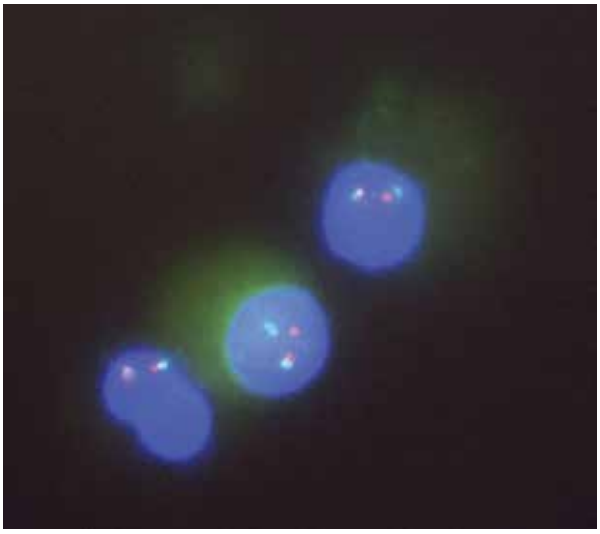

Figure 1. Plasma cell identified by labeling of cytoplasmic immunoglobulin light chains (c-IG). Fluorescence *in situ* hybridization (FISH) with immunoglobulin heavy chain (IGH) breakapart probe (Zytovison, Bremerhaven, Germany): separate green and red signals indicate rearrangement of *IGH*. Non-plasmatic cell has two not rearranged IGH (yellow) signals

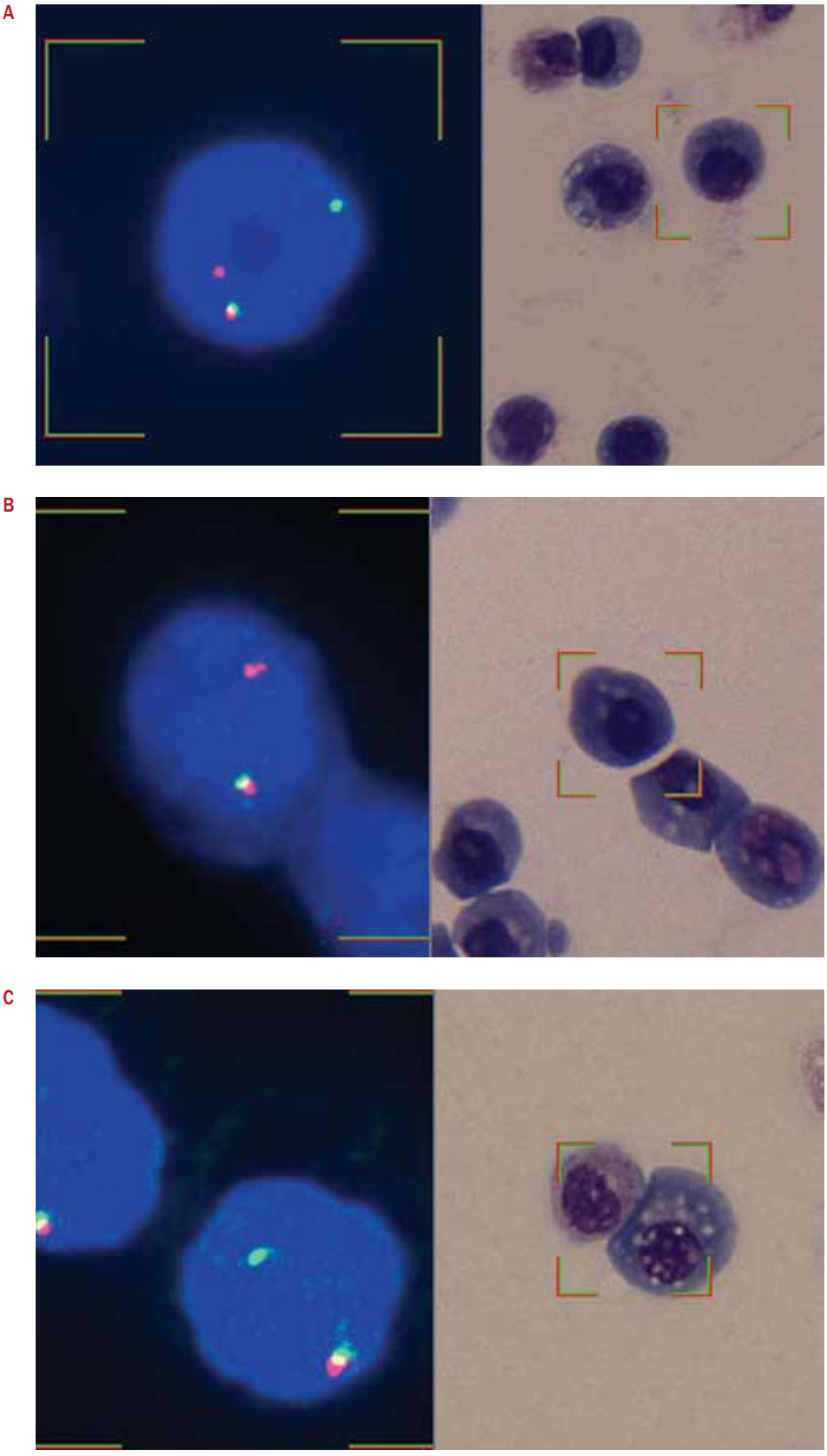

Figure 2. Plasma cells identified by target fluorescence *in situ* hybridization (FISH). FISH result of immunoglobulin heavy chain (IGH) break-apart (BAP) probe (Zytovison, Bremerhaven, Germany) on left, same cell stained with May-Grünwald-Giemsa (MGG) on right: **A.** Typical rearrangement of *IGH*: one 3'IGH (red) signal, one 5'IGH (green) signal and one IGH (yellow) signal (1Y1R1G); **B.** Deletion of 5'*IGH* region: one 3'IGH (red) signal and one IGH (yellow) signal (1Y1R); **C.** Deletion of 3'*IGH* region: one 5'IGH (green) signal and one IGH (yellow) signal (1Y1G)

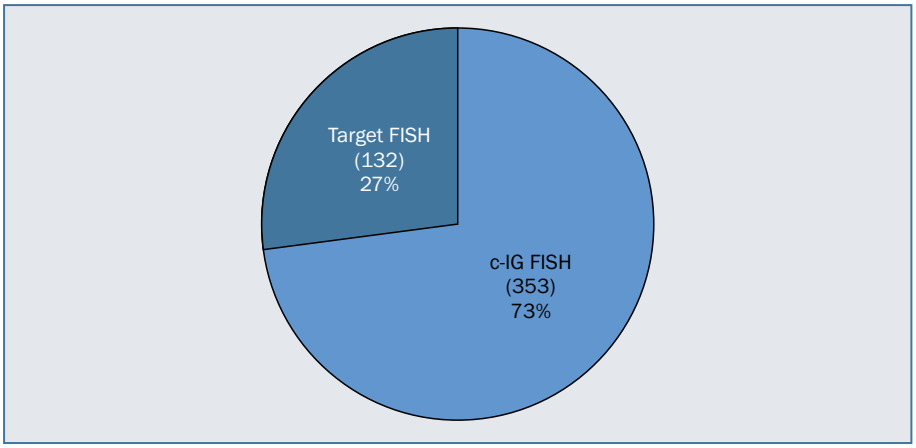

Figure 3. Fluorescence *in situ* hybridization (FISH) myeloma tests performed in Cytogenetic Laboratory, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland between 2018 and 2021. In this period, two methods of plasma cells identification for FISH were used: labeling of cytoplasmic immunoglobulin light chains [immunostaining of cytoplasmic immunoglobulin chains (c-IG) FISH] and target FISH. Total number of tests was 485, c-IG FISH represented 73% of all tests, target FISH represented 27% of all tests

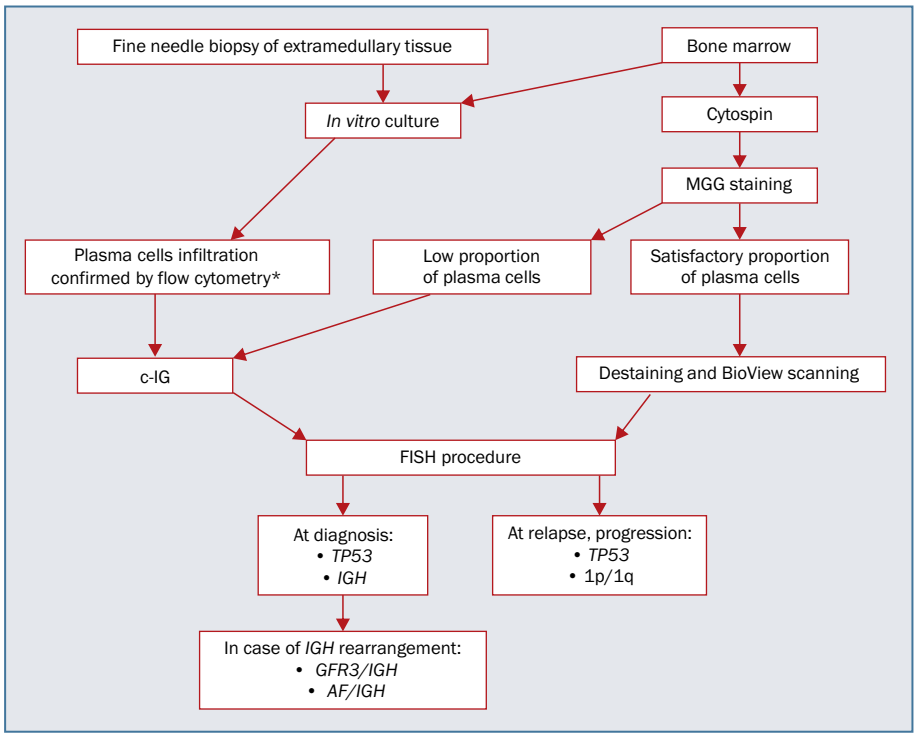

Figure 4. Scheme of myeloma fluorescence *in situ* hybridization (FISH) diagnostic algorithm used in Cytogenetic Laboratory, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw; *flow cytometry confirmation is needed only in biopsy specimens; MGG — May-Grünwald-Giemsa staining; c-IG — immunostaining of cytoplasmic immunoglobulin chains

In newly diagnosed patients, or in patients without any cytogenetic data, our basic panel consists of IGH BAP and TP53/centromere 17 probes. If IGH signal pattern suggests rearrangement, IGH/FGFR3 and IGH/MAF dual fusion probes are used to detect high risk fusions. It should be emphasized that not only typical split signal pattern (1Y1R1G — one yellow, one red, one green signal) point at the rearrangement of the *IGH* (Figure 2A). In 10–17% of PCM patients, partial deletions of the *IGH* locus are observed. These deletions are heterogeneous, most often including monoallelic deletion of 3'*IGH* (constant region) and monoallelic deletion of 5'*IGH* (variable region) [8, 15, 16]. Moreover, these deletions may be accompanied by duplications of the *IGH* regions. Various *IGH* deletion signal patterns can be observed, including 1Y1R, 1Y1G, and 2Y with diminished R or G signal (Figure 2B, 2C). As approximately 20% of these deletions coexist

with translocations, it is important to use dual fusion probes when deletion of the *IGH* is identified [16].

The aforementioned panel of FISH probes has been used in our laboratory since 2017. Prior to that, our panel was more extended. This panel followed valid myeloma FISH recommendations and included testing for *IGH/FGFR3*, *IGH/MAF*, *IGH/CCND1*, 13q14 deletion and *TP53* deletion.

In progression or at relapse of PCM we use CKS1B/CDKN2C and TP53/centromere 17 probes for testing of 1p/1q aberrations and *TP53* deletion.

In addition to the imperative of identifying PCs, intrapatient/intratumoral heterogeneity creates further difficulties in FISH diagnostics [2]. It often happens that there are discrepancies between the proportion of PCs assessed by examination of bone marrow aspirate smears or trephine sections, and the proportion of PCs in samples dedicated to FISH. In some cases, we have observed that despite a high proportion of PCs in morphological smears, FISH samples had too few PCs to allow an analysis. On the other hand however, regardless of a very low proportion of PCs in morphological smears, we have occasionally found an adequate number of PCs on c-IG slides or MGG cytospins.

In conclusion, accurate FISH analysis in PCM is more complicated and time-consuming than in other hematological FISH tests.

The proper FISH diagnostics in plasma cell myeloma should be carried out according to the recommendations of the European Myeloma Network and the European Cytogeneticists Association. Every laboratory which performs myeloma FISH tests should follow the latest advice regarding risk stratification in myeloma.

Author's contributions

RW — sole author.

Conflict of interest

The author declares no conflict of interest.

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None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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