

RESEARCH ARTICLE

The Utilization of Karyotyping, iFISH, and MLPA for the Detection of Recurrence Genetic Aberrations in Multiple Myeloma

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Abstract

Multiple myeloma (MM) is a hematological malignancy characterized by abnormal accumulation of clonal plasma cells in the bone marrow. Recently, multiplex ligation-dependent probe amplification (MLPA) has emerged as an effective and robust method for detection of common genetic alterations in MM patients. Here, we aimed to confirm MLPA utility for this purpose and furthermore to test the feasibility of a combination of karyotyping, interphase fluorescence in situ hybridization (iFISH) and MLPA methods for diagnosis, prognostic assessment and risk stratification of MM. Thirty-five genomic DNA samples isolated from CD138-enriched plasma cells from bone marrow of MM patients were analyzed using the MLPA method. We found that amp (1q) was the most frequent genetic alteration (48.6%) in the tested samples, followed by del (1p) and del (13q) (34.3%). Moreover, concordant results between sensitivity and specificity of iFISH and MLPA for the detection of del (13q) (p-value >0.05) and del (17p) (p-value >0.05) were obtained. In summary, we could provide evidence of MLPA assay utility for the detection of common genetic alterations in MM. The combination of karyotyping, iFISH, and MLPA proved very helpful for clinical risk stratification.

Keywords: Multiple myeloma-karyotyping-interphase Fluorescence in situ hybridization

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Introduction

Multiple myeloma (MM) is a malignant of terminally differentiated B cell which is characterized by the presence of clonal proliferation of malignant plasma cells (PCs) in the bone marrow and excessive production of monoclonal immunoglobulin resulting in multiple organ dysfunctions (Palumbo and Anderson, 2011; Sultan et al., 2016). MM is a heterogeneous disease in which clinical representation, cell morphology, immunophenotype, prognosis, disease progression as well as treatment outcome, some harboring specific genetic alterations. Common genetic lesions in MM are including chromosomal translocation involving immunoglobulin heavy chain (IgH) locus (14q32), copy number variations (CNVs) of variable chromosomes (e.g., hyperdiploidy of odd numbered chromosomes) or specific region of chromosome (e.g., 13q deletion), and the acquired somatic mutations (e.g. p53, *KRAS*, *NRAS*, and *BRAF*) (Braggio et al., 2015; Chesi and Bergsagel, 2013). Several molecular genetic techniques including conventional cytogenetic study (karyotyping) and interphase fluorescent in situ hybridization (iFISH) are

currently used for the diagnosis, risk assessment, and monitoring of the disease during treatment (Calasanz et al., 1997; Drach et al., 1995; Sawyer, 2011; Zhan et al., 2006). While conventional cytogenetic study offers a full view of chromosomes, the assay could detect approximately up to 30% of cytogenetic abnormalities in MM due to the low mitotic activity of MM cells and the low resolution of the technique (Fonseca et al., 2004). iFISH has been designed to overcome several shortcomings of conventional cytogenetic analysis. The assay could identify approximately to 90% of recurrence cytogenetic abnormalities in MM. Moreover, the efficacy of the assay is dramatically increased when applied for a positive selection of CD138 tumor cells (plasma cell-enriched sample). Therefore, iFISH has emerged as the most viable and widely used technique for the detection of cytogenetic abnormalities in MM. Nevertheless, iFISH is a laborious and time-consuming method with high cost, and is only capable to detect gain/loss of sequences larger than 20–50 kb (Talley et al., 2015). Although cytogenetic analysis and iFISH are able to detect a large scale of primary oncogenic events (e.g.,

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balance translocations and chromosomal aneuploidy) and clearly used as a diagnostic test for MM, several key driving mutations associated with disease progression and treatment outcomes (e.g., copy number variation (CNVs) of specific region on chromosome) could not identify by using those conventional techniques. Recently, multiplex ligation-dependent probe amplification (MLPA) technique has been developed as a fast and robust assay for the detection of CNVs up to 50 different genomic sequences simultaneously. MLPA probes are able to recognize target sequences with 50–100 nucleotides in length, which makes it possible to be applied for highly fragmented DNA. Additionally, the assay could detect a small deletion encompassing only a single exon (Schouten et al., 2002). MLPA specific panels have been recently developed for several disease entities such as inherited conditions and hematological malignancies including multiple myeloma (Alpar et al., 2013). In this report, we aimed to use a robust pan-screening multiplex ligation-dependent probe amplification (MLPA) assay to detect common CNVs in newly diagnosed multiple myeloma patients. Furthermore, we determined the feasibility for the combination of standard karyotyping, iFISH, and MLPA for the diagnosis and risk-stratification of multiple myeloma.

Materials and Methods

Patients and samples

Bone marrow samples were obtained from 35 patients diagnosed with symptomatic myeloma during July 2016 to February 2017 at the Faculty of Medicine, Ramathibodi Hospital. Diagnosis criteria was based on the International Myeloma Working Group 2016 (Kumar et al., 2016). Additionally, 5 peripheral blood control samples from healthy donors were also included into this study. In a total of 35 multiple myeloma samples, the male to female ratio was 18/17 (1.05) which the mean and median ages were 60.4 and 59, respectively (range: 38–79 years old). This work was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki (MURA2016/440).

Cytogenetic study

Complete cytogenetic study was performed at Human Genetic Laboratory, Department of Pathology, Ramathibodi Hospital using G-banding technique after short term culture without mitogen activation. On-screen karyotyping was performed on 20–30 metaphases using Ikaros software, MetaSystems, Germany. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2016).

Interphase fluorescent in situ hybridization (iFISH)

iFISH was performed on whole bone marrow mononuclear cells. In brief, chromosome 13 deletion were detected with probe specific for the 13q34 locus (LSI 13q34, Abbott Laboratories, Illinois, USA). Chromosome 17 deletion was detected by using probe specific to 17p13.1 locus (LSI p53, Abbott Laboratories, Illinois, USA). LSI

IGH/FGFR3 dual-color and LSI IGH/CCND1 XT probes (Abbott Laboratories, Illinois, USA) were used to detect t(4;14) (p16.3;q32) and t(11;14)(q13;q32), respectively. Fluorescence images were captured with epifluorescence microscope (Zeiss, Germany) using appropriated filters. One hundred interphase nuclei were scored for each probe. The cut off levels for positive value of each probe were following 10% for chromosomal translocation and 20% for numerical abnormalities according of European Myeloma Network FISH workshops recommendations (Ross et al., 2012).

Plasma cells (PCs)-enriched and genomic DNA extraction

Plasma cells were purified using CD138 plasma cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacture protocol. The purity of isolated plasma cells were confirmed to be >50% in all cases by cell staining (Wright-stained) after cytopsin and visualized under microscope. Genomic DNA (gDNA) were extracted by using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacture instruction. The quality and quantity of isolated gDNA were measured by using Nano Drop 2000 (Thermo Fisher Scientific, USA) according to manufactures instruction. DNA samples were then stored at -20 °C prior used.

Multiplex ligation-dependent probe amplification (MLPA) analysis

Fifty nanograms of gDNA were subjected to MLPA analysis by using SALSA MLPA P425-B1 MM probemix (MRC-Holland, Amsterdam, Netherlands). In detail, the probemix contained 46 probes for the detection of following regions 1p32.3 (*FAF1*, *CDKN2C*), 1p32.2 (*PLPP3* and *DAB1*), 1p31.3 (*LEPR*), 1P31.2 (*RPE65*), 1p21.3 (*DPYD*), 1p21.1 (*COL11A1*), 1p12 (*FAM46C*), 1q21.3 (*CKS1B*), 1q23.3 (*NUF2*, *RP11* and *PBX1*), 5q31.3 (*PCDH1*, *PCDHAC1*, *PCDHB2*, *PCDHB10*, *SLC25A2*, and *PCDHGA11*), 9p24.1 (*JAK2*), 9q34.3 (*COL5A1*), 12p13.31 (*CD27*, *VAMPI*, *NCAPD2*, *CHD4*), 13q14.2 (*RBI* and *DLEU2*), 13q22.1 (*DIS3*), 14q32.32 (*TRAF3*), 15q12 (*GABRB3*), 15q26.3 (*IGF1R*), 16q12.1 (*CYLD*), 16q23.1 (*WWOX*) and 17p13.1 (*TP53*). MLPA reactions including internal quality controls and negative controls were performed according to the manufacturer instructions. The PCR products were analysed using ABI 3130 Genetic analyser (Applied Biosystems, Foster City, CA, USA) and Coffalyser.net software (MRC Holland, Amsterdam, Netherlands) according to the manufacture instruction.

Statistics analysis

Congruency of iFISH and MLPA results were analyzed using McNemar test. The statistical significant difference was considered at p-value less than 0.05.

Results

Cytogenetic study and iFISH for the detection of genetic alterations in multiple myeloma

In this study, 25 of 35 multiple myeloma patients displayed normal karyotype (46,XX and 46,XY) by

Table 1. Summary of Genetic Alterations Detected by Karyotyping, Ifish and MLPA in Individual Multiple Myeloma Patient

Sample	Age/Sex	Karyotyping	iFISH	MLPA
HGU001	57/Female	46,XX[36]	del(13q)	del(1p), amp(5q), amp(9p,q), del(13q), amp(15q), del(16q)
HGU002	62/Male	46,XY	t(4;14), three copies of CCND1	del(1p), amp(9p,q), del(12p), amp(15q)
HGU003	57/Male	45-47,Y,dic(1;6)(p13;q22),?t(1;2)(p36.1;q37),+add(5)(q13),del(6)(q21q25),+del(6),der(7)t(1;7)(q21;p22),del(8)(p21),+9,add(14)(q32),+19,inc[cp5]/46,XY[33]	del(13q), one copy of 14q32 (IGH)	del(1p), amp(1q), amp(9p,q), del(13q), del(14q), amp(15q)
HGU004	38/Male	46,XY[30]	three copies of CCND1	amp(5q), amp(9p,q), amp(15q)
HGU005	66/Female	45,X,-X,del(1)(p13p36.1),add(5)(p15.1),del(6)(q21q25),add(8)(p11.2),der(9)t(1;9)(q21;q34),-13,+17[3]/46,XX[37]	del(13q), trisomy17p	del(1p), amp(1q), del(13q), amp(17p)
HGU006	52/Female	46,XX[30]	three copies of CCND1, three copies of CCND3, three copies of MAF	not detected
HGU007	71/Female	46,XX[30]	del(13q), three copies of CCND1, three copies of CCND3	del(1p), amp(1p), amp(1q), amp(5q), amp(9p,q), del(13q), amp(15q)
HGU008	79/Female	46,XX[41]	unable to analyze	amp(12p), amp(16q)
HGU009	57/Female	46,XX	del(13q), del(17p)	del(1p), amp(1q), del(13q), del(14q), del(16q), del(17p)
HGU010	63/Male	45,X,-Y[8]/46,XY[22]	unable to analyze	not detected
HGU011	59/Female	46,XX[31]	not detected	not detected
HGU012	61/Female	46,XX[30]	unable to analyzed	not detected
HGU013	44/Male	46,XY[32]	polysomy of chromosome 13, two to four copies of FGFR3, two to four copies of CCND1, two to four copies of IGH	del(1p), amp(1p), del(1q), amp(1q), del(5q), amp(5q), del(9q), amp(9p), del(12p), amp(13q), del(14q), del(15q), del(16q), amp(16q), del(17p)
HGU014	50/Male	46,XY[30]	not detected	del(1p), amp(1p), amp(1q), amp(12p)
HGU015	58/Female	46,XX[32]	del(13q), del(17p)	amp(1q), del(5q), del(13q)
HGU016	57/Male	52,XY,+1,dic(1;?) (p12;?),+9,+11,del(13)(q12q14),add(14)(q11.2),+15,del(15)(q22q26.1) or del(15)(q24),+19,+21[31]	del(13q), three copies of CCND1	del(1p), amp(1q), del(5q), amp(9p), del(12p), del(13q), amp(15q), del(15q)
HGU017	66/Male	46,XY[30]	duplication of 13q34 with deletion of 13q14.3, trisomy 17, tetrasomy 17, IGH/FGFR3 fusion gene t(4;14), three to four copies of CCND1 and four copies of IGH	amp(1q), del(13q)
HGU018	69/Male	45,X,-Y[5]/46,XY[26]	translocation of 14q32 (IGH) involving with other chromosome	del(1p), amp(1p), amp(1q), amp(5q)
HGU019	62/Male	46,XY[30]	not detected	no detected
HGU020	69/Female	46,XX[30]	del(13q), t(4;14)	not detected
HGU021	57/Female	46,XX[31]	del(17p)	amp(1q), amp(5q), amp9(p), amp(15q)
HGU022	47/Male	46,XY[14]	not detected	not detected
HGU023	65/Female	-	not detected	del(1p), amp(1q), amp(5q), amp(15q), del(16q), amp(17p)
HGU024	58/Female	46,XX[30]	not detected	not detected
HGU025	69/Male	-	del(13q), t(4;14)	amp(1q), del(13q)
HGU026	49/Female	-	del(13q)	amp(1q), amp(9q), del(13q)
HGU027	59/Female	46,XX[3]	not detected	del(1p), amp(1q), del(1q), del(13q), amp(14q), del(16q)
HGU028	66/Female	-	Two to three copies of TP53, four copies of centromere chromosome 17, fusion gene of t(4;14) and four copies of CCND1	amp(1p), amp(13q)
HGU029	52/Male	46,XY[41]	unable to analyzed	amp(1p), amp(1q), amp(12p), amp(13q), del(16q)
HGU030	64/Male	46,XY	not detected	amp(12p)
HGU031	54/Male	46,XY	not detected	amp(1p), amp(1q)
HGU032	74/Female	-	Three copies of CCND1 and translocation 14q32 (IGH) involving with other chromosome	amp(5q), amp(9p), amp(9q), amp(14q), amp(15q), del(16q)
HGU033	58/Male	46,XY	del(13q)	amp(9p), amp(9q), del(13q), amp(15q)
HGU034	75/Male	46,XY	not detected	not detected
HGU035	71/Male	46,XY[30]	unable to analyzed	not detected

(-), not analyzed

Table 2. Summary of Genetic Lesions Detected by MLPA

Sample	Target region (number of affected probes/total number of probes)									
	Del 1p	Amp 1q	Amp 5q	Amp 9(p, q)	Del 12p	Del 13q	Del 14q	Amp 15q	Del 16q	Del 17p
HGU001	3/10	-	6/6	2/2	-	5/5	-	2/2	2/4	-
HGU002	1/10	-	-	2/2	5/5	-	-	2/2	-	-
HGU003	5/10	7/7	-	2/2	-	5/5	2/2	2/2	-	-
HGU004	-	-	6/6	2/2	-	-	-	2/2	-	-
HGU005	5/10	7/7	-	-	-	5/5	-	-	-	2/3
HGU006	-	-	-	-	-	-	-	-	-	-
HGU007	3/10	7/7	6/6	2/2	-	5/5	-	2/2	-	-
HGU008	-	-	-	-	1/5	-	-	-	1/4	-
HGU009	8/10	3/7	-	-	-	5/5	2/2	-	3/4	3/3
HGU010	-	-	-	-	-	-	-	-	-	-
HGU011	-	-	-	-	-	-	-	-	-	-
HGU012	-	-	-	-	-	-	-	-	-	-
HGU013	9/10	7/7	5/6	2/2	5/5	5/5	2/2	1/2	2/4	2/3
HGU014	2/10	3/7	-	-	2/5	-	-	-	-	-
HGU015	-	7/7	1/6	-	-	5/5	-	-	-	-
HGU016	1/10	2/7	1/6	1/2	1/5	5/5	-	2/2	-	-
HGU017	-	7/7	-	-	-	2/5	-	-	-	-
HGU018	5/10	7/7	1/6	-	-	-	-	-	-	-
HGU019	-	-	-	-	-	-	-	-	-	-
HGU020	-	-	-	-	-	-	-	-	-	-
HGU021	-	6/7	6/6	2/2	-	-	-	2/2	-	-
HGU022	-	-	-	-	-	-	-	-	-	-
HGU023	3/10	1/7	5/6	-	-	-	-	2/2	1/4	1/3
HGU024	-	-	-	-	-	-	-	-	-	-
HGU025	-	7/7	-	-	-	5/5	-	-	-	-
HGU026	-	2/7	-	1/2	-	5/5	-	-	-	-
HGU027	6/10	5/7	-	-	-	4/5	1/2	-	4/4	-
HGU028	1/10	-	-	-	-	1/5	-	-	-	-
HGU029	2/10	3/7	-	-	1/5	1/5	-	-	1/4	-
HGU030	-	-	-	-	2/5	-	-	-	-	-
HGU031	1/10	1/7	-	-	-	-	-	-	-	-
HGU032	-	-	6/6	2/2	-	-	2/2	2/2	1/4	-
HGU033	-	-	-	2/2	-	5/5	-	2/2	-	-
HGU034	-	-	-	-	-	-	-	-	-	-
HGU035	-	-	-	-	-	-	-	-	-	-

Del, deletion; Amp, amplification; (-) not detected.

conventional cytogenetic analysis. We could identify chromosomal abnormalities in 5 of 35 (14.3%) patients. Among this group, complex chromosomal abnormalities were observed in 3 of 5 patients. In addition, we could detect a deletion of Y chromosome in 2 patients (table 1). Nevertheless, conventional cytogenetic technique failed to detect chromosomal abnormalities in 5 patients due to no metaphase cells.

iFISH could detect recurrent genetic alterations in 20 of 35 (57.1%) patients. Among this group, 5 patients were positive for t(4;14)(p16;q32) translocation which all of them harbor additional chromosome abnormalities (3 cases with deletion of chromosome 13, 1 case with amplification

of *TP53*, and 1 case with 3 copies of *CCND1*). However, we could not identify t(11;14)(q13;q32) translocation in this study. Prospective on the detection of chromosomal aneuploidy by iFISH, 13q deletion was the most frequent genetic abnormalities identified in this study (12/35 = 34.3%) followed with 17p deletion (3/35 = 8.6%). Other genetic alterations identified using iFISH were including amplification of *CCND1* and *CCND3* (Table 1). Nevertheless, 10 of 35 (28.6%) patients were negative for iFISH. In this work, we could not analyze 5 samples using iFISH due to not enough plasma cells. Taken together, we could detect several recurrent genetic alterations in bone marrow samples of multiple myeloma patients by using

Table 3. Frequency of Recurrent Genetic Abnormalities Detected by Karyotyping, iFISH and MLPA (n=35)

Abnormality	Gene	Karyotyping % (n)	iFISH % (n)	MLPA % (n)
Normal	-	85.7% (n=30)	42.9% (n=15)	28.6% (n=10)
Abnormal	-	14.3% (n=5)	57.1% (n=20)	71.4% (n=25)
t(4;14)	<i>FGFR3, MMSET</i>	-	14.3% (n=5)	-
t(11;14)	<i>CCND1</i>	-	0% (n=0)	-
Amp (1q)	<i>CKS1B, ANP32F, BCL-9, PDZK1</i> <i>RBI, DIS3, DLEU1</i>	-	-	48.6% (n=17)
Del (13q)	<i>FAM46C, CDKN2C, FAF1</i>	-	34.3% (n=12)	34.3% (n=12)
Del (1p)	<i>JAK2, COL5A1</i>	-	-	34.3% (n=12)
Amp 9(p, q)	<i>GABRB3</i>	-	-	31.4% (n=11)
Amp (15q)	<i>PCHAC</i>	-	-	28.6% (n=10)
Amp (5q)	<i>C-MAF, CYLD, WWOX</i>	-	-	22.9% (n=8)
Del (16q)	<i>CD27</i>	-	-	20.0% (n=7)
Del (12p)	<i>TRAF3</i>	-	-	8.6% (n=3)
Del (14p)	<i>TP53</i>	-	-	8.6% (n=3)
Del (17p)	-	-	8.6% (n=3)	5.7% (n=2)

Del, deletion; Amp, amplification; (-), not analysed.

conventional cytogenetic analysis and iFISH.

MLPA for the detection of recurrent genetic alterations in multiple myeloma

We could identified 93 genetic alterations in 35 diagnosed multiple samples by using MLPA. Interestingly, 71.4% (25/35) of patients had at least one copy number variations (CNVs) (Table 1 and 2). Additionally, there was ranged from 1 to 10 genetic aberrations detected in individual patients. This findings further suggested the persistence of genetically heterogeneity and complexity among multiple myeloma patients. In summary, the most frequently genetic aberrations detected by the current MLPA was the amplification of 1q which is accounted for 17/35 cases (48.6%). The *CKS1B-1* at 1q21.3 was the most frequently 1q amplification (13/17), followed by *CKS1B-2* at 1q21.3, *NUF2-14* at 1q23.3 (12/17) and *RP11-541J2* (9/17), respectively. There were 2 cases (patient number HGU013 and number HGU027) positive for chromosome 1q deletion (Table 1). The chromosome 1p deletion was positive in 12 of 35 (34.3%) which *PLPP3-2* at 1p32.2 and the *COL11A1-45* at 1p21.1 are the most frequently 1p deletion (6/12), followed by *CDKN2C-3* at 1p32.3, *DABI-14* at 1p32.2, *DPYD-20* at 1p21.3 and *FAM46C-2* genes at 1p12 (5/12). In addition, we could identify 7 patients (patient number HGU007, HGU013, HGU014, HGU018, HGU028, HGU029, and HGU031) with chromosome 1p amplifications.

In this report, chromosome 5q amplification was

detected in 8/35 cases (22.9%). The *PCDHB10-1* was the most frequently 5q amplification (8/8), followed by *PCDHA1-1b*, *PCDHAC1-1a*, *PCDHGA11-1b* (7/8) and *SLC25A2-1* (6/8), respectively. Additionally, we found that 3 patients (patient number GHU013, HGU015 and HGU016) have chromosome 5q deletion with similar single probe deletion pattern.

Chromosome 9 abnormalities including 9p and 9q amplifications were positive in 11/35 cases (31.4%). The *JAK2-6* at 9p24.1 was the most frequently affected sequence (10/11), followed by *COL5A1-40* at 9q34.3 (8/11). Furthermore, we found that one patient (patient number HGU013) shows positive result for 9q deletion with single probe deletion pattern.

Chromosome 12p deletion was detected in 3/35 cases (8.6%). The *NCAPD2-32* was the most frequently 12p deletion (3/3), followed by *CD27-3*, *VAMP1-4b*, *CHD4-40* and *CHD4-2* (2/3). Moreover, 4 patients (patient number HGU008, HGU014, HGU029 and HGU030) were positive for 12p amplification.

Chromosome 13q deletion was detected in 12/35 cases (34.3%). The *RBI-26* at 13q14.2 was the most frequently 13q deletion (12/12) followed by *RBI-8*, *DLEU2-intr1* at 13q14.2, *DIS3-18*, *DIS3-6* at 13q22.1 (11/12). Additionally, 3 patients (patient number HGU013, HGU028 and HGU029) were positive for 13q amplification.

The chromosome 14q deletion was detected in 3/35 cases (8.6%). The *TRAF3-3* and *TRAF3-11* were the most frequently 14q deletions (3/3). Furthermore, two patients

Table 4. Comparison of iFISH and MLPA Results

		iFISH			
		Del 13q		Del 17p	
		positive	negative	positive	negative
MLPA	positive	11	1	1	1
	negative	1	22	2	31
McNemar's test		p = 1.0000		p = 1.0000	

Table 5. Sensitivities and Specificities of MLPA Compared with iFISH

		Del 13q		Del 17p	
		Sensitivity	Specificity	Sensitivity	Specificity
MLPA		91.70%	95.70%	33.30%	96.90%
iFISH		91.70%	95.70%	50.00%	94.00%

Table 6. Frequency of Recurrent Genetic Alterations Detected by iFISH and MLPA in Multiple Myeloma

Genetic alteration	Alpar et al., 2013(13)		Boyle et al., 2015(25)		Zang et al., 2015(31)		In this study	
	iFISH	MLPA	iFISH	MLPA	iFISH	MLPA	iFISH	MLPA
t (4;14)	15.00%	-	-	-	(IgH)	-	14.30%	-
t (11;14)	14.00%				54.20%		0%	
t (14;16)	6.00%						-	
t (8;14)	0%						-	
Del (1p)	9.00%	35.00%	17.00%	15.00%	-	50.80%	-	34.30%
Amp (1q)	48.00%	48.00%	34.00%	31.00%	59.30%	54.20%	-	48.60%
Amp (5q)	32.00%	28.00%	-	-	-	22.00%	-	22.90%
Amp 9 (p, q)	-	-	-	-	-	-	-	31.40%
Del (12p)	-	20.00%	-	-	-	20.30%	-	11.40%
Del (13q)	64.00%	57.00%	49.00%	48.00%	44.10%	61.00%	34.30%	34.30%
Del (14p)	-	-	-	-	-	-	-	8.60%
Amp (15q)	-	-	-	-	-	-	-	28.60%
Del (16q)	-	19.00%	27.00%	22.00%	-	23.70%	-	20.00%
Del (17p)	7.00%	9.00%	7.00%	4.60%	14.10%	11.90%	8.60%	5.70%

Del, deletion; Amp, amplification; (-), not analysed

(patient number HGU027 and HGU032) were positive for 14q amplification.

The chromosome 15q amplification was detected in 10/35 cases (28.6%). The *GABRB3* at 15q12 was the most frequently 15q amplification (10/10) followed by the amplification of *IGFIR-18* at 15q26.3 (9/10). Additionally, two patients (patient number HGU013 and HGU016) were positive for 15q deletion with single probe deletion pattern.

The chromosome 16q deletion was detected in 7/35 cases (20.0%). The *CYLD-2* at 16q12.1 was the most frequently 16q deletion (5/7) followed by *WWOX-1a* and *WWOX-7* at 16q23.1 (3/7) and *CYLD-19* at 16q12.1 (2/7), respectively. In additionally, 2 patients (patient number HGU008 and HGU013) were positive for 16q amplification with single probe amplification pattern.

The chromosome 17p deletion was detected in 2/35 cases (5.7%). The *TP53-10* and *TP53-7* were the most frequently 17p deletion (2/2) followed by *TP53-4b* (1/2). Moreover, 2 patients (patient number HGU005 and HGU023) were positive for 17p amplification.

Comparison of MLPA assay and conventional iFISH method

We further determined the consistency of the results obtained from the conventional iFISH method and MLPA (the summary of recurrent genetic alterations detected by karyotyping, iFISH, and MLPA are list in table 3). The concordant results of iFISH and MLPA for the detection of 13q deletion and 17p deletion by McNemar test were observed in this study. There were no significant differences between iFISH and MLPA results of 13q deletion (p-value = 1.0000) and 17p deletion (p-value = 1.0000) (table 4). The sensitivity and specificity of iFISH and MLPA to detect 13q deletion were 91.7% and 95.7%. The sensitivity and specificity of iFISH to detect 17p deletion were 50.0% and 94.0%, respectively. Additionally, the sensitivity and specificity of MLPA to determine 17p deletion were 33.3% and 96.9% (table 5). This further indicated the potential used of MLPA for the detection of common genetic alterations in multiple myeloma.

Table 7. Sensitivities and Specificities of MLPA and iFISH to Detect 13q and 17p Deletions in This Present Study Compared with Previous Publications

Study		13q deletion		17p deletion	
		Sensitivity	Specificity	Sensitivity	Specificity
This study	MLPA	91.70%	95.70%	33.30%	96.90%
	iFISH	91.70%	95.70%	50.00%	94.00%
Alpar et al., 2013(13)	MLPA	88.50%	100.00%	100.00%	98.70%
	iFISH	100.00%	82.90%	85.70%	100.00%
Boyle et al., 2015(25)	MLPA	97.00%	77.00%	100.00%	99.00%
	iFISH	100.00%	71.00%	100.00%	97.00%
Zang et al., 2015(31)	MLPA	88.50%	60.60%	77.80%	100.00%
	iFISH	63.90%	87.00%	100.00%	96.20%

Discussion

Genetic alterations in MM including chromosomal translocation-involved immunoglobulin heavy chain (IGH) and hyperdiploidy (trisomy chromosome) of the odd numbered chromosomes (3, 5, 7, 9, 11, 15, 19, and 21) are recognized as hallmarks and pathogenesis of the disease. Those provoke plasma cells to transform and cooperating genetic events including copy number variations (CNVs), loss of heterozygosity (LOH), acquired mutations (e.g., *TP53*, *MAF*, and *KIT*), and epigenetic modifications could contribute to disease progression and full-blown malignancy (Prideaux et al., 2014). At present, those genetic mutations are represented as the important diagnostic, prognosis, monitoring, risk stratification markers, and could be a potential therapeutic targets for the treatment of MM. There are abundance in current available techniques to molecularly detect genetic alterations in multiple myeloma including the standard karyotyping and iFISH. Similar to previous reports (Lai et al., 1995; Smadja et al., 1998; Zandecki et al., 1996), the majority of multiple myeloma cases exhibited normal karyotype patterns (46,XX and 46,XY) that derived from normal myeloid cells in samples. Additionally, concordance to previous reports (Debes-Marun et al., 2003; Sawyer et al., 1995; Smadja et al., 2001) that karyotyping is sensitive to detect only numerical abnormalities of chromosome in sample from multiple myeloma patients was observed in this study. Interestingly, we could identify monosomy of Y chromosome in 2 patients. However, the prognosis significant of monosomy of Y chromosome in multiple myeloma patients still unclear (Shin et al., 2017). These findings further supported the important application of karyotyping for the screening of chromosomal aneuploidy which is critical for the disease risk-stratification. Nevertheless, the assays have several shortcomings including low sensitivity, low resolution, time-consuming, and require a very high experience workers.

iFISH have been developed to overcome several disadvantages of conventional cytogenetic for the detection of recurrent genetic alterations in multiple myeloma. Importantly, the technique could detect the low proliferative tumor cells (interphase cell) and iFISH is able to detect some small chromosomal rearrangements which are frequently observed in multiple myeloma. Furthermore, the sensitivity of iFISH is dramatically increased when applied on the plasma cell-enriched sample. In this work, the most frequently chromosomal abnormality detected by iFISH was the deletion of chromosome 13q (34.3%) followed with the deletion of chromosome 17p (8.6%). While the prevalence of 17p deletion was concordant to previous reports (Alpar et al., 2013; Boyle et al., 2015; Zojer et al., 2000), lower in frequency of 13q deletion was observed in this study (Table 3). This might be affected by the limited in number of tested population and we did not apply the plasma cells-enriched samples for iFISH assay. Similar to previous studies (Fonseca et al., 2009; Sewify et al., 2014), iFISH could detect the amplification of *CCND1* in 25.7% (9/35) and *CCND3* in 5.7% (2/35) of tested samples. For the detection of chromosomal translocation by iFISH, t(4;14)(p16;q32) was only cryptic

chromosomal translocation detected in this study with similar in frequency to previous reports (Chesi et al., 1998; Chesi et al., 1997). At present, there are several FISH commercial available probes which are designed to cover all well-defined clinical significant genetic alterations in multiple myeloma (as were recommended by the Mayo Clinic, Rochester, MN, USA)(Rajan and Rajkumar, 2015). Thus, iFISH is highly recommended for the detection of recurrent genetic alterations in multiple myeloma.

Recently, several studies reported that MLPA could be applied for the screening of CNVs as well as unbalance genetic lesions in multiple myeloma (Alpar et al., 2013) (Boyle et al., 2015; Zang et al., 2015). The robustness of the technology has been confirmed previously by analysis of various types of hematological and other malignancies (Homig-Holzel and Savola, 2012). In this report, we used the well-recognized commercial available MLPA kit (SALSA P425-B1 multiple myeloma) for the analysis of common CNVs in DNA samples isolated from CD138-enriched bone marrow mononuclear cells of newly diagnosed multiple myeloma patients. In our initial experiments, we found that complete MLPA profiles could be performed by using genomic DNA isolated from CD138-enriched plasma cells. In contrast, ambiguous MLPA signals were observed when the amplification started with genomic DNA isolated from whole bone marrow mononuclear cells. The data strongly suggested the beneficially application of CD138 microbeads to enrich targeted tumor cells from normal mononuclear cells background. Although we applied CD138 isolation prior to MLPA reaction in all cases, incomplete MLPA results were observed in particular cases such as sample number HGU020 was failed to amplify 13q region (inconclusive result with iFISH). Moreover, in sample number HGU015 and HGU021 were not able to detect 17p deletion by using MLPA. This could be resulted from the limitation of the assay to detect the low amount and quality of tumor DNA.

Interestingly, while MLPA could detect 13q deletion in case number HGU027 and 17p deletion in case number HGU013, iFISH failed to detected those aberrations in both cases. This could explain by the number of MLPA probes dispersing along the region of interested (FISH has one probe to target one region of each chromosome). In addition, the discrepancy between iFISH and MLPA data was resulted from the different in probes used in both techniques. For the detection of 13q deletion, iFISH has probe specific for 13q14.3 locus (LSI RB-1). Whereas MLPA contains two probes which were designed for the specific detection of exon 8 and exon 26 of *RB1* gene. Additionally, *DLEU-2* probe (13q14.2) in MLPA is located at about 2 Mb far from iFISH LSI RB1 target region. Therefore, some patients with 13q14.2 deletion may not be detected by iFISH in this study.

Several genetic alterations have been reported and recognized as the biomarkers for multiple myeloma. Recent study reported that 13q deletion is the most frequent aberration in MM (table 6) (Alpar et al., 2013; Boyle et al., 2015; Zang et al., 2015). However, we found that 1q amplification is the most frequent genetic aberrations in this study. Note, we observed that sensitivity and specificity of iFISH and MLPA for the detection of 13q

deletion (p-value >0.05; no significant) and 17p deletion (17p) (p-value >0.05; no significant) were concordant to recent reports (table 7) (Alpar et al., 2013; Boyle et al., 2015; Zang et al., 2015). However, the limitation of MLPA were observed in this work including the assay could not detect balanced translocations and not able to generate complete MLPA profile in a case with low number of tumor cells.

In summary, karyotyping, iFISH and MLPA analysis are mutually complementary for the detection of genetic aberrations in multiple myeloma. Those techniques are very helpful for the diagnosis, risk stratification, and prognosis in multiple myeloma.

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