Quick detection of overexpressed genes caused by myeloma-specific chromosomal translocations using multiplex RT-PCR

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Abstract. Multiple myeloma (MM) is a malignancy of the plasmocyte and is associated with various symptoms such as anemia, immunodeficiency, bone lesions and kidney insufficiency. Although prognosis was poor until some years ago, recent advances that introduced newer molecular targeting agents such as bortezomib and thalidomide have resulted in a better prognosis for MM. However, clinical manifestations and the relationship between cellular and molecular findings, including chromosomal translocation and the related overexpression of oncogenes such as CCND1 (cyclin D1) and FGFR3 (fibroblast growth factor receptor 3), remain unclear. It has been reported that a specific translocation may influence the prognosis of MM. Although translocations and overexpressed genes should be examined in ordinary clinical investigations, limited definitive assays for translocation involve the use of FISH (fluorescent *in situ* hybridization) or SKY (special karyotypic) methods. We therefore, attempted to establish a quick detection method for major translocated genes such as FGFR3, CCND1, CCND3 and MAF using multiplex RT-PCR (MP-RT-PCR). MP-RT-PCR can be performed within several to 24 h after bone marrow samples are taken. Two of 21 bone marrow blood samples from MM patients were analyzed using MP-RT-PCR and double-color FISH, and the results of both methods were compatible. Future utilization and elaboration of this method may help our understanding of the cell biology and clinical features of MM.

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the clonal expansion of malignant plasma cells in the local bone marrow environment. Patients with MM suffer from anemia, immunodeficiency, renal insufficiency (known as myeloma kidney), hyperviscosity syndrome, hypercalcemia and bone lesions due to the presence of space-occupying malignant myeloma cells in the bone marrow, reduced production of normal immunoglobulins (Ig), deposition of malignant paraprotein, hyper-paraproteinemia produced by myeloma cells and osteolytic cytokines secreted from myeloma cells (1-3). Although prognosis of MM was relatively poor until a few years ago (average survival span was estimated at approximately three years), the introduction of newer therapeutic agents such as thalidomide (4-6), lenalidomide (7-9) and bortezomib (10,11) has improved the prognosis for MM patients.

In regards to myeloma cell biology, the myeloma-specific chromosomal translocations leading to fusion of Ig loci such as 14q32 for the heavy chain, 2q11 for the λ light chain and 22q11 for the κ light chain, including several oncogenic genes such as CCND1 (cyclin D1) at 11q13, FGFR3 (fibroblast growth factor receptor 3) and MMSET (multiple myeloma SET domain) on 4q32.3, CCND3 (cyclin D3) at 6p21.1, MUM1 (multiple myeloma oncogene 1)/IRF4 (interferon regulatory factor 4) on 6p25, c-myc on 8q24, c-maf on 16q23, and IRT1 (the immune receptor translocation-associated protein 1)/IRTA2 on 1q21-31, are considered important to elucidate the subclassification of myeloma cell characteristics and multistep genomic carcinogenesis in myeloma (12-16). The major and four primary translocated genes found in the majority of the cells from MM patients are CCND1, FGFR3/MMSET, CCND3 and c-maf (12-16). However, the specific clinical features of MM caused by individual chromosomal translocations and overexpressed genes are unclear. On the other hand, clinical observations have indicated that MM patients with

Table I. Clinical characteristics of myeloma cases.

Case no.	Age (at sampling)	Gender	M-protein (Ig type)	Clinical stage (ISS)		Clinical stage (DS)	Prior therapy
				Onset	Sampling		
1	75	F	G-κ	I	III	IIA	MP, VAD
2	74	M	BJP-λ	II	n.c.	IIA	MP
3	70	M	G-κ	unknown	II	IIIA	VAD, BD
4	52	M	A-λ	I	n.c.	IIIA	VAD, PBSCT
5	59	M	BJP-κ	I	n.c.	IIIA	none
6	78	F	BJP-λ	II	n.c.	IIIB	MP
7	71	M	A-κ	I	n.c.	IA	none
8	69	M	A-λ	unknown	III	IIA	MP, CPA+PSL
9	58	F	G-λ	III	n.c.	IIIA	none
10	54	M	BJP-κ	III	II	IIIB	VAD, BD
11	58	F	G-λ	III	II	IIIA	VAD
12	80	F	A-λ	I	n.c.	IIA	none
13	58	M	BJP-κ	III	n.c.	IIIB	VAD, BD
14	89	M	A-λ	III	n.c.	IIIA	none
15	64	F	D-λ	II	n.c.	IIIA	none
16	88	F	A-κ	II	II	IIIA	DEX
17	67	M	A-λ	III	I	IIA	VAD
18	79	M	BJP-λ	II	n.c.	IA	none
19	84	F	G-λ	III	n.c.	IIIA	none
20	65	M	BJP-λ	Amyloidosis			
21	67	M	A-λ	III	I	IIA	VAD, hdCPA, PBSCT

F, female; M, male; BJP, Bence-Jones protein; ISS, International staging system; DS, Durie-Salmon; n.c., not changed; MP, melphalan and prednisone; VAD, combination chemotherapy with vincristine, adriamycin and dexamethasone; BD, bortezomib and dexamethasone; PBSCT, peripheral blood stem cell transplantation; CPA, cyclophosphamide; PSL, prednisone; DEX, dexamethasone; hdCPA, high-dose cyclophosphamide.

t(11;14)(q13;q32) and translocation of CCND1 show a better prognosis (17-19), while MM patients with t(4;14)(q32.3;q32) and FGFR3 overexpression have a worse prognosis (20-23).

The detection of various characteristic chromosomal translocations should use recent advances of karyotypical analyses such as those employed in interphase DCFISH (double-color fluorescence *in situ* hybridization) or SKY (special karyotypic) methods (24-26), although these methods involve relatively higher effort and costs compared to standard examinations in clinical laboratories. If detection of these various chromosomal translocations affects the clinical management of MM, quick detection methods may contribute to the selection of an appropriate therapeutic regimen that takes into account special clinical features in order to obtain a better prognosis for each MM patient. Here, we introduce a quick detection method for four major overexpressed genes due to myeloma-specific chromosomal translocations using multiplex reverse transcriptase (RT)-PCR (MP-RT-PCR) that can be performed within several to 24 h from the time the samples are obtained. Although the precise conditions should be analyzed using numerous samples, these types of trials may help our understanding of myeloma cell biology and improve the future management of MM patients.

Materials and methods

Subjects and sample preparation. Bone marrow samples from 21 MM patients who visited or were admitted to the Division of Hematology, Kawasaki Medical School Hospital, Kurashiki, Japan, were used in this study. All patients were Japanese. Clinical characteristics of the patients are shown in Table I. Samples were only taken from subjects from whom signed informed consent was obtained, and this study was approved by the Ethics Committee of the Kawasaki Medical School. Mononuclear cells from 1-2 ml of heparinized bone marrow blood samples were separated using the Ficoll-Hypaque gradient centrifugation methods, and CD138-positive and negative cells were purified by positive and negative selection using anti-human CD138 monoclonal antibody bound magnetic-beads (Miltenyi Biotec Inc., Auburn, CA, USA). Total RNA from purified CD138-positive and negative fractions and from various human myeloma cell lines established in the Kawasaki Medical School and possessing various myeloma-specific translocations were extracted using the RNeasy mini kit (Qiagen, Hilden, Germany), and cDNAs were synthesized using the PrimeScript II® first strand cDNA synthesis kit (Takara, Shiga, Japan), all of which

Table II. List of primer sets for the MP-RT-PCR method used in this study.

Gene	Product size (bp)	Primer sequences	
GAPDH	727	Forward	CGAGATCCCTCCAAAATCAA
		Reverse	TGCTGTAGCCAAATTCGTTG
FGFR3	515	Forward	CTGAAAGACGATGCCACTGA
		Reverse	CCAAAGGACCAGACGTCACT
CCND3	307	Forward	TGGATGCTGGAGGTATGTGA
		Reverse	GAATGAAGGCCAGGAAATCA
CCND1	206	Forward	TGTGTGCAGAAGGAGGTCC
		Reverse	CCTTCATCTTAGAGGCCACG
MAF	99	Forward	AACTGGCAATGAGCAACTCC
		Reverse	CCGGTTCCTTTTTCACTTCA

were performed according to the manufacturer's instructions (28,29).

MP-RT-PCR for the detection of major overexpressed genes in myeloma. The 20 μl total volume of the MP-RT-PCR reaction was comprised of 2 μl 10X buffer; 2 μl dNTP (2 mM); 2 μl $MgCl_2$ (25 mM); 0.2 μl of 5 U/μl Taq polymerase (equivalent to 1 Unit of Taq); 1.0-4.4 μl template cDNA (up to the total RNA amount of the samples), 1 μl (2 pmol) primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase as a control), 2 μl (4 pmol) FGFR3, 0.4 μl (0.8 pmol) CCND1, 2 μl (4 pmol) CCND3 and 4 μl (8 pmol) c-maf primers with water. The sequences of the primers are listed in Table II.

MP-RT-PCR was performed as an initial denaturation at 94˚C for 4 min, followed by 36 cycles of denaturation (94˚C, 30 sec), annealing (55˚C, 30 sec), extension (72˚C, 60 sec), and a final extension at 72˚C for 5 min. After visualization of the MP-RT-PCR products on a 2.0% agarose gel stained with ethidium bromide, gel images were obtained using a Dolphin-DOC gel-image analyzer (Kurabo Ltd., Osaka, Japan).

The samples for the cell line control were made using cDNAs from various human myeloma cell lines established at the Kawasaki Medical School such as KMM-1 having a translocation of c-myc and CCND3, KMS-12PE and BM having a CCND1 translocation and KMS-11 with the FGFR3 translocation (27-32). In addition, cDNA from each cell line was subjected to MP-RT-PCR and ensured that the cell line-specific overexpressed genes were amplified (data not shown).

DCFISH analysis. DCFISH was performed as previously described (31,33-36). Briefly, for the detection of t(4;14) and t(11;14), we used the the LSI IGH/FGFR3 and LSI IGH/CCND1 Dual Fusion Translocation Probes (Abbott/Vysis, Downers Grove, USA), respectively. Nuclei of tumor cells were prepared from bone marrow samples taken from patients. Control samples for interphase analysis were prepared from cytogenetically normal bone marrow cells obtained from 5 patients with mild leukocytosis or leukocytopenia. For quantitative analysis, hybridization signals were evaluated in 100 interphase nuclei with a hybridization efficiency of >90%.

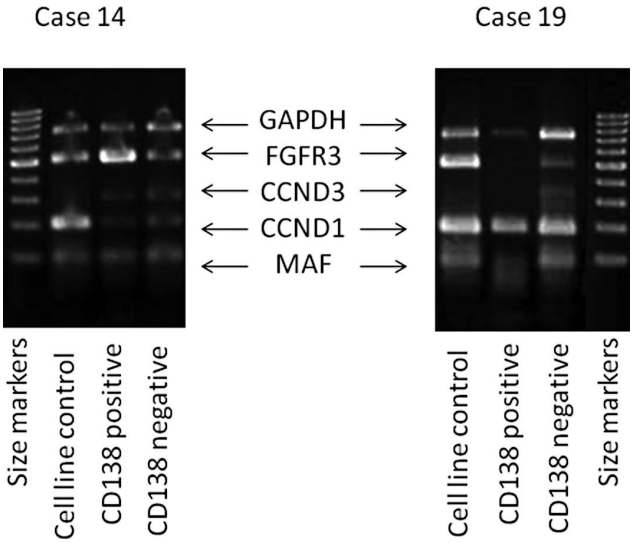

Figure 1. Gel images of cases 14 and 17 regarding products of the multiplex RT-PCR (MP-RT-PCR) method for detection of myeloma-specific translocated genes. MP-RT-PCR was performed as described in Materials and methods. Size markers (Fermentas International Inc., Ontario, Canada) show 100 (the lowest) to 1,000 (the highest) bp, every 100 bp. GAPDH, glyceraldehyde-3-phosphate dehydrogenase used as a control; FGFR3, fibroblast growth factor receptor 3; CCND3, cyclin D3; CCND1, cyclin D1.

Results

Gel images of MP-RT-PCR. Gel images of MP-RT-PCR from the cell line control, CD138-positive and CD138-negative samples of cases 14 and 19 are shown in Fig. 1. The lane representing the cell line control exhibited similar amplification of FGFR3 and CCND1 with faint products of MAF and CCND3.

In Case 14, although the CD138-negative sample showed almost equal amplification of all genes with a slightly higher amplification of GAPDH and FGFR3, the CD138-positive sample showed strong FGFR3 amplification with faint GAPDH, CCND1, CCND3 and MAF genes. On the other hand, the gel image of Case 19 showed strong amplification of CCND1 not only in the CD138-positive sample, but also in negative samples (probably due to small contamination of CD138-positive cells in the 'negative' fraction). It was therefore,

Table III. Number of cells at interphase showing color signals.

	IGH (green)/FGFR3 (red)		IGH (green)/CCND1 (red)	
Case 14	YYRG	32	YGR	2
	YRRGG	4	YRGG	2
	YRG	3	RRGG	43
	YYRG	2	RGG	2
	YRRGG	1	RRRRGGG	1
	YYRRGG	1		
	RRGG	6		
	RGG	1		
Y-positive/total cells (%)		86		8
Case 19	YRGG	4	YRGG	37
	YYGG	1	YRGG	10
	RRGG	16	YGG	2
	RRGGG	28	RRGGG	1
	RRRGGG	1		
Y-positive/total cells (%)		10		98

Y, signal showing a yellow color; R, signal showing a red color; G, signal showing a green color.

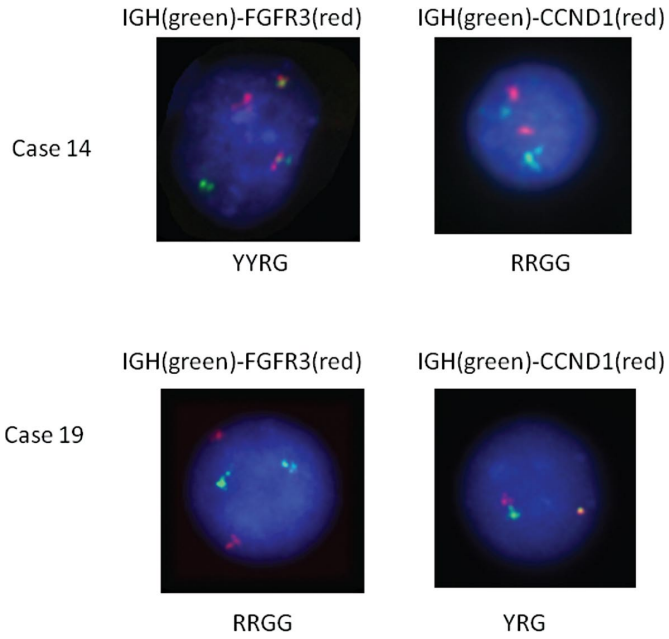

Figure 2. Representative interphase nuclei for double-color fluorescent *in situ* hybridization (DCFISH) methods for cases 14 and 19. Left-upper panel shows IGH (green)-FGFR3 (red) signals in the nucleus of CD138-positive myeloma cell. Right-upper, left-lower and right-lower panels represent IGH-CCND1 in Case 14, and IGH-FGFR3 and IGH-CCND1 in Case 19 DCFISH nuclei, respectively. In regard to signals found in the nucleus, Y means a signal showing a yellow color, R is a signal showing a red color, and G is a signal showing a green color. Thus, YYRG (in the left-upper panel) means there are two Y signals, a single R signal and a single G signal.

assumed that Case 14 possessed the FGFR3 translocation and overexpresssed this gene, while Case 19 had the CCND1 translocation and overexpression.

DCFISH results of cases 14 and 19. The results of DCFISH analyses performed using CD138-positive samples from cases 14 and 19 are shown in Fig. 2. The panel of IGH (immunoglobulin heavy chain) (green)-FGFR3 (red) DCFISH for Case 14 showed YYRG expression. Y means a yellow signal and indicates a fusion signal of IGH and FGFR3, R is a single red signal, and G is a single green signal. However, the panel of IGH (green)-CCND1 (red) for Case 14 showed RRGG without a Y signal. We examined 100 interphase nuclei and found that cells having a Y signal represented 86% of the total cells in the IGH-FGFR3 analysis and 8% in the IGH-CCND1 analysis as shown in Table III. These results were sufficiently significant to support the claim that the sample from Case 14 possessed the IGH-FGFR3 translocation without the IGH-CCND1 translocation.

On the other hand, IGH-FGFR3 in Case 19 only showed a Y signal in the IGH-CCND1 analysis, and 98% of interphase nuclei showed the IGH-CCND1 translocation with 10% exhibiting the Y signal in the IGH-FGFR3. These results support the opinion that Case 19 possessed the IGH-CCND1 translocation.

Digital analysis of MP-RT-PCR. The densitometric analysis of MP-RT-PCR is shown in Fig. 3. All bands amplified from CD138-positive samples of individual cases were calculated as a relative expression using the intensity of the GAPDH band as 1.0. The results of Case 14 are shown by black-filled circles. The results of Case 19 are shown by gray-filled circles. The results of MR-RT-PCR for Case 19 only revealed the amplified band of CCND1 among other translocation-related bands as shown in Fig. 1, and this case was only plotted in CCND1. However, Case 14 revealed a strong FGFR3 band and other translocation-related bands that were faint in appearance. Thus, black-filled circles were plotted for all of the genes, and FGFR3 was the highest among the four genes.

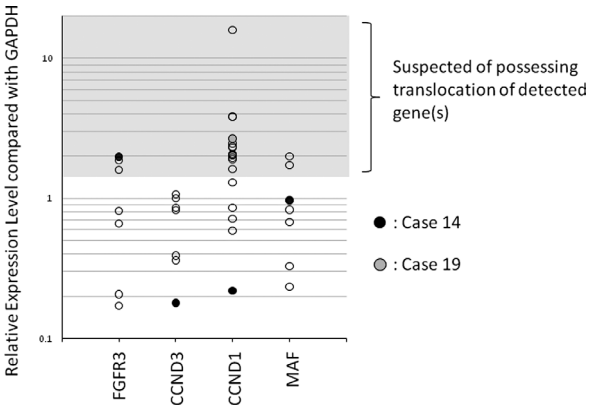

Figure 3. Densitometric analyses of multiplex RT-PCR (MP-RT-PCR) for 21 MM patients. Black-filled circles indicate results for Case 14, and gray-filled circles represent the results for Case 19.

The gray-shaded samples were suspected of having a specific translocation and translocation-induced overexpression of genes, although all of the samples were not analyzed by DCFISH in this study. On the basis of MP-RT-PCR, it was concluded that no sample showed overexpression of CCND3, while overexpression was detected in 3 of 21 (14.3%) samples for FGFR3, 12 of 21 (57.1%) samples for CCND1, and 2 of 21 (9.5%) samples for MAF.

Discussion

After introduction into the clinic of newer molecular targeting drugs such as bortezomib (10,11), thalidomide (4-6) and its relative, lenalidomide (7-9) for MM treatment, the prognosis for these patients has improved dramatically. However, the mechanisms of carcinogenesis involved in myeloma and the roles of myeloma-specific chromosomal translocation and overexpression of genes due to the translocation remain unclear (12-16). Reports have shown that specific translocations may be related to the prognosis of MM for cases in which the management of MM was conducted before the introduction of the newer molecular targeting agents (17-22).

The cellular and molecular roles of these translocation-dependent overexpressed genes and their contributions to the formation of the pathophysiological status of MM have not yet been clarified (12-16). If these translocation-status genes are related to the prognosis, the quick detection of these overexpressed genes may result in benefits for the management and treatment of MM patients. Definitive evidence of these translocations can be obtained using FISH and SKY methods (24-26), although these procedures take time and involve high costs when utilized for ordinary clinical study. Special laboratories are required to complete these assays at the research level, and companies may be prevented from accepting these laboratory investigations due to foundation and time-consuming reasons.

In this study, we aimed to develop a quick method for detecting overexpressed genes in myeloma samples using MP-RT-PCR. Results can be obtained after several hours or an overnight period from the time the bone marrow (or even the peripheral blood) samples of MM patients are collected. Our method only detects major translocation-dependent overexpressed genes such as CCND1, CCND3, FGFR3 and MAF. In addition to the two cases shown in Figs. 1 and 2 and Table III, two other cases were also confirmed to have chromosomal translocations following the use of MP-RT-PCR with DCFISH analysis. In addition, results concerning the frequencies of translocations (FGFR3, 14.3%; CCND3, 0%; CCND1, 57.1%; MAF, 9.5%) appear to have overestimated CCND1 and underestimated FGFR3. This may be important for the improvement of this method in the future.

Furthermore, it is thought that not all myeloma-specific translocations are equally related to myeloma carcinogenesis. Rare cases have shown the involvement of both CCND1 and FGFR3 translocations, and these may be independent primary abnormalities of myeloma cells. However, other translocations such as those involving c-myc are sometimes found for cases in which myeloma cells possess other translocations. As far as the status of the patient is concerned, we sometimes could detect two overexpressed genes through the MP-RT-PCR method. However, this represents a point that needs to be resolved in the future.

Although several aspects should be resolved regarding the quick and precise detection of overexpressed genes due to myeloma-specific chromosomal translocations, the MP-RT-PCR method can be used widely in many clinical departments and companies performing laboratory medicine. The method should develop following wide use and estimation using clinical data and FISH (and/or SKY) data of the same selected samples. Chemotherapies for myeloma have entered a new era, and this will result in a greater understanding of the cellular and molecular aspects of myeloma and its specific chromosomal translocations. The MP-RT-PCR method utilized in this study may help this advancement. It may be that the comprehensive understanding of both the accumulation of data using these methods and clinical evaluations will lead to a better recognition of myeloma biology, and may contribute to further clinical benefits concerning the management of MM.

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