Frequent occurrence of *CCND1* deregulation in patients with early stages of plasma cell dyscrasia

Kazuhisa Miura,[1] Shinsuke Iida,[1,6] Ichiro Hanamura,[1] Miyuki Kato,[1] Shogo Banno,[1] Takashi Ishida,[1,3] Shigeru Kusumoto,[3] Genji Takeuchi,[3] Hiroshi Miwa,[4] Masakazu Nitta,[4] Hiroshi Inagaki,[2] Tadaaki Eimoto,[2] Kenichi Nomura,[5] Masafumi Taniwaki[5] and Ryuzo Ueda[1]

[1]Department of Internal Medicine & Molecular Science and [2]Department of Clinical Pathology, Nagoya City University Graduate School of Medical Science, 1 Kawasumi, Mizuho-chou, Mizuho-ku, Nagoya 467-8601, [3]Division of Hematology, Shizuoka Saiseikai General Hospital, 1-1-1 Oshika, Shizuoka 422-8527, [4]Division of Hematology, Department of Internal Medicine, Aichi Medical University, Nagakute-chou, Aichi-gun, Aichi 480-1195 and [5]Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Hirokouji, Kawaramachi, Kamikyo-ku, Kyoto 602-8566

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Chromosomal translocations involving the immunoglobulin heavy chain gene (*IgH*) and nonrandom protooncogene loci are the hallmark of genetic alterations found not only in multiple myeloma (MM), but also in premalignant stages of MM, including monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM). We studied the frequency of *IgH* (14q32) rearrangements and their partner chromosomes in 16 Japanese patients with MGUS (13 cases), and SMM (3 cases) by means of interphase double-color fluorescence *in situ* hybridization (DCFISH) applied to purified plasma cells and using CD138-bead selection. *IgH* rearrangement was recognized in nine of the patients (56.3%). Protooncogene loci juxtaposed to *IgH* were identified in seven cases including *CCND1* (11q13) in six cases and *FGFR3* (4p16) in one. Four out of the six t(11;14)-positive cases showed nuclear staining of the cyclin D1 protein, whereas none of the seven t(11;14)-negative cases did. Moreover, neither *MUM1*(6p25)-*IgH* nor *MAFB*(20q11)-*IgH* fusion signals were observed. This suggests to us that cyclin D1 deregulation due to the presence of t(11;14) is involved in the early development of plasma cell neoplasms, and that this event alone is not enough for the development of symptomatic myeloma. (Cancer Sci 2003; 94: 350–354)

Monoclonal gammopathy of undetermined significance (MGUS) affects a small percentage of the population over 50 years of age, and is known to develop into multiple myeloma (MM) and related disorders such as macroglobulinemia and primary amyloidosis at a risk of 1%/year.[1,2] As reflected by the presence of monoclonal immunoglobulin protein in serum or urine, some of the bone marrow plasma cells in patients with MGUS clonally proliferate and carry chromosomal aberrations including aneuploidy and chromosomal deletions/translocations, suggesting that the first genetic abnormality in early plasma cell dyscrasia is chromosomal instability.[3–5] Karyotypic profiles in MM cells appear complicated, but recently developed cytogenetic approaches such as spectral karyotyping (SKY) and fluorescence *in situ* hybridization (FISH) have helped unravel their complexity.[6–8] For example, deletion at the long arm of chromosome 13q (13q−) and chromosomal translocations involving chromosome 14q32 loci (14q+) are two common nonrandom abnormalities which have been identified in MM. More recently, similar chromosomal aberrations have been shown to exist in early forms of plasma cell dyscrasia such as MGUS and smoldering myeloma (SMM).[4,5,7] The 13q− abnormality, which is found in approximately 20–30% of patients with MM, has been widely accepted as an unfavorable prognostic factor for MM treated with conventional chemotherapy or high-dose chemotherapy.[9–11] It is also found in MGUS patients and it has been suggested that it constitutes a subgroup, which tends to progress to MM in a relatively short time.[12] As for 14q+, it is found in nearly 80–90% of MM and in 50–60% of MGUS patients.[7,8,13] Overall clinical courses, including survival time, of MM patients with or without 14q+ chromosomes do not differ significantly.[14] However, recent studies identifying the partner chromosomes of the 14q32 loci have demonstrated the presence of clear differences in clinical features including patients' prognosis.[15,16] In particular, MM patients carrying t(11;14)(q13;q32) or t(4;14)(p16;q32) have been reported to show, respectively, favorable and unfavorable prognosis compared to those without either of these abnormalities.[15] Such differences are presumed to originate from those of corresponding protooncogenes deregulated by juxtaposition with immunoglobulin heavy chain gene (*IgH*) enhancer loci. In other words, *CCND1* and *FGFR3* expressing MM can be characterized as resulting in, respectively, better and poorer prognosis for MM.[17,18] However, only a few studies have been conducted regarding the 14q+ chromosome and its partners in early plasma cell dyscrasia.[4,5] A detailed study from the Intergroupe Francophone du Myelome (IFM) in France has reported the frequent presence (46%) of 14q+ chromosomes as well as the occurrence (15%) of t(11;14)(q13;q32) in 100 patients with MGUS/SMM, although t(4;14)(p16;q32) was detected in only 2% of the cases, even though t(4;14) was identified in approximately 15–20% of MM patients.[4] Another study from the United States, however, also reported a frequency of the 14q+ chromosomes in MGUS/SMM of 46% (27/59 cases), while the respective frequencies of t(11;14), t(4;14) and t(14;16)(q32;q23) were 25%, 9% and 5% of their cases, figures which are similar to those reported for advanced MM cases.[5] In the study presented here, we therefore investigated the 14q+ chromosomes closely associated with early plasma cell dyscrasia in order to delineate the initial molecular pathogenesis of MM in Japanese patients.

Materials and Methods

Patients. At the time of diagnosis (n=9) and/or during routine clinical follow-up (n=7), 1 ml of bone marrow aspirates was obtained from 13 patients with MGUS and three patients with SMM with their informed consent, as shown in Table 1. Patient no. 14 was diagnosed as having SMM, as she had a serum M-protein (IgG) value of 3745 mg/dl in spite of low plasma cell percentage in her marrow. Diagnosis of MGUS and SMM was based on the criteria described by Kyle *et al.*[1,2] Median age at the time bone marrow aspirates were obtained was 68 years (range 31–88 years), and the male-to-female ratio was 11:5.

Purification of plasma cells. Positive selection of the plasma cells from bone marrow mononuclear cells was performed with an immunomagnetic method using an anti-CD138 antibody and with the aid of a VarioMACS (Miltenyi Biotec, Auburn, CA)

[6]To whom correspondence should be addressed. E-mail: iida@med.nagoya-cu.ac.jp

according to the manufacturer's instructions. Purity of the plasma cells was morphologically evaluated by means of May-Giemsa staining of the cytospin smears and 16 samples, which showed a purity exceeding 90% of the total number of cells. The cells were then fixed in methanol:acetic acid (3:1, v/v) followed by analysis by FISH.

Double color fluorescence *in situ* hybridization (DCFISH). DCFISH was performed as described previously by Hanamura *et al*.[19] In brief, each BAC/PAC/YAC-derived DNA was labeled with a nick translation kit (Vysis, Inc., purchased from Fujisawa Pharmaceutical Co., Osaka) using either Spectrum Orange or Spectrum Green. Hybridization and washing protocols were those made available by the company (Vysis) on the Internet and the slides were counterstained with 4′,6′-diamino-2-phenylindole dihydrochloride (DAPI). For the *IgH* splitting procedure, we used a LSI *IgH* dual color break-apart probe (Vysis). The cut-

Table 1. Patient characteristics and FISH study of 14q32 rearrangement

No.	Age/sex	Disease status	M protein type	%BMPC	*IgH* % splitting	Cyclin D1 positivity by immunostaining	% Fusion signal				G-banded karyotype	Follow-up since diagnosis (months)	Period (months) until MM progression
							t(11;14)	t(4;14)	t(6;14)	t(14;20)			
1	68M	MGUS	Mλ	0.2	13.0	Negative	19.6	ND	5.0	6.0	ND	0	—
2	63M	MGUS	Gλ	0.6	20.2	Positive	65.5	ND	5.0	0	ND	58	—
3	76M	MGUS	Gκ	0.8	43.7	Negative	36.7	ND	5.0	7.5	47,XY, +mar1	0	—
4	55M	MGUS	Gκ	1.2	47.0	Negative	38.4	ND	5.0	2.4	ND	52	—
5	63M	MGUS	Aκ	1.2	13.0	Negative	13.8	ND	1.0	1.0	ND	67	—
6	88F	MGUS	Aκ	3.0	4.0	Negative	17.0	ND	6.0	7.0	46,XX	0	—
7	31F	MGUS	Gκ	3.4	5.0	Negative	4.0	ND	6.0	4.0	46,XX	0	—
8	82M	MGUS	Gκ	6.0	3.9	Negative	13.2	ND	0	5.0	ND	69	—
9	73F	MGUS	BJP	6.5	45.0	Positive	45.7	ND	1.4	2.3	46,XX	0	—
10	80M	MGUS	Mκ	7.0	43.0	Positive	58.9	ND	3.0	5.0	46,XY	0	—
11	67M	MGUS	Gκ	7.5	53.8	ND	9.4	8.0	0	0	46,XY	0	—
12	59M	MGUS	Gκ	8.0	16.9	ND	19.2	ND	4.9	4.0	45,X,–Y	0	—
13	74F	MGUS	Gλ	9.2	53.0	Positive	89.0	ND	3.0	0	ND	81	—
14	59F	SMM	Gκ	0.6	16.0	ND	10.0	ND	4.0	3.0	ND	100	—
15	56M	SMM	Aκ	17.2	54.0	ND	20.0	5.7	5.0	1.0	46,XY	1	3
16	83M	SMM	Gλ	25.2	68.0	ND	10.0	62.2	0	0	ND	0	6

%BMPC, % of bone marrow plasma cells; ND, not done.

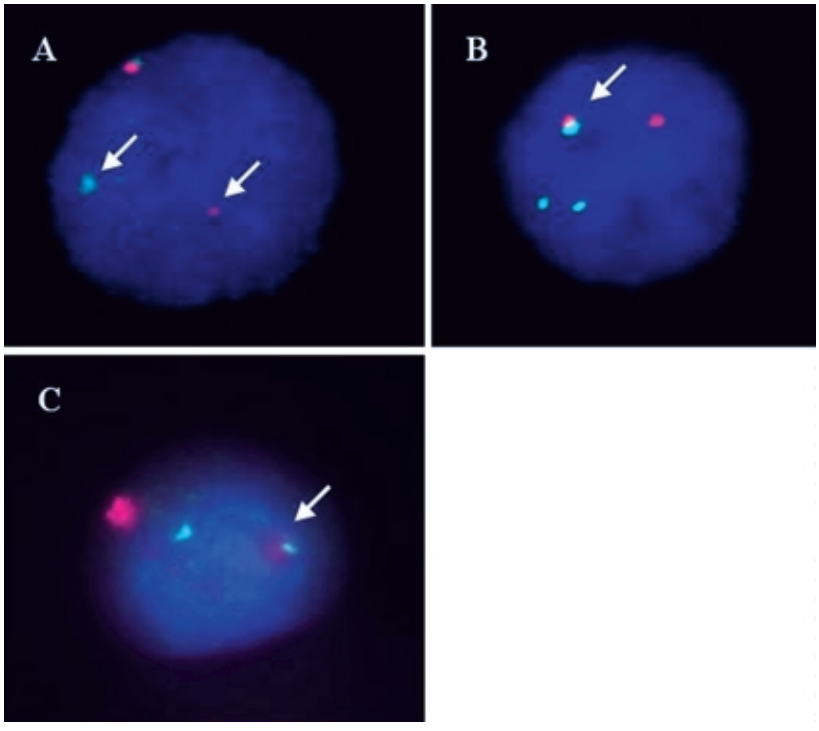

Fig. 1. DCFISH analysis identifying 14q+ chromosomes. A. DCFISH analysis showing split signals (indicated by arrows) between constant (spectrum orange) and variable (spectrum green) regions of the *IgH* locus in case 15. These split signals indicate the presence of chromosomal translocation at the *IgH* locus. B. *CCND1-IgH* fusion signal (arrow) in case 4 detected by DCFISH analysis using spectrum orange-labeled *CCND1* and spectrum green-labeled *IgH* constant region probes. C. *FGFR3-IgH* fusion signal (arrow) in case 16 detected by DCFISH analysis using spectrum orange-labeled *FGFR3* and spectrum green-labeled *IgH* constant region probes.

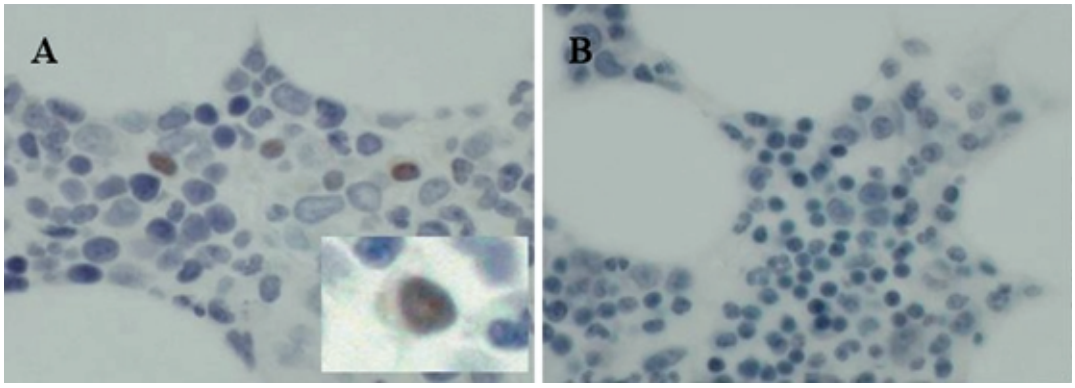

Fig. 2. Immunostaining of cyclin D1 protein in the bone marrow clot section with 5D4 mAb. A. Positive staining of the scattered plasma cells found in case 13, whose purified plasma cells carried t(11;14). The inset shows intense nuclear localization of the protein in a plasma cell. B. Negative nuclear staining of the cyclin D1 protein in case 4, in spite of the presence of MGUS cells carrying t(11;14).

off value for the assessment of the *IgH* translocation by this approach was determined at 20.1%, because the mean splitting value+2SD was 16.6+3.5% for the interphase nuclei derived from peripheral blood mononuclear cells of the five normal volunteers. For the detection of t(11;14)(q13;q32), which produces the fusion between *IgH* and the *CCND1* gene, LSI *IgH/CCND1* dual-color, dual-fusion translocation probe was used (Vysis). Other probes used for FISH in this study were BAC417P24 for the *IgH* constant region probe, PAC644L1 for the *MAFB* gene at 20q11, PACs 120I21 and 61B14 for the *MUM1* gene at 6p25 and cosmid pC385.12 for the *FGFR3* gene at 4p16.[19–22] Before attempting to detect the fusion signals of the specimen, we determined the cut-off index for each of the gene fusions by evaluating peripheral blood mononuclear cells derived from at least five normal volunteers. The cut-off values were then defined as mean+2SD (standard deviation) by counting nonspecific fusion signals in 200 interphase nuclei. In this study, the respective cut-off values to distinguish positivity for *CCND1-IgH*, *FGFR3-IgH*, *MUM1/IRF4-IgH* and *MAFB-IgH* were set at 21.7%, 18.6%, 12.8% and 11.6%.

Detection of cyclin D1 protein overexpression by immunohistochemistry. Formalin-fixed, paraffin-embedded sections of marrow clots were subjected to immunostaining using 5D4 monoclonal antibody (mAb).[23] As previously described by Banno *et al.*, specific nuclear staining was considered as indicating deregulation of this protein, which is closely associated with t(11;14) as determined in a large-scale study of mantle cell lymphoma described by Yatabe *et al*.[23, 24]

Results

High frequency of 14q+ chromosomes in early plasma cell dyscrasia. To evaluate the incidence of 14q+ chromosomes, representing chromosomal rearrangement between *IgH* and partner protooncogene loci, we first performed DCFISH analysis using a LSI *IgH* dual color break-apart probe. This break-apart strategy resulted in the detection of interphase nuclei, whose *IgH* constant region and variable region signals were split as a result of *IgH* translocations involving the switch sequences. This strategy identified *IgH* translocations in nine out of the 16 cases (56.3%) with a frequency ranging from 20.2% to 68.0% (median: 47.0%) (Fig. 1A).

***CCND1-IgH* fusion signals derived from t(11;14)(q13;q32) are frequently identified in early plasma cell dyscrasia.** To identify the translocation partner loci by means of the DCFISH strategy, we searched for the presence of fusion signals between the *IgH* locus and *CCND1*, *FGFR3*, *MUM1/IRF4* and *MAFB* gene loci. Of the nine 14q+ chromosome positive cases, six harbored *CCND1-IgH* (range of positive fraction; 36.7%–89.0%) and one *FGFR3-IgH* (positive fraction; 62.2%) fusion signals (Fig. 1, B and C). In the remaining two cases, the partner chromosomal loci have not been identified yet. As for the *MUM1/IRF4-IgH* and *MAFB-IgH* fusion signals, none of the samples showed positivity in our analysis.

Early plasma cell dyscrasia with t(11;14) shows the specific nuclear staining pattern of the cyclin D1 protein. We next investigated whether the deregulated expression of the protooncogene product was associated with specific chromosomal translocations. Since the *CCND1-IgH* fusion signals were seen in six of the 16 cases, we used immunostaining analysis to study cyclin D1 protein overexpression in six t(11;14)-positive and eight negative cases. As shown in Fig. 2, four out of six *CCND1-IgH* fusion positive cases showed a nuclear staining pattern, which is a hallmark of the deregulated expression of the cyclin D1 protein, as in the case of mantle cell lymphoma,[23, 24] whereas none of the eight *CCND1-IgH* fusion negative cases showed such positivity.

Discussion

We found a high incidence of *IgH* rearrangement (56.3%) in Japanese cases with MGUS/SMM, although only a small number of cases were analyzed. The incidence was somewhat higher than those reported previously by Avet-Loiseau *et al.* and Fonseca *et al*.[4, 5] The most commonly deregulated protooncogene as a result of the presence of 14q+ was the *CCND1* gene, which was identified in 66.7% (6/9) of the 14q+ chromosomes and 37.5% (6/16) of the MGUS/SMM cases. As for the discrepancy observed in patient no. 2, where only 20.2% of cells had an *IgH* split signals, but 65.5% of cells had *IgH-CCND1* fusion signals, we assume that the *IgH* break has occurred in variable (*VH*) sequences or the fusion signals were the result of an insertion of the whole *IgH* sequence into the *CCND1* locus as previously reported by Yoshida *et al*.[21] These findings are similar to other published data, indicating the relative importance of the *CCND1* gene in the pathogenesis of MGUS/SMM, as well as of primary amyloidosis, when compared to the incidence in symptomatic MM cases.[4, 5, 25, 26] It may thus be hypothesized that there are several distinct pathways for normal plasmablasts to transform into MM, i.e., some plasmablasts harboring t(11;14) develop to the MGUS stage followed by slow progression to MM, while others harboring t(4;14) tend to skip the MGUS stage and directly develop to *de novo* MM in a short time. The nuclear staining pattern of cyclin D1, which represents deregulated expression of this protein, is specifically found in cases with t(11;14). This indicates that the deregula-

tion of the *CCND1* gene definitely contributes to the molecular pathogenesis of MGUS/SMM, but it is not enough for the full transformation of the plasmablasts into symptomatic MM. However, it remains unknown why two out of six t(11;14) cases showed negativity for cyclin D1 staining, since RNA from these two cases with t(11;14) is not available. The *FGFR3* locus was also involved in a case with SMM in our study, which was in keeping with previously published data showing that a small fraction of MGUS/SMM cases possess t(4;14).[4, 5] As for the *MUM1/IRF4* and *MAFB* genes, none of the cases in our study harbored fusion signals with the *IgH* locus. This may simply be because these chromosomal translocations tend to occur as secondary events associated with the progression to symptomatic or clinically aggressive MM, since *MUM1-IgH* fusions derived from t(6;14) were found in 21.1% of the Japanese MM cases analyzed by DCFISH in our previous study.[21]

In a panel of our cases, neither the specific clinical characteristics nor the laboratory findings were recognized in the cases with 14q32 rearrangements or with t(11;14) (data not shown). During our short follow-up period, one case (no. 16) with t(4;14) developed stage IIIA MM 6 months after our FISH study. Real-time polymerase chain reaction using a Light Cycler detected overexpression of the *FGFR3* mRNA in this patient during disease progression, although the RNA derived from the SMM stage was not available for analysis (data not shown). Another case (no. 15) showed 14q32 translocation with an unknown partner chromosome, and development to stage IIA MM 3 months later. This seems to suggest that MGUS cases positive for t(4;14) or other specific 14q32 translocations tend to develop into MM in a short time, whereas the relationship between protooncogenes deregulated as a result of the presence of the 14q+ chromosome and the clinical course of early plasma cell dyscrasia remains to be clarified. To determine whether MGUS with t(4;14) comprises a high risk group for transformation into symptomatic MM, a long-term follow-up of a large number of cases with MGUS needs to be performed.

What are the primary molecular events responsible for the development of MGUS? Although the appearance of the 14q+ chromosome may be the initial event, seven out of the 16 cases in this study harbored no 14q+ chromosome. Another common genetic event in MGUS, 13q deletion, is reported to occur in 15–50% of patients.[4, 5, 27] However, previous studies have also pointed out that not all of the clonal plasma cells in MGUS possess 14q+ chromosomes.[4, 5] In fact, Fonseca *et al.* recently observed that the percentage of clonal plasma cells in MGUS patients, which are considered clonal as they carry chromosomal aneuploidy, is higher than the percentage with 14q+ chromosomes or with 13q deletions, suggesting that chromosomal instability is the primary event of plasma cell dyscrasia.[5] To address this important issue, we need to further investigate molecules such as double strand break repair (DSBR) gene products, whose aberrations lead to chromosomal instability, in clonal plasma cells derived from patients with MGUS/SMM and symptomatic MM.[28, 29]

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