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Ectopic Expression of *MAFB* Gene in Human Myeloma Cells Carrying (14;20)(q32;q11) Chromosomal Translocations

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Chromosome 14q+, which represents a chromosomal rearrangement involving the immunoglobulin heavy chain gene (*IgH*) locus, is a genetic hallmark of human multiple myeloma (MM). Here, we report the identification of (14;20)(q32;q11) chromosomal translocations found in MM cells. Double color fluorescence *in situ* hybridization analyses pinpointed the breakpoints at the 20q11 locus in two MM cell lines within a length of at most 680 kb between the *KIAA0823* and *MAFB* gene loci. Among the transcribed sequences in the vicinity of the breakpoints, an ectopic expression of the *MAFB* gene, which is located at 450–680 kb telomeric to one of the breakpoints and encodes a member of the MAF family basic region/leucine zipper transcription factor, was demonstrated to be associated with t(14;20). This finding, together with that of a previous study describing its transforming activity, suggests that the *MAFB* gene may be one of the targets deregulated by regulatory elements of the *IgH* gene as a result of t(14;20).

Key words: Multiple myeloma — t(14;20)(q32;q11) — *MAFB* — Ectopic expression

Complex chromosomal rearrangements represent genetic aberrations involved in the multistep oncogenesis of human multiple myeloma (MM).[1–3] In particular, the importance of the chromosomal translocations involving immunoglobulin (*Ig*) gene loci, such as *IgH* on 14q32 (14q+ chromosome) and *Igλ* on 22q11, has been well documented.[1–4] Consequently, the specific protooncogenes located at donor chromosomal loci such as 11q13, 8q24, 6p25, 4p16 and 16q24, harboring respectively the *CyclinD1, c-MYC, MUM1, FGFR3* and *c-MAF* genes, are deregulated by the effect of regulatory elements of the *Ig* genes.[2, 3, 5–10] Each chromosomal translocation seems to have a distinct role in the development of MM. For instance, t(11;14)(q13;q32) arises during the early phase of plasma cell dyscrasia, which is clinically represented by monoclonal gammopathy of undetermined significance (MGUS), in contrast to t(8;14)(q24;q32) and t(4;14)(p16;q32), which are assumed to accelerate the progression from MGUS through MM.[11, 12] However, not all of the responsible genes activated on the 14q+ chromosomes in MM have been identified. This prompted us to focus on the remaining partner chromosomes of the *IgH* loci in an attempt to ascertain new elements associated with MM development.

This is the first report to clarify, by means of fluorescence *in situ* hybridization (FISH) analysis, the detailed locations of the two 20q11 breakpoints involved in t(14;20)(q32;q11). We also found that this specific genetic alteration resulted in an ectopic expression of the *MAFB* protooncogene, which is a member of the MAF family basic region/leucine zipper transcription factor and is located at 450–680 kb telomeric to one of the breakpoints, probably due to the influence of the 3′α enhancers of the *IgH* gene.

MATERIALS AND METHODS

A total of 16 MM cell lines (U266, SK-MM-1, ODA, AMO1, NCU-MM-1, XG-7, FR4, NOP-1, KM-4, KM-5, KM-7, SACHI, JJN3, ILKM-2, ILKM-3 and ILKM-8) were used.[7, 9, 13] These cell lines have been characterized previously except for SACHI, which was established from the pericardial fluid of a 47-year-old female patient with plasma cell leukemia (PCL) generating IgG-λ type M-protein. G-Banding analysis revealed its complex karyotype

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featuring hyperdiploidy with 14q+ chromosomes. FISH analysis of the SACHI cells identified the donor chromosomes of the *IgH* loci as 8q24 and 20q11 loci (Taniwaki *et al*., unpublished observation). The presence of t(14;20)(q32;q11) in SK-MM-1 was demonstrated by spectral karyotyping analysis.[1)] Twenty-two patients with MM, whose percentage of tumor cells exceeded 13.6% of the total nucleated cells in bone marrow aspirates, were included in this study after their informed consent had been obtained.

Information for yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs) mapped to the 20q11 locus was obtained through the homepages of the MIT Whitehead Institute (http://www-genome.wi.mit.edu/) and the Sanger Centre Human Genome Data Base (http://www.sanger.ac.uk/HGP/Chr20). The CEPH YACs were purchased from Research Genetics Inc. (Huntsville, AL) and their contig was constructed on the basis of the presence or absence of the distinct STS markers. The PAC contig was constructed according to the sequence overlap and on the basis of hybridization results with PAC-end probes prepared with the bubble-PCR method when sequence data were not available.[14)]

Chromosome preparation for DCFISH analyses has been described previously.[4, 9, 10)] Each PAC/BAC/YAC DNA itself was labeled with either Spectrum Orange or Spectrum Green using a nick translation kit (Vysis Inc., purchased from Fujisawa Pharmaceutical Co., Osaka) according to the manufacturer's instruction. BAC417P24 DNA purchased from BAC/PAC Resources (Oakland, CA) was used as a probe derived from the *IgH* constant region at the 14q32 locus. To determine the chromosomal loci of specific signals on the metaphase spreads, we used whole chromosome painting (WCP) probes which could detect chromosomes 2, 14, 20 and 22 (Vysis) when needed. Hybridization and washing protocols were the same as those available from the company (Vysis) on the internet. Each slide was counterstained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). For the interphase DCFISH used for the patient marrow samples, signals containing PAC644L1/BAC417P24 fusions were evaluated in 100 nuclei per slide. The cut-off index to assess fusion signals was determined by evaluating peripheral blood mononuclear cell samples derived from five normal volunteers.

Northern blot analysis was performed as described previously.[7, 14)] Total RNA was isolated with the guanidine isothiocyanate/cesium chloride ultracentrifugation method. Human *MAFB* probes were amplified from the PAC RPCI4-644L1 by PCR, as this gene has no intron, by using a primer pair, F5′ (5′-GACCGCTTCTCCGACGACCA-3′) and R6′ (5′-CCCTCTCGCTCAAGTCAAAC-3′), whose sequences were reported by Wang *et al*.[15)] The size of the probe was 479 bp. Expressed sequenced tag (EST) clones, mapped telomeric to SACHI's breakpoint, were purchased from Research Genetics Inc. and their inserts were used as probes. The quality and amount of each RNA were assessed by subsequent hybridization with β-*ACTIN* probe.

RESULTS AND DISCUSSION

The chromosome 20q11 locus is known to be rearranged or deleted in various types of human malignancies. It has attracted the attention mainly of hematologists because a region extending from 20q11.2 to 20q12 is commonly deleted in myeloid leukemias, suggesting the existence of an as yet unidentified tumor suppressor gene within this region.[15–17)] Accordingly, YAC contigs have already been constructed by several groups and were available when we focused on t(14;20), which is occasionally encountered in human MM. In order to determine the approximate locations of the 20q11 breakpoints of SK-MM-1 and SACHI cell lines, we decided to use DCFISH analysis. In brief, two Mega-YACs' DNAs located between loci D20S884 and D20S96 were labeled (Fig. 1A), one with Spectrum Green and the other with Spectrum Orange, and hybridized to metaphase spreads of the two cell lines. When the green and red signals were split onto different chromosomes, we could determine the locations of the breakpoints between the two YACs, which were used as probes. This strategy resulted in the eventual confirmation of SK-MM-1's breakpoint within y953C12 itself and that of SACHI between y953C12 and y808C5 (Fig. 1, A, B and Fig. 2, A, B). These YACs were then mapped at the 20q11.2–12 locus. To narrow down the breakpoints, PAC/BAC contigs encompassing the region between the D20S174 and *PLC1* gene loci, whose locations were mapped to centromeric and telomeric ends of y953C12 and y808C5, respectively, were constructed based on the sequence information provided by Sanger Centre Human Genome Data Base. As for the PAC/BAC clones whose complete sequences were not available, we confirmed each overlap by means of Southern hybridization analyses using PAC/BAC-end probes obtained with the bubble-PCR method,[9)] shown as open circles in Fig. 1. Further FISH analyses using PAC/BAC probes could narrow down the locations of these two breakpoints to within PACs 600E6 for SK-MM-1 and 191L6 for SACHI cells, as shown in Fig. 1. This means that the distance between the breakpoints was no more than 680 kb (460–680 kb), as estimated from the PAC/BAC data with respect to size and sequence.

In an attempt to make sure that these 20q11 breakpoints were juxtaposed to the *IgH* gene at the chromosome 14q32 locus as a result of t(14;20), we next employed DCFISH using either y808C5 or PAC644L1, which were mapped

telomeric to SACHI's breakpoint, and BAC417P24 probes spanning the *IgH* constant region at 14q32, as shown in Fig. 2C. The reason for this strategy was that the protooncogenes, dysregulated by the 3′α enhancers of *IgH* as a result of the presence of 14q+ chromosomes in MM, were reported generally to be located telomeric to chromosomal breakpoints.[2, 3] In SK-MM-1 cells, signals derived from y808C5/PAC644L1 moved to chromosome 14q32 and fused to BAC417P24, indicating that the telomeric tip to its breakpoint at 20q11 was juxtaposed to the constant region of the *IgH* gene (Fig. 2D). In a SACHI cell, two fused signals between y808C5/PAC644L1 and BAC417P24 were observed in a metaphase spread, while they were mapped onto two marker chromosomes, whose derivations originated from neither chromosome 14 nor 20 (Fig. 2E).

Since the *IgH* 3′α enhancer has been reported to influence the transcription of specific protooncogenes over sev-

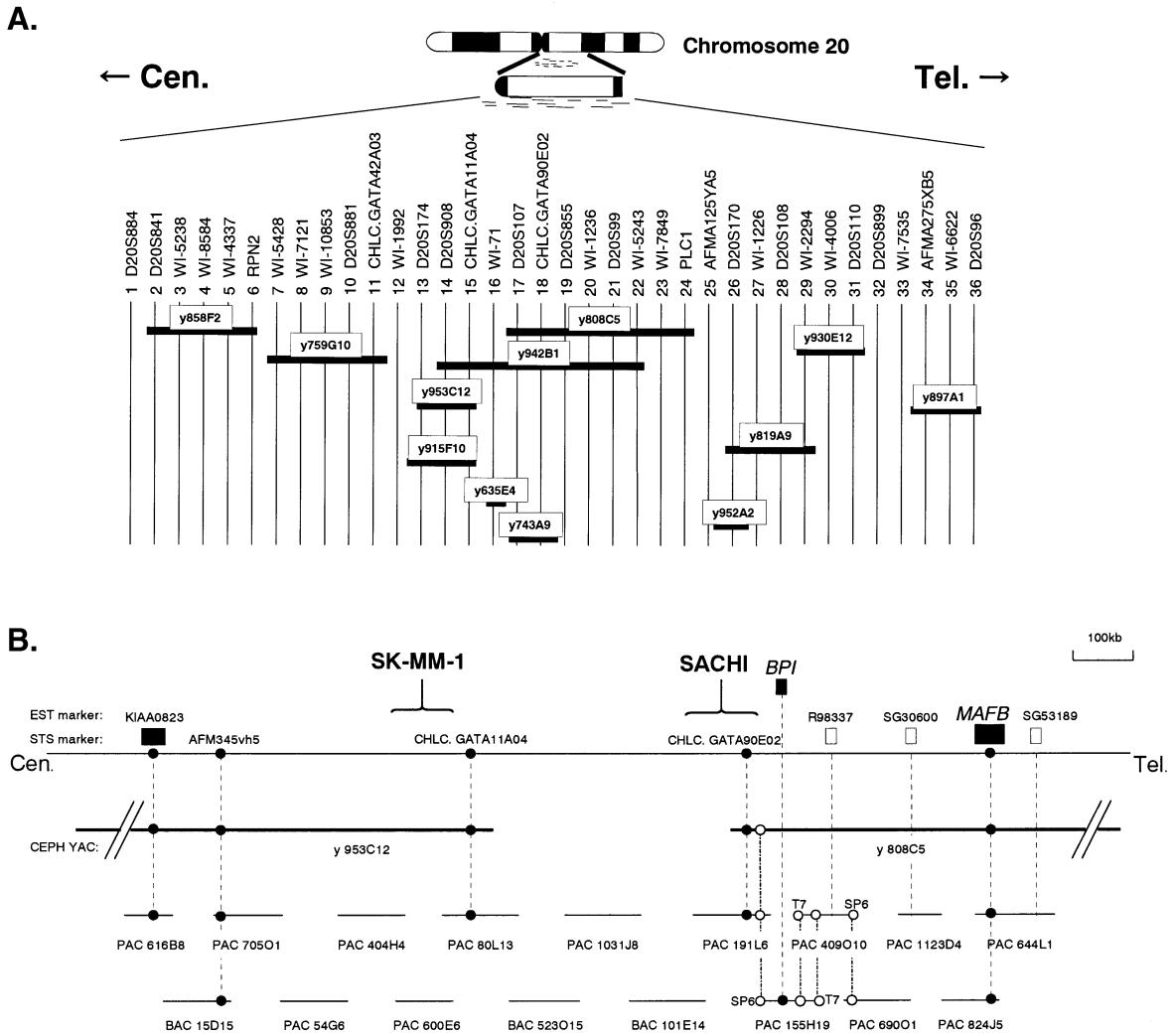

Fig. 1. Relationship of chromosomal breakpoints identified in two MM cell lines to *MAFB* gene. A. A YAC contig spanning 20q11–12 locus. YACs are shown as bold lines, the length of which reflects the number of included STSs (sequenced tagged sites), not the actual size. The physical distances between adjacent STSs have not been determined directly. Information for YACs was obtained through the web site of the MIT Whitehead Institute. B. A PAC/BAC contig at the 20q11.2–12 locus encompassing two chromosomal breakpoints identified in MM cell lines and the *MAFB* gene is shown. Closed circles represent STS and open circles PAC-end derived markers. Solid boxes show the locations of known genes and open boxes those of EST markers. Approximate locations of the chromosomal breakpoints confirmed in SK-MM-1 and SACHI cell lines are indicated along the top. All PAC/BAC clones between PACs 616B8 and 644L1 are linked based on the sequence overlap and on the hybridization results with PAC-end probes shown as open circles. Cen., centromeric side; Tel., telomeric side.

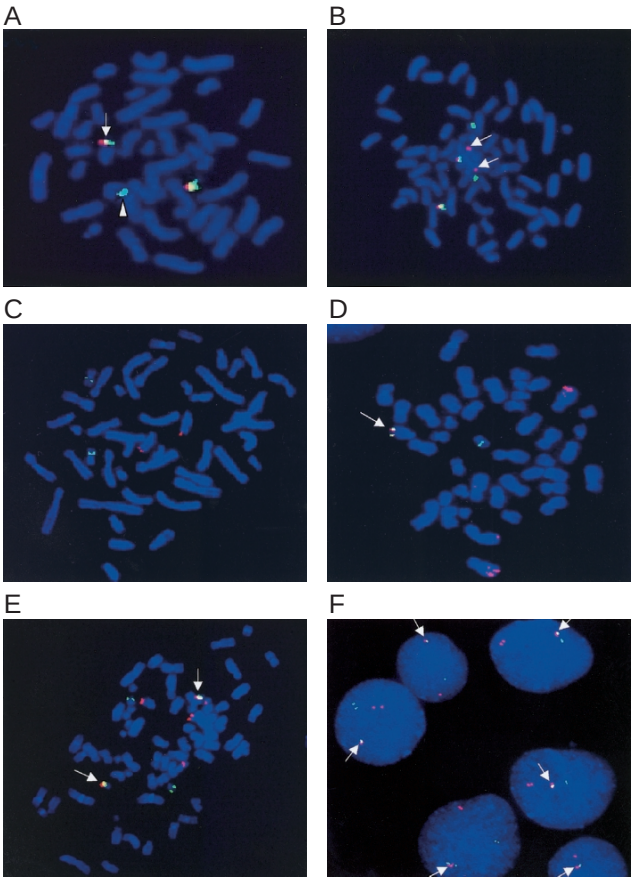

Fig. 2. DCFISH analyses showing the location of 20q11 breakpoints and fusion signals from the telomeric region to the breakpoints and *IgH* loci. A. SK-MM-1 has its 20q11 breakpoint within y953C12. YAC-DNAs derived from y953C12 and y808C5 were labeled with Spectrum Green and Spectrum Orange, respectively. A part of the green signals remains on chromosome 20 as indicated by the arrowhead, while the other part fused with a red signal (a yellow signal) moved to 14q+ chromosomes as indicated by the arrow. B. SACHI has its 20q11 breakpoints between y953C12 and y808C5. The signals derived from y953C12 (green) and y808C5 (red) were split in the metaphase spread. The two red signals indicated by arrows moved to marker chromosomes of unidentified origins, and the two yellow fusion signals were derived from normal chromosome 20. C. DCFISH analysis, using BAC417P24 (*IgH*) labeled with Spectrum Orange and PAC644L1 (*MAFB*) labeled with Spectrum Green, of a metaphase spread derived from a normal peripheral blood lymphocyte. D. A yellow fusion signal between red BAC417P24 and green PAC644L1 signals is observed on a 14q+ chromosome of a SK-MM-1 cell. Two *IgH*-derived red signals are also seen on chromosome 8. E. Two yellow fusion signals between BAC417P24 (*IgH*) and PAC644L1 (*MAFB*) were observed on a metaphase spread of a SACHI cell, although they were localized on unidentified marker chromosomes. F. Interphase DCFISH analysis using BAC417P24 (*IgH*) and PAC644L1 (*MAFB*) probes clearly shows yellow fusion signals as indicated by arrows in the nuclei prepared from SK-MM-1 cells. Fusion signals are indicated by arrows.

eral hundred kilobases in MM cells,[2, 5, 18] we hypothesized the presence of the common target protooncogene(s) in these two cell lines, specifically at a location telomeric to the 20q11 breakpoints. We then surveyed transcripts mapped telomeric to the breakpoints of both cell lines at the 20q11 locus according to the sequence data and gene profile of the PAC/BAC clones obtained from the Sanger Centre Human Genome Data Base in addition to the exon trapping analyses of PAC155H19, whose genomic sequences had not been fully completed. This region showed a relatively low gene density. Only three ESTs and two known genes, *BPI* (bactericidal/permeability-increasing protein) and *MAFB*, have been reported between the SACHI's breakpoint and PAC644L1, which are located at no more than 800 kb downstream to the breakpoint, as shown in Fig. 1. For the purpose of identifying candidate gene(s) dysregulated by t(14;20), we examined the expression of each of their cDNAs by means of northern blot analyses of MM cell lines including SK-MM-1 and SACHI cells. The mRNA expression of ESTs R98337, SG53189 and *BPI* was not detected in the MM cell lines we used (data not shown). This was supposed to be due to either the high fidelity of promoter functions, which were shut down in the plasma cell stage, or the low sensitivity of our northern analyses. But, EST SG30600 was expressed as a 4.0 kb message at similar levels in all MM cell lines, as well as in various hematopoietic cell lines including those consistent with the mature B cell stage (data not shown). Interestingly, the *MAFB* gene mapped at 450–680 kb telomeric to the SACHI's breakpoint was expressed in both SK-MM-1 and SACHI cells (Fig. 3). In our panel of 16 MM cell lines, three cell lines, SK-MM-1, SACHI and KM-5, expressed 3.0 kb transcripts as shown in Fig. 3A (some data omitted). In particular, remarkable overexpression of both 3.0 kb mRNA and 1.8 kb mRNA of an alternatively transcribed form was observed in the SACHI cells when compared to that in SK-MM-1 and KM-5. Since the expression of the *MAFB* gene is restricted to immature myeloid/monocytic lineages in hematopoietic cells, mRNA expression in these three MM cell lines is ectopic.[15, 19, 20] We also analyzed various hematopoietic cell lines including B-lymphoid lines, ranging from pro-B to mature B cell stages, for *MAFB* expression, but no expression was seen (Fig. 3B). We next attempted to clarify by means of DCFISH analyses the mechanisms underlying the ectopic *MAFB* expression in KM-5 cells. However, the *MAFB* locus did not fuse to any other *Ig* gene loci, including the *IgH*, *Igλ* and *Igκ* loci, in this cell line, nor could any amplification of the gene be found when Southern blot analysis was used (data not shown), so the responsible mechanism in this cell line remains to be clarified.

At present, we cannot exclude the possibility of other target gene(s) existing besides *MAFB*, because the ectopic

expression pattern found in MM cells carrying t(14;20) is obviously aberrant, thus indicating the possibility of *MAFB* being one of the target genes of this chromosomal aberration. As is often the case with *IgH* rearrangements with *c-MAF* and *CyclinD1* loci reported in MM cells, the distance between the SACHI's breakpoint and the *MAFB* gene is not too far for the *IgH* 3′α enhancer to have an effect, either.[5, 8] The *MAFB* gene, also known as *KRML*, belongs to the MAF family basic region/leucine zipper (bZip) transcription factors.[21] Like other large MAF proteins such as c-MAF involved in MM with t(14;16),[8] it has both a carboxy-terminal bZip domain, which mediates DNA binding and dimer formation, and an amino-terminal acidic domain associated with transactivating capability.[15, 21] MAFB proteins have dual functions in the transcription of downstream genes and whether they function as transactivator or transrepressor depends on the target sequences of MAF-responsive elements (MARE) and the interacting proteins, such as c-Fos and Ets-1. *MAFB* seems to be ubiquitously expressed in various tissues, while its expression in hematopoietic cells is restricted to myelomonocytic lineage and macrophages. It has recently been demonstrated to play crucial roles in the proliferation of myelomonocytic progenitors and subsequently the promotion of differentiation into the monocytic lineage, and in the prevention of erythroid differentiation in myeloid progenitors.[19, 20] Although we have not yet fully sequenced the *MAFB* cDNAs derived from SK-MM-1 and SACHI cells, transcripts with aberrant sizes were not observed, at least in northern analysis. However, wild-type *MAFB* itself has been shown to transform chicken embryonic fibroblasts when it is artificially overexpressed,[21] indicating that it acts as an oncogene under certain circumstances. We conclude that the ectopic *MAFB* expression in plasma cells as a result of t(14;20) may disturb physiological process of proliferation and differentiation of B lymphocytes, depending on the presence of interacting proteins, and may eventually lead to unlimited proliferation. In our experiments, it has been difficult to find any correlation between expression level of the *MAFB* gene in three MM cell lines and specific malignant phenotypes, because these cell lines carry not only t(14;20) but also other 14q+ chromosomes, which lead to the deregulated expression of various protooncogenes such as *c-MYC* and *MUM1*.[7] Accordingly, the effect of *MAFB* expression in B cell lineage remains to be clarified through further transfection and transgenic studies.

By means of DCFISH using PAC644L1 and BAC417P24 probes, we further explored the frequency of the fusion between *MAFB* and *IgH* loci in human MM. Although metaphase spreads were prepared and analyzed for the 14 MM cell lines other than SK-MM-1 and SACHI, no cell lines harboring *MAFB*/*IgH* fusions were found, thus indicating a 12.5% frequency of t(14;20) in MM cell lines. Because of the low mitotic index of the MM cells, interphase DCFISH was used to analyze fresh MM samples, as it was effective in the case of SK-MM-1 and SACHI, as shown in Fig. 2F. The fusion signals evalu-

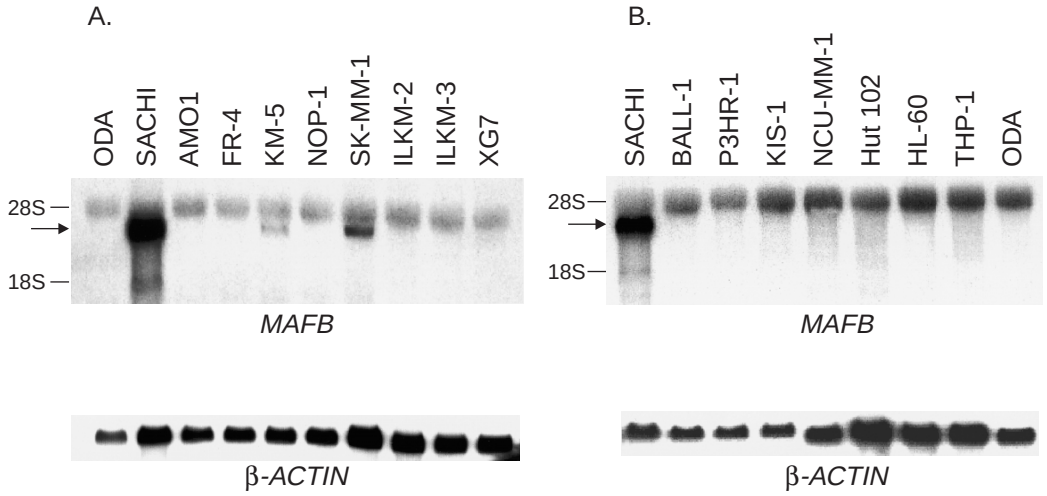

Fig. 3. Ectopic *MAFB* gene expression in MM cell lines carrying t(14;20). *MAFB* expression in a panel of human MM cell lines (A) and in various hematopoietic tumor-derived cell lines (B). *MAFB* mRNA expression was evaluated by means of northern analysis using a 479 bp *MAFB* cDNA probe. Note that 3.0 kb *MAFB* mRNA is detected only in SACHI, KM-5 and SK-MM-1 cell lines as indicated by arrows. A spliced variant of 1.8 kb *MAFB* mRNA is faintly observed only in SACHI cell line. BALL-1, Pre-B cell line; P3HR-1 and KIS-1, mature B cell lines; HUT-102, mature T cell line; HL-60, myeloid line; THP-1, monocytic line. The expression level of β-*ACTIN* mRNA is shown below. Ten micrograms of total RNA was loaded onto each lane. Dashes on the left indicate 28S and 18S ribosomal RNA markers.

ated in controls accounted for 7.40±2.07% (mean±1 SD). Of the 22 cases analyzed, none possessed *MAFB/IgH* fusions in statistically significant fractions (>mean+3 SD) of the total nuclei. Accordingly, t(14;20) seems to be a nonrandom but relatively rare chromosomal alteration in human MM, although further studies covering a large number of MM cases are need to clarify the significance of this alteration, especially in association with clinical and biological behaviors, as it has been suggested that some specific 14q+ markers may be relevant to certain clinical phenotypes.[2, 3, 10)]

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