Detection of t(4;14)(p16.3;q32) Chromosomal Translocation in Multiple Myeloma by Reverse Transcription-Polymerase Chain Reaction Analysis of IGH-MMSET Fusion Transcripts

Ursula Malgeri, Luca Baldini, Vittorio Perfetti, Sonia Fabris, Maurizio Colli Vignarelli, Gualtiero Colombo, Valentina Lotti, Silvana Compasso, Silvia Bogni, Luigia Lombardi, Anna Teresa Maiolo, and Antonino Neri

Hematology Service [U. M., L. B., S. F., V. L., S. C., B. L., L. M. A. T. M., A. N.] and 3rd Division of Internal Medicine [G. C.], Università degli Studi di Milano, Ospedale Maggiore IRCCS, 20122 Milan, and Department of Internal Medicine [V. P.] and Biotechnology Research Laboratories [M. C. V.], Policlinico San Matteo IRCCS, 27100 Pavia, Italy

Abstract

We and others have recently identified a novel recurring t(4;14)(p16.3;q32) translocation in multiple myeloma (MM) that leads to an apparent deregulation of the FGFR3 and WHSC1/MMSET genes. Because the presence of IGH-MMSET hybrid transcripts has been found in MM cell lines with t(4;14), they may represent a specific tumor-associated marker in MM. In this study, we developed a reverse transcription-PCR (RT-PCR) assay for detecting chimeric transcripts from all of the 4p16.3 breakpoints identified thus far, and we used it to investigate a representative panel of 53 MM patients and 16 patients with monoclonal gammopathy of uncertain significance; in addition, t(4;14) was investigated in all of the MM patients by means of two-color fluorescence in situ hybridization. IGH-MMSET transcripts were found in 11 of the 53 (20%) MM cases and 1 of 16 (6%) cases of monoclonal gammopathy of uncertain significance.

There was complete concordance between the RT-PCR and fluorescence in situ hybridization analyses of the MM cases. The results of this study indicate that RT-PCR is a sensitive and reliable method of detecting t(4;14) and suggest that it may be useful for monitoring the disease in a significant proportion of patients.

Introduction

MM is a malignant proliferation of bone marrow plasma cells that is characterized by a wide spectrum of clinical entities. A number of recent advances in the molecular biology of MM have provided new insights into the pathogenesis of this still incurable disease (1). Despite the apparently low incidence revealed by cytogenetic analyses, we and others have demonstrated that translocations involving the immunoglobulin loci, predominantly the heavy chain locus (IGH) at 14q32, are frequently associated with MM (2–5). Translocations to the IGH locus usually occur within the switch regions and involve a large number of chromosome loci, particularly 11q13, 4p16.3, 16q23, and 6p25, in which the cyclin D1, FGFR3 and MMSET, c-MAF, and MUM1/IRF4 putative target genes are located, respectively (5–11).

The t(4;14)(p16.3;q32) chromosomal translocation has not been described previously in reports based on conventional cytogenetics, but probably because of the involvement of the telomeric portions of chromosomes 4p16.3 and 14q32; however, we and others have identified this translocation in MM by cloning the “illegitimate” switch-rearranged IGH alleles suggestive of translocation events. Molecular analyses of the 4p16.3 breakpoints (4, 7, 9) have shown that they occur 50–100 kb centromeric to the fibroblast growth factor receptor 3 gene (FGFR3; Ref. 12) and within the 5′ regions of a novel gene called WHSC1/MMSET (9, 13). The FGFR3 gene is a member of the tyrosine kinase receptor family (fibroblast growth factor receptors 1–4), whose ability to bind a large number of related mitogenic fibroblast growth factors leads to the activation of complex signaling pathways regulating cell proliferation, differentiation, and migration in many different tissues (14). The gene is overexpressed in the translocated cases, and activating point mutations have been found in the deregulated FGFR3 genes of some MM cell lines (4, 7, 15), suggesting that it may play a critical role in tumorigenesis. The WHSC1/MMSET gene, which has been proposed as a candidate for Wolf-Hirschhorn malformation syndrome (13), contains several functional domains, is expressed in early development (particularly in rapidly growing tissues), and is thought to play a role in transcriptional regulation. It extends over about 120 kb on the genome, and its 5′ untranslated region lies approximately 50 kb centromeric of FGFR3. The 4p16.3 breakpoints directly involve the WHSC1/MMSET gene because they occur within 5′ introns or upstream of its coding sequence, probably in the transcription regulatory regions. Finally, the gene is expressed at relatively low levels in MM cell lines but is overexpressed in the majority of those carrying the translocation (9, 13). The t(4;14)(p16.3;q32) translocation may therefore lead to the deregulation of two different potential oncogenes in MM.

The detection of t(4;14) is difficult by means of conventional cytogenetics or Southern blotting because of the poor sensitivity of karyotype analyses and the relatively large dispersion of 4p16.3 breakpoints. We and others have recently developed a two-color FISH assay using IGH- and 4p16.3-specific probes, which has revealed this lesion in 12–17% of the studied MM cases (5, 8). Chesi et al. (9) have shown that the presence of t(4;14) in MM cell lines leads to the formation of IGH-MMSET hybrid transcripts, which may therefore represent a specific marker of the translocation. In this study, we developed a RT-PCR assay for the detection of IGH-MMSET transcripts, investigated their presence in total mRNAs from pathological samples of 53 MM patients and 16 patients with MGUS, and compared the results of the RT-PCR and two-color FISH analyses in MM patients. Our data indicate that RT-PCR is a sensitive and reliable method for the detection of the t(4;14) translocation in MM.

Materials and Methods

Patients. The study involved 53 MM patients and 16 patients with MGUS whose diagnoses were made according to the criteria described by Durie and...
Salmon (16). The bone marrow aspirate from each patient was examined by means of conventional light microscopy and immunophenotype analyses. Complete clinicopathological data were available for all of the patients and are summarized in Table 1. The pathological samples from 35 MM patients (including three cases with plasma cell leukemia) were obtained at diagnosis; the samples from the remaining 18 MM patients were obtained during phases of disease relapse or progression. The Durie-Salmon clinical stage of all of the MM patients shown in Table 1 relates to that recorded at the time of diagnosis. The pathological samples from all of the MGUS patients were obtained at diagnosis.

RNA Extraction and RT-PCR Analysis. The mRNAs from KMS-11 (kindly provided by Dr. T. Otsuki, Okayama, Japan), OPM-2 and NCI-H929 cell lines (DSMZ, Braunschweig, Germany), and the bone marrow cell suspensions of the studied patients were extracted using the Trizol reagent (Life Technologies, Inc., Grand Island, NY). First-strand cDNA was synthesized as described previously (4). The PCR amplifications were made by diluting 5 μl of first-strand cDNA from each case into a 25-μl PCR mixture containing specific primers (20 pmol), MgCl₂ (1 mM), deoxynucleotide triphosphates (200 mM), and Taq DNA polynucleas (0.5 unit; Roche Diagnostic, Milan, Italy). The approximate location of primers used in the study is indicated in Fig. 1B, and their sequences (5′ to 3′) are as follows: (a) JH6, ACCACGGTCACCGTTCCTCA (sense primer); (b) ms5f, ACCGCCTTTGTAATGACTTG (sense primer); (c) ms2, CTTGGCAAGGCTCAGGTGAC (sense primer); (d) ms2f, ATTCAGCCTCGGAAAGATC (sense primer); (e) ms6r, CTCATTTCCTCCTGAATTTGTT (antisense primer); (f) m5r, AAGAACGTGACTGATACGTG (antisense primer); and (g) m5f, TAAGTTGGTATAGCTGTGA (antisense primer). In the case of the reactions using the JH6-ms6f or ms1-ms6r primers, 35 amplification cycles were performed at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. For the nested-PCR, 1 μl of a first amplification reaction carried out with the JH6-ms6f or ms1-ms6r set of primers was used in 30 amplification cycles performed at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s with the internal ms2 and ms5r primers.

DNA Sequencing. The PCR-amplified DNA fragments were sequenced directly using the appropriate primers. The fragments were purified by means of agarose gel extraction and sequenced in both directions using the Big Dye directly using the appropriate primers. The fragments were purified by means of conventional light microscopy and immunophenotype analyses. Complete clinicopathological data were available for all of the patients and are summarized in Table 1. The pathological samples from all of the MGUS patients were obtained at diagnosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MM patients</th>
<th>MGUS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>53</td>
<td>16</td>
</tr>
<tr>
<td>M/F</td>
<td>30/23</td>
<td>9/7</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>57 (35-79)</td>
<td>53 (27-76)</td>
</tr>
<tr>
<td>Median follow-up (months; range)</td>
<td>26 (2-146)</td>
<td>48 (13-97)</td>
</tr>
<tr>
<td>Protein type</td>
<td>IgG</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>IGD</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>k/λ</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Marrow plasmacytosis (%)</td>
<td>32 ± 15</td>
</tr>
<tr>
<td></td>
<td>Durie-Salmon stage</td>
<td>18</td>
</tr>
</tbody>
</table>

*NA, not applicable.

In an attempt to assess a RT-PCR capable of detecting all of the breakpoint types, we performed two separate PCRs on reverse-transcribed cDNAs from the three cell lines using the JH6-ms6f or ms1-ms6r set of primers (Fig. 1B). IGH-MMSET fusion transcripts were detected with both sets of primers (Fig. 1C and the scheme in Fig. 1D). A single amplified fragment of the expected size was detected in the OPM-2 cell line in each reaction, whereas two fragments were constantly found in the NCI-H929 cells (the lower and stronger fragment was of the expected size); this pattern was also detected using the ms5r but not the ms4r antisense primer (see the scheme in Fig. 1B; data not shown). In the KMS-11 cells, amplification with JH6-ms6r or ms1-ms6r primers showed the presence of a fragment of the expected size plus a faint upper fragment that was still present when the ms5r but not the ms4r primer was used (data not shown). The fragments of the expected size were separated by agarose gel and analyzed by direct sequencing. The fainter upper fragments observed in NCI-H929 and KMS-11 were sequenced and found to contain an additional 104-nucleotide stretch between exon 4 and exon 5 (data not shown). A data bank search indicated that this 104-bp sequence is located 7654 nucleotides downstream of exon 4 of the MMSET gene [position 24666-24562 of the L190b4 cosmid clone described by Baxendale et al. (17)]; this sequence is flanked by canonical acceptor and donor splice sites and may therefore represent a novel putative MMSET exon (which we called exon 4a). MMSET transcripts, including those containing the putative exon 4a, are constantly detected by RT-PCR in both normal and neoplastic leukocytes (data not shown); the analysis of the open reading frame containing exon 4a revealed that it leads to a putative MMSET truncated protein of 273 amino acids (data not shown).

Because t(4;14) represents a specific molecular marker suitable for the molecular monitoring of the minimal residual disease in MM, we tried to develop a more sensitive nested PCR assay by using the internal ms2 and ms5r primers specific for the ms1-MMSET transcript. KMS-11, NCI-H929, or OPM-2 cells were serially diluted with the KG1 cell line negative for the translocation; under our nested PCR conditions, we were able to detect the amplified fragment specific for each cell line in 1 positive cell in 10⁶ negative cells (data not shown).

RT-PCR Analysis in MM Patients. RT-PCR analysis was performed in 53 patients using the JH6-ms6r (data not shown) or ms1-ms6r (Fig. 2A) primers in separate reactions. Hybrids transcripts were found in 11 cases with both sets of primers. MB4-1, MB4-2, and MB4-3 breakpoints were found in six cases, three cases, and one case, respectively; in the remaining case (LB109 in Fig. 2A), two amplified fragments of similar intensity were detected, with the lower fragment having the size specific for the MB4-3 type. The amplified fragments were sequenced in all of the positive cases, confirming the type of breakpoint in the first 10 patients; in case LB109, the sequence of the lower fragment was specific for the MB4-3 type, whereas the sequence of the upper fragment contained the 104-nucleotide stretch from the putative exon 4a. The latter findings suggest that the breakpoint in this case is 5′ to exon 4a, a hypothesis that is further supported by the
absence of this fragment in the OPM-2 cell line, for which the breakpoint has been localized 886 bp downstream of exon 4a (2).

**FISH Analysis in MM Patients.** FISH analysis was performed in all of the 53 MM patients as described previously (8). Twenty-three of the cases included in this study have already been investigated by FISH in our laboratory, and 4 cases were found to be positive (8); of the remaining 30 cases, we found 7 positive cases (data not shown). All of these 11 cases were positive for the presence of the IGH-MMSET hybrid transcripts, thus indicating that the specificity and sensitivity of the two approaches are comparable in MM.

**RT-PCR Analysis in MGUS Patients.** The presence of the IGH-MMSET fusion transcripts was investigated in bone marrow samples taken from 16 MGUS patients. No hybrid transcripts were detected in first PCR with both the JH6-ms6r and IJH1-ms6r combinations of primers. Nested PCR analysis (Fig. 2B) revealed the presence of an amplified fragment (breakpoint MB4-2 type) in only one case [1 of 16 cases (7%)].

**Correlation with Clinicopathological Features.** We did not find any significant correlations between the presence of t(4;14) and the clinicopathological characteristics of our MM patients. Of the 35 patients evaluated at diagnosis, 8 cases were positive for the translocation (3 of 18 patients with stage I disease, 3 of 14 patients with stage II/III disease, and 2 of 3 patients with plasma cell leukemia); of the 18 patients evaluated during disease relapse or progression, 3 were found to be positive for the translocation. No correlation was found with age, monoclonal component, sex, or lactate dehydrogenase levels (≤460 or >460 units/liter) and β2-microglobulin (≤2.6 or >2.6 mg/ml). The MGUS patient positive for the translocation is still in this clinical phase after 60 months of follow-up.

---

**Fig. 1.** RT-PCR assay for the detection of the IGH/MMSET hybrid transcripts associated with the t(4;14)(p16.3;q32) translocation in MM. A, schematic representation of the WHSCUMMSET and FGFR3 loci on 4p16.3. The white and black boxes indicate untranslated and translated MMSET exons, and the arrows indicate the approximate location of 4p16.3 breakpoints in the MM cell lines and primary tumors characterized thus far (4, 7, 9). B, schematic representation of the t(4;14) junction, der(4), showing the three different types of 4p16.3 breakpoints (see the text): the MMSET exons (□) and the IGH region (I1, I2, I3, I4, I5) are indicated. The approximate locations of the primers used (see “Materials and Methods”) are shown below the map. C, RT-PCR analysis of the IGH/MMSET hybrid transcripts in the KMS-11, NCI-H929, and OPM-2 cell lines using the IJH1 and ms6r (Lane 1) or JH6 and ms6r (Lane 2) primers; the length of the HaeIII-digested φX174 molecular markers is indicated in bp. D, schematic representation of the IGH/MMSET hybrid transcripts shown in C and their respective lengths in bp. E, nested PCR analysis. cDNA obtained from serial dilutions of the NCI-H929 cells and negative control KG1 cells was amplified with the IJH1 and ms6r primers (left). The nested PCR amplifications using the IJH2 and ms5r internal primers are shown on the right. The length of the amplified fragments (the one at the top contains the putative exon 4a; see the text) is indicated in bp.

---

**Fig. 2.** RT-PCR detection of the IGH/MMSET hybrid transcripts. A, analysis of MM patients; all of the positive cases are shown together with some representative negative cases. B, nested PCR analysis in representative MGUS patients; case 11 showed the presence of a MB4-2 breakpoint. Left, the length of the HaeIII-digested φX174 molecular markers is shown.
Discussion

The t(4;14)(p16.3;q32) translocation is a recurrent lesion in MM that leads to the apparent deregulation of the FGFR3 and WHSC1/MMSET genes, which are closely associated on 4p16.3 (4, 5, 7–9). In particular, the 4p16.3 breakpoints occur within the 5′ introns or regulatory regions of the MMSET gene, leading to the juxtaposition of IGH sequences in the same transcriptional orientation in both the der(4) and der(14) chromosomes (9). It has also been demonstrated in MM cell lines that these recombinations generate both IGH-MMSET and Ij4-MMSET chimeric transcripts, but the IJH-MMSET and Ij4-MMSET hybrid transcripts arising from the IGH promoters relocated on the der(4) chromosome seem to be more frequently expressed (9). Gene deregulation due to a similar mechanism of promoter substitution has been reported previously in the t(3;14)(q27;q32) translocation involving the BCL-6 gene in diffuse large cell lymphomas (18). Regardless of the functional consequences of these alterations in the deregulation of MMSET expression, the IGH-MMSET transcripts may be a reliable marker for the detection of t(4;14) in MM; this is particularly important because, as we and others have recently shown (5, 8), FISH is the only valid means of detecting this translocation.

To the best of our knowledge, there are no previously published reports concerning the investigation of chimeric IGH-MMSET transcripts in MM and/or MGUS patients. In this study, we began by attempting to develop a RT-PCR assay to detect the chimeric transcripts from all of the 4p16.3 breakpoints identified thus far, and then we used it to investigate a representative panel of 53 MM and 16 MGUS patients; we also performed comparative FISH analyses in all of the MM cases. Eleven of the 53 (20%) MM cases were found to be positive for IGH-MMSET transcripts, and the use of two-color FISH detected the presence of a t(4;14) translocation in all of them. These findings in a larger series confirm our previous data concerning the frequency of FISH-detected t(4;14) in MM (5 of 30 MM cases (17%), Ref. 8) and indicate that the sensitivity and specificity of the two approaches are comparable. Given that RT-PCR is an easier, less expensive, and routinely available procedure in most (if not all) laboratories, our data strongly suggest that it should be considered the method of choice for detecting the t(4;14) translocation in MM. These considerations are even more valid in the case of MGUS because chromosomal analyses (including the more sensitive FISH) are still difficult to perform as a result of the small number of clonal plasma cells (usually less than 5%). Furthermore, because MGUS is considered to be a preneoplastic stage that has a 25% chance of progression to overt myeloma, the early identification of genetic lesions may have important implications in terms of clinical management. Our RT-PCR analysis revealed the presence of IGH-MMSET transcripts in only 1 of 16 (6%) of our MGUS patients, and, although our MM and MGUS series are not numerically comparable, it seems that the incidence of t(4;14) is lower in MGUS than in MM. These findings are quite similar to those recently reported by Avet-Loiseau et al., whose FISH studies have revealed the translocation in 2% (2 of 100) of their MGUS patients (19) but in 12% (12 of 102) of their MM patients (5). They have suggested that in MGUS patients, t(4;14) may directly precipitate clonal plasma cells into true myeloma cells, but our single MGUS patient harboring t(4;14) showed no progression of the disease after a 60-month follow-up period.

The analysis of IGH-MMSET transcripts in MM also makes it possible to characterize the relative position of 4p16.3 breakpoints. Our study confirms that they do not occur within the coding sequences of the MMSET gene and that most of them (6 of 11) occur 5′ to exon 3. The chimeric IGH-MMSET allele in patients with a MB-1 breakpoint may produce an overexpressed full-length MMSET protein, whereas those in patients with MB4-2 or MB4-3 breakpoints may give rise to putative truncated MMSET proteins lacking the 238 or 323 NH2-terminal amino acids, respectively (9). We also found that appreciable levels of MMSET transcripts are detected by RT-PCR in translocated MM cell lines and tumors as well as in normal and neoplastic leukocytes (data not shown); this finding suggests that the major consequence of the t(4;14) translocation with regard to the MMSET gene may be homotypic deregulation. The functional role of normal and tumor-associated MMSET forms remains to be investigated.

The RT-PCR detection of t(4;14) may also have implications in terms of the molecular monitoring of minimal residual disease in MM: its specificity and sensitivity could make it a fast and simple alternative to current immunoglobulin-based methods in the subset of patients harboring the translocation.

References

Detection of t(4;14)(p16.3;q32) Chromosomal Translocation in Multiple Myeloma by Reverse Transcription-Polymerase Chain Reaction Analysis of IGH-MMSET Fusion Transcripts

Ursula Malgeri, Luca Baldini, Vittorio Perfetti, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/15/4058

Cited articles
This article cites 19 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/15/4058.full#ref-list-1

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/15/4058.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.