Multiple Myeloma

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Novel mutation and RNA splice variant of fibroblast growth factor receptor 3 in multiple myeloma patients at diagnosis

SIMONA SOVERINI, CAROLINA TERRAGNA, NICOLETTA TESTONI, DEBORAH RUGGERI, PATRIZIA TOSI, ELENA ZAMAGNI, CLAUDIA CELLINI, MAURO FIACCHINI, MICHELE CAVO, MICHELE BACCARANI, SANTE TURA, GIOVANNI MARTINELLI
Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna, Italy

Correspondence: Giovanni Martinelli, MD, Istituto di Ematologia ed Oncologia Medica "Seràgnoli", Policlinico Sant'Orsola-Malpighi via Massarenti 9, 40138 Bologna, Italy. Phone: international +39.051.6363829. Fax: internationl +39.051.6364037. E-mail: gmartino@kaiser.alma.unibo.it

Background and Objectives. The karyotypically silent t(4;14)(p16.3;q32) translocation can be found in approximately 15-20% of multiple myeloma (MM) patients and results in the ectopic expression of fibroblast growth factor receptor 3 (FGFR3) from der4. Point mutations in specific FGFR3 domains can be found in the translocated allele, and have been recently proven to be oncogenic. These mutations produce a constitutively activated receptor, which shows dimerization and autophosphorylation even in the absence of ligand. We investigated the presence of FGFR3 expression and activating mutations in a series of newly diagnosed MM patients.

Design and Methods. We validated a new sensitive and specific Taqman real-time reverse-transcription polymerase-chain-reaction (RT-PCR) set up to evaluate FGFR3 mRNA expression, and applied it to 78 newly diagnosed patients; in positive cases, FGFR3 mRNA transcripts were sequenced. Fluorescence *in situ* hybridization (FISH) was done in 32 cases with sufficient material.

Results. Real-time RT-PCR revealed FGFR3 mRNA expression in 10/78 (13%) patients. In two cases, sequence analysis revealed novel FGFR3 mutations. In a patient with FISH evidence of the t(4;14), a CGC to TGC transition was detected in codon 248. In a patient without the t(4;14), three additional, abnormal-sized transcripts were detected, corresponding to truncated transcripts originating from cryptic splice donor sites located within exon 7.

Interpretation and Conclusions. We describe a novel FGFR3 mutation (with a demonstrated deregulatory mechanism), as well as a case of alternative splicing in the absence of t(4;14), detected in newly diagnosed MM patients overexpressing FGFR3. This implies that FGFR3 mutation can occur at an early stage of myelomagenesis and even in the absence of the t(4;14).

Key words: multiple myeloma, FGFR3, realt-time RT-PCR, alternative splicing.

Although no specific genetic lesions are regularly associated with multiple myeloma (MM), recent studies have shown that the most frequent chromosomal abnormality is 14q+.[1] In approximately 15-20% of cases, this marker originates from a karyotypically silent t(4;14)(p16.3;q32) translocation resulting in ectopic expression of fibroblast growth factor receptor 3 (FGFR3) from der4.[2-4] FGFR3 is a transmembrane tyrosine-kinase receptor normally expressed at barely detectable levels in adult hematopoietic tissue.[5] Germline point mutations in specific FGFR3 domains are associated with autosomal dominant disorders of human skeletal development such as achondroplasia, thanatophoric dysplasia types I and II (TD-I and -II), and hypochondroplasia.[6] These mutations produce a constitutively activated receptor, which shows dimerization and autophosphorylation even in the absence of ligand.[7] Interestingly, some of the FGFR3-activating mutations that are known to cause dwarfism have been found to be associated with the t(4;14) and the high expression of FGFR3 found in certain MM patients.[2,3] Recently, Chesi *et al.*[8] and Li *et al.*[9] demonstrated that FGFR3 mutations associated with MM are oncogenic. Such mutations are thought to occur at a relatively late stage of myelomagenesis.[8]

The frequency of FGFR3 mRNA high expression in MM patients is currently unknown. In the present study, we validated a new sensitive and specific Taqman real-time reverse-transcription polymerase-chain-reaction (RT-PCR) set up to quantify FGFR3 mRNA expression. We applied the technique to a series of newly diagnosed MM patients. Sequencing of the FGFR3 mRNA transcripts in positive cases led to the identification of a novel FGFR3 mutation, as well as a case of alternative splicing in the absence of t(4;14), both detected at the time of diagnosis.

Design and Methods

Study design

We validated a novel TaqMan real-time RT-PCR set up for the quantification of FGFR3 mRNA, and applied it to diagnostic bone marrow (BM) samples from 78 MM patients at diagnosis. Bone marrow infiltration by plasma cells was in all cases over 75%. To evaluate whether FGFR3 expression was associated with activating mutations, we amplified and sequenced five distinct cDNA fragments containing the codons usually affected by amino acid substitutions. In 32 cases with sufficient material, fluorescence *in situ* hybridization (FISH) analysis was also done, as described elsewhere,[10] to evaluate the presence of the t(4;14).

Real-time RT-PCR set-up and validation

For real-time RT-PCR, mononuclear cells from samples from the patients and 7 healthy donors were obtained by Ficoll-Hypaque density gradient centrifugation and stored at -20°C in guanidinium thiocyanate until use. Total cellular RNA was obtained as described elsewhere.[11] RNA was spectrophotometrically quantified at 260 nm and its integrity was assessed by electrophoresis on a 2% agarose gel. Reverse transcription to cDNA was done using 1 μg of total cellular RNA in a 20 μL final volume containing 25 mM random hexamers as primers, as previously described.[12] TaqMan real-time RT-PCR was performed on an ABI PRISM 7700 Sequence Detector (ABI/Perkin Elmer, Foster City, CA, USA) according to described principles.[13] Triplicate experiments were done for each sample, and threshold cycle (Ct) values were averaged. According to the comparative Ct method,[14] the average Ct values for FGFR3 were normalized with respect to the average Ct values for an endogenous reference (the ABL housekeeping gene), to yield the ΔCt. The average ΔCt value obtained from 7 healthy donors' BM samples (chosen as the calibrator) was then subtracted from the average ΔCt value for each patient, to give the ΔΔCt.

For FGFR3, a set of primers and probe positioned on exon 6 was designed (forward:GCCTGGTCATGGAAAGCGT; reverse:CGGATGCTGCCAAA CTTGTT; probe: FAM-ACCGCGGCAACTACACCTGCGT-TAMRA); for ABL, a forward primer was positioned on exon 1a (forward:TCCTCCAGCTGTTATCTGGAAGA), and a reverse primer and probe were both positioned on exon 2 (reverse: TGGGTCCAGCGAGAAGGTT; probe: FAM-CCAGTAGCATCTGACTTTGAGCCTCAGGG-TAMRA). Primers and probes were all selected using Primer Express software (Perkin Elmer, Foster City, CA, USA). Real-time amplification was done using a 25 μL reaction mixture as previously described.[12]

RT-PCR for IgH-MMSET translocation and spanning the t(4;14)

In order to confirm the validity of FGFR3 Taqman quantification as a screening assay in the patient without FISH evidence of t(4;14) we performed RT-PCR analysis of the IgH-MMSET translocation as reported by Chesi *et al.*[4]

Sequence analysis

To search for activating mutations in BM samples showing FGFR3 high expression, we amplified and sequenced five distinct cDNA fragments containing the codons usually affected by amino acid substitutions: namely, codon 248; the entire transmembrane domain (i.e. codons 371, 373, 375, and 380); codon 540; codon 650; and codon 807 (cod.248: forward, AACCCCACTCCCTCCATCTCC; reverse, CAAGGAGAGAACCTCTAGCT; TD: forward, AGGAGCTGGTGGAGGCTG; reverse, GGAGATCTTGTGCACGGTGG; cod.540: ACTGACAAGGACCTGTCGGAC; reverse, GCCCTGCGTGCAGGCGCC; cod.650: GCATCCACAGGGACCTGG; reverse, TGAGTGTAGACTCGGTCA; cod.807: forward: CCTGTCGGCGGCCTTTCGAGCAGTAC;reverse,CACCAG CAGCAGGGAGGGCTGGCTAG). PCR experiments were performed in 50 mLfinal volume containing 10 μL of cDNA, 1U of Taq Gold DNA polymerase (PE Applied Biosystems), 10×PCR buffer, 100 mM of each dNTP, 2.5 mM M_gCl_2, and 1 mM of each primer. An initial denaturation step of 5 min at 95° C was followed by amplification for 50 cycles (denaturation: 30 sec at 95°C; annealing: 40 sec at 60°C; extension: 50 sec at 72°C) and final extension for 7 min at 72°C. The amplified fragments were purified by electrophoresis on a 1.25% agarose/TAE gel prior to DNA extraction from gel slices (Ultrafree-DA, Millipore). Sequence analysis was performed by an automated method (ABI Prism 3700 DNA Analyzer, PE Applied Biosystems), according to the manufacturer's instructions. Genomic DNA was obtained as described.[15] Amplification of the genomic DNA region encompassing codon 248 was performed using the same PCR protocol and the following primers: forward: GGAGAACAAGTTTGGCAGCA (exon 6); reverse: TTGCGTGAGGATTTGGATCTA (intron 7).

Results

FGFR3 high expression was detected in the original diagnostic BM samples of 10/78 (13%) patients. Patients who overexpressed FGFR3 had a ΔΔCt mean value of -7.5 (range, -10.5 to -3.1) as compared with 0.5 (range, -0.9 to 1.6) for those who did not. We found a strong concordance between the high expression of FGFR3 by RT-PCR Taqman analysis and the results of FISH analysis in 31 of 32 patients analyzed by both methods. In the

remaining patient, RT-PCR analysis of the IgH-MMSET translocation performed as reported[4] confirmed the presence of translocation and the suspicion of an internal deletion (which could give rise to lack of recognition by the FISH probe).

Apparently, our real-time assay for FGFR3 expression is able to recognize all cases with evident or cryptic t (4;14) translocation. No statistical differences were found in age, gender, immunoglobulin isotype, stage at diagnosis, BM plasma cell infiltration, serum β_2-microglobulin, C-reactive protein and creatinine levels between patients who expressed a high level of FGFR3 and those who did not.

Sequence analysis revealed mutations in the samples of 2 of these 10 patients, one with and one without t(4;14), as evaluated by FISH.

Firstly, in a patient for whom FISH analysis had confirmed the presence of the t(4;14), we detected a single base-pair mutation in codon 248 in the form of a C to T transition (CGC→TGC) leading to an Arg to Cys amino acid substitution (Figure 1). Approximately 52% of the entire FGFR3 mRNA has been sequenced by our PCR analysis.

Secondly, in a patient without t(4;14), as evaluated by FISH (but positive for a breakpoint into the MMSET gene detected by PCR), primer-specific amplification of sequences encompassing codon 248 revealed not only the expected fragment (WT) corresponding to the FGFR3-IIIb isoform, but also three additional abnormal-sized transcripts (AT-I, -II and -III in Figure 2A). Sequence analysis showed that these should correspond to truncated transcripts originating from cryptic splice donor sites located within exon 7, at positions 829 (AT-I), 868 (AT-II), and 892 (AT-III). As a consequence, these shorter transcripts had deletions of 39, 63 and 102 nucleotides, respectively (Figure 2B).

Thus, the translation products of AT-I, -II and -III can be predicted as truncated proteins lacking 13, 21 and 34 amino acids in the third immunoglobulin-like domain (Figure 2C). Sequence analysis of the genomic DNA sequences surrounding the cryptic splice donor sites did not show any mutation or deletion. Further analysis has been set up and is presently ongoing in order to detect the frequency of the FGFR3 splice variant.

Discussion

It has been shown that specific mutations in the overexpressed FGFR3 found in some MM patients exert an oncogenetic effect. Nevertheless, little is currently known about the frequency of FGFR3 expression and mutation in MM patients. Having

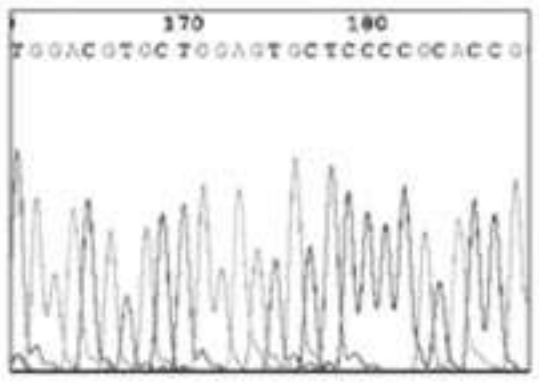

Figure 1. C to T transition in FGFR3 codon 248. Sequence analysis of the RT-PCR product from the first patient, showing the single base-pair mutation in codon 248 (CGC →TGC).

set up a sensitive and reliable real-time RT-PCR method capable of quantifying FGFR3 mRNA expression, we found that in a series of 78 newly diagnosed MM patients, 10 (13%) expressed FGFR3 at high level.

Sequencing of the transcripts of the positive cases uncovered a novel FGFR3 mutation and a case of alternative splicing. Firstly, in a patient for whom FISH analysis had confirmed the presence of the t(4;14), we detected a C to T transition leading to an Arg to Cys amino acid substitution at codon 248. When arising within the germline, this mutation causes the most severe form of dwarfism (i.e. TD-I),[7] and has been demonstrated to result in strong ligand-independent activation of the receptor. To our knowledge, this mutation had never been reported in MM patients.

We also found a case of alternative splicing leading to truncated FGFR3 transcripts, which presumably originated from cryptic splice donor sites located within exon 7. Sequence analysis of the genomic DNA sequences surrounding the cryptic splice donor sites did not show any mutation or deletion. However, these sequences were identical in seven (CAAG/GTG), four (CAAG) and eleven bases (CAAG/GTGGGCC) to the normal exon 7/intron7 boundary (...CAAG/GTGGGCCGG...) and shared the presence of a common CAAG tetranucleotide (GenBank sequence L78726). This suggests that the three abnormal-sized transcripts arose from the activation of cryptic splicing sites and occurred mechanistically in *trans*. The cryptic splice donor site at position 829 has already been reported in colorectal cancer.[16] The functional significance of this aberrant splicing pattern is still unclear and ideally a kinase

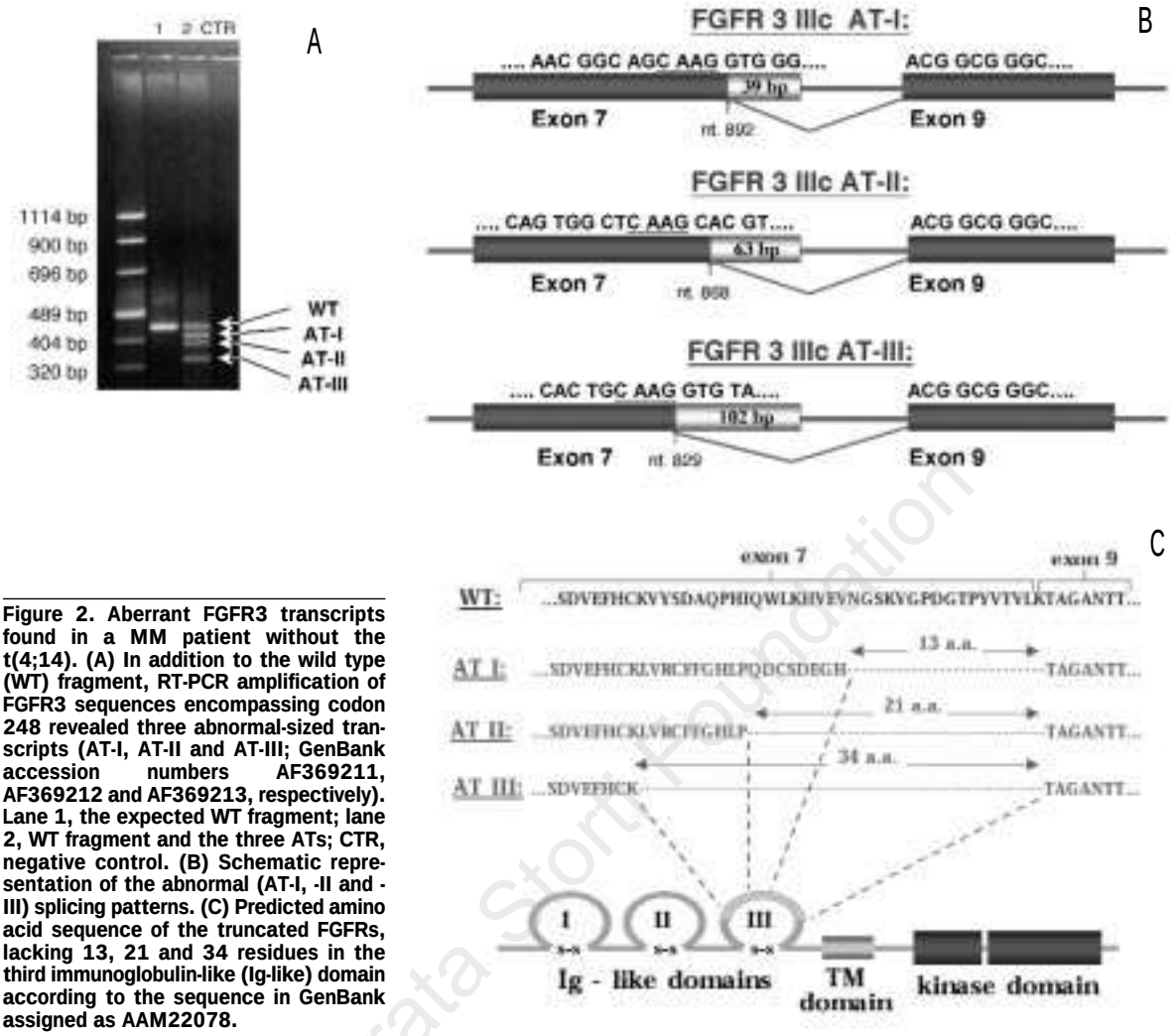

Figure 2. Aberrant FGFR3 transcripts found in a MM patient without the t(4;14). (A) In addition to the wild type (WT) fragment, RT-PCR amplification of FGFR3 sequences encompassing codon 248 revealed three abnormal-sized transcripts (AT-I, AT-II and AT-III; GenBank accession numbers AF369211, AF369212 and AF369213, respectively). Lane 1, the expected WT fragment; lane 2, WT fragment and the three ATs; CTR, negative control. (B) Schematic representation of the abnormal (AT-I, -II and -III) splicing patterns. (C) Predicted amino acid sequence of the truncated FGFRs, lacking 13, 21 and 34 residues in the third immunoglobulin-like (Ig-like) domain according to the sequence in GenBank assigned as AAM22078.

assay on the patient's purified myeloma cells would be useful to address the significance of the splice variants. Unfortunately, we do not have cells collected from the patient at diagnosis in order to perform this analysis.

It remains to be seen whether the same pattern can be found in other MM patients. Interestingly, the alternative splicing was found in a patient without the t(4;14) as evaluated by FISH.

Chesi *et al.*[8] have suggested that FGFR3 mutations occur during tumor progression and are strictly associated with the t(4;14). However, our findings demonstrate that mutations and alternative splicings in the overexpressed FGFR3 can also occur in the absence of this translocation. Furthermore, these variants were detected at the time of diagnosis of MM, suggesting that FGFR3 mutations may actually occur at a much earlier stage of myelomagenesis than has hitherto been thought.

In conclusion, the present study suggests that as many as 10% or more MM patients may overexpress FGFR3 at diagnosis. Our finding in diagnostic samples of a novel mutation and a case of alternative splicing that occurred in the absence of the t(4;14) questions the notion that FGFR3 mutations may be a relatively late myelomagenetic event linked to the t(4;14). This concept should be borne in mind in further studies investigating the frequency and biological significance of FGFR3 mutations.

Contributions and Acknowledgments

SS and CT designed the study and were the principal investigators. Moreover, they set up the PCR procedures and drafted the paper. NT and DR were

responsible for cytogenetic analyses. PT, EZ and CC contributed to the analysis and interpretation of data and revised the article critically. GM was responsible for funding and direct supervision. MC, ST and MB gave the final approval for submission- We are grateful to Robin M.T. Cooke for editing. GM: primary responsible for the manuscript writing; SS: responsible for the Figures.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received May 8, 2002; accepted August 20, 2002.

What is already known on this topic

A subset of patients with multiple myeloma have the t(4;14)(p16.3;q32) translocation, which results in ectopic expression of wild-type fibroblast growth factor receptor 3 (FGFR3). In some cases, FGFR3 kinase-activating mutations can subsequently occur that might have a role in disease progression.

What this study adds

This study suggests that as many as 10% or more multiple myeloma patients may over-express FGFR3 at diagnosis, and shows that FGFR3 mutation can occur even in the absence of the t(4;14).

Potential implications for clinical practice

Mutation analysis of genes can nowadays be performed with relatively simple approaches. However, before including evaluation of FGFR3 mutations in clinical practice, prospective studies are required to show any clinical relevance of these mutations in patients with multiple myeloma.

Mario Cazzola, Editor-in-Chief (Pavia, Italy)