FGFR3 Gene Mutations Associated With Human Skeletal Disorders Occur Rarely in Multiple Myeloma

To the Editor:

Fibroblast growth factor receptor 3 (FGFR3) is one of four distinct tyrosine-kinase receptors (FGFR1-4) that are capable of binding a repertoire of at least nine related mitogenic fibroblast growth factors (FGFs). FGFRs encode proteins that all contain three glycosylated extracellular Ig-like domains, a transmembrane domain (TM), and a split cytoplasmic tyrosine-kinase domain. Point mutations in distinct domains of the FGFR3 gene are associated with autosomal dominant human skeletal disorders, such as achondroplasia, thanatophoric dysplasia types I and II, and hypochondroplasia. Recent reports indicate that the point mutations associated with these disorders produce constitutively activated FGFR3, which shows autophosphorylation in the absence of ligand and is no longer regulated by FGF binding. 

We and others have recently provided the first evidence of FGFR3 gene involvement in human cancer. In particular, the FGFR3 gene located at 4p16.3 is translocated to chromosome 14q32 as a result of a novel and karyotypically undetectable t(4;14)(p16.3;q32) chromosomal translocation in multiple myeloma (MM), a malignant proliferation of plasma cells. Molecular studies have shown this lesion in five MM-derived cell lines and in four primary tumors. Although the breakpoints on 4p16.3 are located approximately 50 to 120 kb centromeric to FGFR3, the gene is overexpressed in these cases, but absent or barely detectable in cell lines without the translocation. Interestingly, FGFR3 gene mutations associated with distinct human skeletal disorders have also been identified in some MM tumors carrying the t(4;14)(p16.3; q32): in particular, the Y373C mutation in the KMS-11 cell line,7,8 the K650E mutation in the OPM2 cell line,7 and the K650M mutation in a primary MM tumor.7 These findings prompted us to look for FGFR3 mutations known to be associated with skeletal disorders in a representative panel of MM, including 80 primary cases (60 patients at first diagnosis, 12 at relapse, and 8 affected by plasma cell leukemia) and 10 MM-derived cell lines (including the KMS-11 and OPM2 cell lines). The analysis was performed by means of the polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) direct sequencing of genomic DNA. We amplified five distinct genomic FGFR3 fragments containing codons affected by mutations: codon 248, the entire TM domain of the introns are indicated by lines. The 3’ untranslated region of the gene has been previously reported.12,13 The sequences of the primers are as follows: 248F (exon 6), 5’-CTTACGGCTATGCGCC-3’, and 248R (exon 7), 5’-CCTTACGGCTATGCGCC-3’; TDS (exon 10), 5’-AGGAGCTGGTGAGGCGTA-3’, and TD3 (exon 10), 5’-GGAGATCTTGTGCACGGTGG-3’; 14S (exon 14), 5’-GGCTCAGGGAGAGTGCAC-3’, and 261R (exon 19), 5’-CAACACAGAGTGAGGCGTAC-3’. The sequences of the primers are as follows: 248F (exon 6), 5’-CTTACGGCTATGCGCC-3’, and 248R (exon 7), 5’-CCTTACGGCTATGCGCC-3’; TDS (exon 10), 5’-AGGAGCTGGTGAGGCGTA-3’, and TD3 (exon 10), 5’-GGAGATCTTGTGCACGGTGG-3’; 14S (exon 14), 5’-GGCTCAGGGAGAGTGCAC-3’, and 261R (exon 19), 5’-CAACACAGAGTGAGGCGTAC-3’.

Fig 1. Schematic representation of the primers from the human FGFR3 gene used in the study. The FGFR3 exons are indicated by white boxes, and the introns are indicated by lines. The 3’ untranslated region of the gene is indicated by the dashed box. The approximate locations of the primers, the length of the amplified fragments, and the approximate positions of codons 248, 540, 650, and 807 are indicated. The nucleotide sequence of FGFR3 cDNA and the intron-exon organization of the gene have been previously reported.12,13 The sequences of the primers are as follows: 248F (exon 6), 5’-CTTACGGCTATGCGCC-3’, and 248R (exon 7), 5’-CCTTACGGCTATGCGCC-3’; TDS (exon 10), 5’-AGGAGCTGGTGAGGCGTA-3’, and TD3 (exon 10), 5’-GGAGATCTTGTGCACGGTGG-3’; 14S (exon 14), 5’-GGCTCAGGGAGAGTGCAC-3’, and 261R (exon 19), 5’-CAACACAGAGTGAGGCGTAC-3’.

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migrating fragments different from the normal control are indicated by arrows. (Fig 1). The mutations at codon 650 were also investigated by means of a restriction enzyme analysis of the PCR-amplified fragment using (codons 371, 373, 375, and 380), codon 540, codon 650, and codon 807 (Fig 2); in both cases, a novel single basepair mutation involving codon 384 in the form of a T to C transition (TTC-CTC) led to a conservative Phe → Leu amino acid substitution (data not shown). Interestingly, this mutation abrogates a Mbo II restriction site and creates a new Mnl I site that allows restriction enzyme analysis of the PCR-amplified fragment. The apparently similar intensity of the normal and mutated bands in both cases, as well as the detection of the mutation in 2 of 100 normal individuals by means of restriction enzyme analysis, suggest that it may represent a rare genetic polymorphism. Finally, the FGFR3 gene was apparently not expressed in the LP-1 cell line; it remains to be seen whether this particular variant may affect FGFR3 biological activity.

Although no specific genetic lesions have been found to be associated with MM (unlike other types of lymphoid neoplasms), cytogenetic and more recent molecular analyses suggest that chromosomal translocations involving the Ig locus on chromosome 14q32 may play an important role in gene deregulation. In this context, the recent identification of the t(4;14)(p16.3;q32) in MM, associated with an apparent deregulation of the FGFR3 gene, may provide some insights into the pathogenesis of this neoplasia. Although more work is needed to assess the role and frequency of the t(4;14) in MM, it can be suggested that deregulation of FGFR3 gene expression may lead to a constitutive oncogenic signal for the growth and/or survival of malignant plasma cells. This possibility is supported by the evidence that the bone marrow environment and, in particular, the stromal cells with which the plasma cells interact are able to produce FGFs. The FGFR3 mutations reported in MM probably represent somatic events, suggesting that FGFR3 gene may be deregulated by different mechanisms. However, we were unable to detect FGFR3 mutations associated with skeletal disorders in our series of samples, except in the cell lines previously reported. This finding suggests that such mutations represent rare events in MM and support the hypothesis that they may occur after the translocation and deregulation of the FGFR3 gene, thus contributing to tumor progression by means of ligand-independent activation.

ACKNOWLEDGMENT

We are grateful to Dr T. Otsuki, Dr F. Malavasi, and Dr A. Solomon for providing us with the some of the MM-derived cell lines (KMM1, KMS-11, KMS-12, LP-1, and UTMC-2) used in this study and to G. Ciceri for technical assistance. The cell lines U266, Sultan, ARH-77, and RPMI 8226 were obtained from ATCC and the OPM2 cell line was obtained from DSMZ. This work was supported by a grant from the Associazione Italiana Ricerca sul Cancro (AIRC) to A.N. and a grant “Ricerca Corrente 1994” from the Ministero Italiano della Sanità to Ospedale Maggiore IRCCS.

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REFERENCES

4. Webster MK, Donoghue DJ: Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. EMBO J 15:520, 1996
5. Webster MK, d’Avis PY, Robertson SC, Donoghue DJ: Profound ligand-independent kinase activation of fibroblast growth factor receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. Mol Cell Biol 16:4081, 1996
Because IL-2 and other common cytokines are known to dramatically influence the sensitivity of the T cells to IL-2, the use of anticoagulants in peripheral blood mononuclear cell (PBMC) preparations could affect IL-2R expression and function. We have previously observed that calcium chelators were used instead of heparin, the levels of all three IL-2R chains on T cells appeared to be increased, and overnight storage of heparinized blood also seemed to upregulate IL-2R subunit expression.

These observations are very important, because they not only seem to settle a long-standing controversy on IL-2R expression but also imply that the use of calcium chelators as anticoagulants instead of heparin could dramatically influence the sensitivity of the T cells to IL-2. Therefore, we compared the levels of IL-2R chains on T cells from healthy donors and from hemochromatosis patients, using whole blood and PBMCs. These results are in contrast with the much higher levels of IL-2R subunits on T cells observed by others using whole blood and fluorescent isothiocyanate (FITC)-labeled commercial monoclonal antibodies, they showed that all three IL-2R chains are usually detectable on either CD4 or CD8 T cells from healthy donors and from hemochromatosis patients. These results are in contrast with the much higher levels of IL-2R subunits on T cells observed by several investigators, including ourselves.

David et al. tentatively explained the discrepancy by invoking effects of anticoagulant and of storage. Indeed, if Ca²⁺ chelators were used instead of heparin, the levels of all three IL-2R chains on T cells apparently increased, and overnight storage of heparinized blood also seemed to upregulate IL-2R subunit expression.

In three separate experiments, blood from five healthy control subjects (all laboratory personnel) was drawn at 10 AM in three different tubes from Sarstedt containing either sodium heparin (final concentration, 0.3 mg/mL), potassium-EDTA (final concentration, 1.6 mg/mL), or sodium-citrate (final concentration, 10.6 mM/L). The largest part of each tube was immediately processed for mononuclear cell (PBMC) separation, using Histopaque 1077 (Sigma, Bornem, Belgium), whereas the rest was kept at room temperature. At 2 pm, 50 µL of whole blood and 50 µL of PBMCs (containing 200,000 cells), derived from each of the three anticoagulant tubes, were incubated for 20 minutes at 4°C with 0.1 µg of the nonconjugated reference monoclonal anti-Tac (IL-2Rα-specific; obtained from Dr Thomas Waldman, National Institutes of Health, Bethesda, MD) and with 2R-B (IL-2Rβ-specific; from Dr Takashi Uchiyama, Institute for Virus Research, Kyoto University, Kyoto, Japan). As an isotypic (IgG1) control, we used purified 56D3 directed against an irrelevant parasitic antigen (provided by Dr J. Brandt, Institute of Tropical Medicine, Antwerp, Belgium). After washing with phosphate-buffered saline (PBS), containing 0.5% bovine serum albumin, 1 µL of FITC-conjugated F(ab')2 goat antimouse IgG (Tago, Burlingame, CA) was added for another 20 minutes. After washing again, the remaining binding sites on the FITC-conjugate were blocked with 5 µL of mouse serum. Next, 5 µL of phycoerythrin (PE)-labeled anti-CD4 and 5 µL of peridinin-chlorophyll A protein (PerCP)-labeled anti-CD3 (both from Becton Dickinson, Erembodegem, Belgium) were added for the last 20 minutes. The tubes with whole blood were then subjected to the Becton Dickinson lysing solution. All preparations were washed once and fixed with 1% paraformaldehyde. The samples were analyzed on a FACScan (Becton Dickinson) using the LYSYS I software.

Based on the scatter and the CD3/CD4 expression, the CD4⁺ and CD4⁻ T lymphocytes were gated separately and the distribution of the first fluorescence was represented in a histogram for each subset. An example of this analysis is shown in Fig 1. It is evident that, within both the CD4⁺ and CD4⁻ T-cell populations, the expression profile of IL-2Rα is rather broad and tends to be bimodal (a negative and a positive subpopulation), whereas the curve of IL-2Rβ is unimodal and shows a shift to the right, which is most evident in the CD4⁻ subset. We chose to express the results for both chains as percentage of positive cells, after establishing a narrow threshold at a relative fluorescence intensity of 10, based on the background of the control monoclonal. A summary of the results is shown in Table 1. No significant difference was observed in the level of IL-2Rα and β chains on CD4⁺ or CD4⁻ T cells, according to the anticoagulant used and regardless of whether the cells were stained in the context of whole blood or PBMCs. Comparing the mean fluorescent intensity of all gated cells (instead of the percentage of positive cells) showed similar results and confirmed that the low level of IL-2Rβ expression on CD4⁺ T cells significantly differed from background (data not shown).

We next wanted to know whether the anticoagulant influences the sensitivity to IL-2. To this end, we cultured the three preparations of PBMCs at a final concentration of 10⁶/mL in RPMI, supplemented with antibiotics (GIBCO, Paisley, UK) and 10% bovine calf serum (HyClone, From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
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