Immunohistochemistry accurately predicts FGFR3 aberrant expression and t(4;14) in multiple myeloma

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Abstract

The t(4;14) translocation detected by fluorescence in situ hybridization (FISH) is an independent prognostic factor for an adverse outcome of multiple myeloma (MM). As t(4;14) uniquely results in FGFR3 expression, decalcified, paraffin-embedded bone marrow biopsies were immunostained for FGFR3 and its expression was correlated with the t(4;14) status. FISH detected t(4;14) in 16 (19%) of 85 MM patient specimens and immunocytochemistry detected aberrant FGFR3 expression in 13 (15%). Twelve (75%) t(4;14) positive cases expressed FGFR3 and 12 (92%) FGFR3 positive cases harbored a t(4;14). FGFR3 expression and t(4;14) were strongly correlated (p<0.0001). FGFR3 expression by immunohistochemistry was associated with the IgA isotype (p=0.0059), a shorter progression free survival (median 11.5 vs. 25.8 months, p=0.0172) and a shorter overall survival (median 19.2 vs 46.3 months, p=0.0309).

Introduction

Chromosomal translocations involving the immunoglobulin heavy chain gene (IgH) locus are found frequently in multiple myeloma (MM), a malignancy of terminally differentiated B-cells. One of the most common translocations, t(4;14), seen in 10-20% of cases, is associated with a poor prognosis. The molecular pathogenesis of t(4;14) is thought to involve aberrant immunoglobulin class switching recombination, leading to a reciprocal translocation between chromosomes 14q32 and 4p16.3 that repositions FGFR3 to der(14) and creates a fusion gene with MMSET on der(4) under the influence of strong enhancers from the IgH region.
FGFR3 is one of a family of five tyrosine kinases through which fibroblast growth factors signal. These receptors are characterized by an extracellular domain with two or three immunoglobulin-like domains, a transmembrane domain and a cytoplasmic tyrosine kinase domain. On ligand binding, FGFR3 undergoes dimerization and tyrosine autophosphorylation, resulting in cell proliferation and differentiation. In MM, FGFR3 dysregulation appears to occur exclusively as a consequence of translocation. We and others have demonstrated that aberrant FGFR3 expression induces lymphoid malignancies in mice, and that aberrant expression in myeloma cell lines results in cell proliferation and survival. Recent studies from our group suggest that the inhibition of FGFR3 by a small molecule induces differentiation and apoptosis of myeloma cell lines and primary MM cells, suggesting FGFR3 is a therapeutic target.

The t(4;14) can be detected by fluorescence in situ hybridization (FISH) or gene expression profiling, and FGFR3 expression has been assessed at the mRNA level by RT-PCR. However, FGFR3 protein expression, which could be a more clinically applicable diagnostic tool, has not been systematically examined. We therefore evaluated paraffin immunocytochemistry (IHC) for FGFR3 expression, and correlated its expression with t(4;14) by FISH and clinical outcome.

Study design

Patients

Eight-five consecutive MM cases accessioned and diagnosed by Durie/Salmon criteria between 1999-2002 were studied. The clinical and laboratory features are summarized in Table 1. The median age of the 53 men and 32 women was 53 years. Forty-three
patients had an IgG paraprotein, 23 IgA, 3 IgD, 14 light chain disease and 2 were nonsecretory. With institutional ethic board approval, bone marrow aspirates and aspirate section/biopsies were obtained from active disease. All patients were treated with melphalan based high-dose chemotherapy and autologous stem cell transplant. The median follow-up after stem cell transplant was 20 months. Approval was obtained from University Health Network (UHN) research reviewer board and informed consent was provided by the patients in the study.

**Immunocytochemistry**

B5 fixed bone marrow aspirate sections and biopsies were decalcified and paraffin-embedded. Sequential 5 μm thick sections were cut, mounted, dewaxed and immunostained for CD138 and FGFR3 by the avidin-biotin-peroxidase method (ABC kit, Vectastain; Vector). The mouse monoclonal anti-FGFR3 antibody (sc-13121; Santa Cruz Biotechnology) is raised against the amino acids 792–806 mapping at the carboxy terminus of the precursor form of human FGFR3.

Briefly, the sections were boiled in 10 mM citrate buffer pH 6.0 for 25 min, stored overnight in the buffer at room temperature, treated with 2% hydrogen peroxide and 10% methanol for 20 min, incubated with 10% goat serum for 1 hr and then incubated overnight at 4°C with one of the primary antibodies. Antibodies to CD138 and FGFR3 were used at dilutions of 1:100 and 1:800 respectively. This incubation was followed by a 30 min incubation with a biotinylated linking reagent (ID Labs. Inc.) and a subsequent 30 min incubation with horseradish peroxidase-conjugated Ultra Streptavidin labeling reagent (ID labs, Inc.). The immunoreaction was visualized with freshly prepared
NovaRed Solution (Vector Labs.) and the slides counterstained with Mayer’s hematoxylin.

The positive control for aberrant expression of FGFR3 was a xenograft tumor from a t(4;14) positive myeloma cell line (KMS11) injected mouse. As a negative control, non-immune mouse serum was substituted for the primary antibody. We considered cells FGFR3 positive if the membrane and/or cytoplasm of at least 20% of the plasma cells stained. Ten normal bone marrow biopsies (containing <5% plasma cells) and 10 B-cell chronic lymphocytic leukemia (CLL) infiltrated bone marrow biopsies served as additional controls.

cIg-FISH analysis
Ficoll-gradient centrifugation enriched mononuclear cells were stained by interphase FISH combined with immunofluorescent cytoplasmic light chain staining (cIg-FISH) to optimize the scoring specificity as previously described.

Statistical analysis
Statistical evaluation used Fisher’s exact test, chi-square test and the nonparametric Wilcoxon test. Progression free survival (PFS) and overall survival (OS) were calculated from the transplant date by the Kaplan-Meier method. Differences between survival curves were analyzed by Log-rank test.
Results

Immunocytochemistry

Anti-CD138 stained all myeloma cells in the evaluated cases. In 13 (15%) of 85 cases, more than 50% of the myeloma cells had moderate to strong membrane and/or cytoplasmic FGFR3 immunoreactivity (Figure 1A). The bone marrow hematopoietic cells were FGFR3 negative as were all 10 normal and 10 CLL bone marrow controls. Plasma cells (<5%) from normal marrows or CLL marrows were CD138 positive but negative for FGFR3.

Correlation of FGFR3 expression, t(4;14) and clinical features

In 16 (19%) of 85 cases, cIg-FISH detected t(4;14) fusion signals in 54-98% of the clonal plasma cells. Twelve (92%) of the 13 FGFR3 IHC positive cases had a t(4;14) and 12 (75%) of the 16 t(4;14) positive cases overexpressed FGFR3. The t(4;14) and FGFR3 expression were strongly correlated (p<0.0001). FGFR3 expression was associated with the IgA isotype (p=0.0059) but there was no correlation with age, gender, disease stage, lytic bone lesions, bone marrow plasmacytosis, albumin, creatinine or beta-2 microglobulin levels (Table 1). Patients with FGFR3 expression by IHC had a significantly shorter OS than those without expression (median 19.2 vs. 46.3 months, p=0.00309) (Figure 1B). The PFS of FGFR3 IHC positive patients was also significantly shorter (median 11.5 vs. 25.8 months, p=0.0172)
Discussion

The t(4;14) is an independent prognostic factor in MM and, as it is predictive for an adverse outcome, a simple, robust and rapid method to detect t(4;14) would be clinically useful. In this study, we found IHC can detect FGFR3 expression in myeloma cells and is strongly correlated with t(4;14) detected by interphase cIg-FISH. To our knowledge, this is the first report of aberrant expression of FGFR3 detected by IHC in a large MM series. Furthermore, FGFR3 expression in MM is associated with the IgA isotype and a shorter OS or PFS, consistent with the features of t(4;14) detected by interphase-FISH.

In this series, 80% of the MM cases were negative for both FGFR3 expression and t(4;14), 14% were positive for both FGFR3 and t(4;14), 4.7% were negative for FGFR3 but t(4;14) positive and 1.5% were positive for FGFR3 but t(4;14) negative. Therefore, the sensitivity of IHC is 75% and the specificity is 98% in predicting t(4;14).

We found that 25% of the t(4;14) positive cases are FGFR3 negative by IHC, in agreement with reports by Keats et al and Santra et al who identified 26% and 32% of their MM cases respectively lacked FGFR3 expression at mRNA level while expressing the IgH/MMSET transcript. An interstitial FGFR3 deletion could explain this finding but in this series, t(4;14) FISH analysis did not detect a FGFR3 deletion in cases lacking FGFR3 expression. Other cryptic mechanisms that block FGFR3 expression at transcriptional or translational levels may account for this discrepancy.

That 1(7.7%) of our 13 cases with FGFR3 expression was t(4;14) negative is unexpected and contrasts with a previous study in which t(4;14) were detected in all cases expressing FGFR3 at the mRNA level. Although rare, it is possible that FGFR3
activation may be due to gene mutations, or other mechanisms not involving the IgH locus. A recent study by gene expression profiling to analyze t(4;14) positive and t(4;14) negative MM cases, found 25% of t(4;14) negative MM patients overexpressed MMSET. Furthermore, a small percentage (~4%) of t(4;14) negative patients express FGFR3 at low level on gene expression profiling (Bergsagel, personal communication). It is also possible that our FGFR3 positive, t(4;14) negative case is an artifact due to nonspecific staining by the anti-FGFR3 monoclonal antibody. However, this is unlikely, since by using Western blot in 8 human myeloma cell lines, we found the anti-FGFR3 monoclonal antibody only reacted with the t(4;14) positive cell lines but not with the t(4;14) negative cell lines (data not shown). Our findings suggest that in a subset of MM, alternative pathways activate FGFR3.

In conclusion, paraffin immunocytochemistry reliably detects for aberrant expression of FGFR3 in MM, and is strongly correlated with the presence of the t(4;14) translocation and an adverse clinical outcome. Since paraffin immunocytochemistry is routinely available, robust and inexpensive, we suggest FGFR3 immunocytochemistry of myeloma cells become a panel of routine evaluation, as if present, FGFR3 represents a potential target for kinase inhibitor therapy.
References


Table 1. Characteristics of patients according to FGFR3 expression by IHC

<table>
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<tr>
<th>Characteristics</th>
<th>Total (n=85)</th>
<th>FGFR3</th>
<th>P-value</th>
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<tr>
<td></td>
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<td>Negative (n=72)</td>
<td>Positive (n=13)</td>
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<tr>
<td>Sex, F:M (F%)</td>
<td>32:53(37.7)</td>
<td>24:48(33.3)</td>
<td>8:5(61.5)</td>
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<td>Age, years</td>
<td>53(32-71)</td>
<td>53(38-71)</td>
<td>58(32-67)</td>
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<td>β2-Microglobulin, mmol/l</td>
<td>207(116-2690)</td>
<td>207(116-2690)</td>
<td>291(128-437)</td>
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<td>CRP, mg/l</td>
<td>3.5(2.0-122.0)</td>
<td>3.8(2.0-122.0)</td>
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<td>Calcium, mmol/l</td>
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<td>88(51,1759)</td>
<td>92(52,216)</td>
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<td>Hb, g/dl</td>
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<td>11.3(5.2-15.8)</td>
<td>10.0(5.6-12.9)</td>
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**Figure 1A. Detection of FGFR3 and t(4;14) in MM.** Immunohistochemistry study with anti-FGFR3 antibody showed a positive reaction with the tumor tissue from the t(4;14) KMS11 cell line injected mouse (a), a negative reaction with a bone marrow biopsy from a t(4;14) negative myeloma patient (b), and a positive reaction with a bone marrow biopsy from a t(4;14) positive myeloma patient (c). clg-FISH showed a fusion signal indicating a t(4;14) translocation in a myeloma cell (d).
Figure 1B. Overall survival of MM patients according to FGFR3 expression by IHC.
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