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Global real-time quantitative reverse transcription-polymerase chain reaction detecting proto-oncogenes associated with 14q32 chromosomal translocation as a valuable marker for predicting survival in multiple myeloma

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ABSTRACT

CCND1, *FGFR3* and *c-MAF* mRNA expression of tumor samples from 123 multiple myeloma patients were analyzed by global RQ/RT-PCR. *CCND1*, *FGFR3* and *c-MAF* were positive in 44 (36%), 28 (23%) and 16 (13%) of patients, respectively. In 7 patients, both *FGFR3* and *c-MAF* were positive. The expression of *c-MAF* was independent unfavorable prognostic factors for overall survival (OS). Autologous stem cell transplantation improved progression-free survival of *CCND1*-positive patients. Bortezomib, thalidomide or lenalidomide extended OS of *FGFR3* and/or *c-MAF*-positive patients. Thus, *CCND1*, *FGFR3* and *c-MAF* mRNA expression can predict survival and is useful for planning stratified treatment strategies for myeloma patients.

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1. Introduction

Multiple myeloma (MM) designates incurable plasma cell neoplasia, but there is a great deal of patient heterogeneity with median survival of 3–4 years; however, survival ranges from a few weeks after diagnosis to more than 10 years [1,2]. Underlying genetic features of the tumor cells largely dictate the clinical heterogeneity of MM, much of which is characterized by recurrent chromosomal translocations involving the immunoglobulin heavy-chain (IgH) locus on chromosome 14q32 [2,3]. Five major oncogenes are commonly involved in 14q32 translocations: *cyclin D1* (*CCND1*) (11q13), *fibroblast growth factor receptor 3* (*FGFR3*)/*multiple myeloma SET domain* (*MMSET*) (4p16.3), *musculoaponeurotic fibrosarcoma oncogene homolog* (*c-MAF*) (16q23), *cyclin D3* (*CCND3*) (6p21) and *MAFB* (20q11) [1–3]. The presence of t(11;14)(q13;q32) is associated with CD20 expression [4], lymphoplasmacytic morphology [5,6], hyposecretory disease [6], and either improved survival or no influence on survival in patients treated with high-dose chemotherapy and autologous stem cell transplantation (ASCT) [7–10]. The presence of t(4;14)(p16;q32) is associated with IgA-type MM, resistance to treatment with alkylating agents on relapse and is a marked independent negative prognostic indicator [7,11–15]. The presence of t(14;16)(q32;p23) is also an unfavorable prognostic indicator [15]. Thus, 14q32-associated chromosomal translocations could be

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valuable diagnostic markers for predicting treatment efficacy and survival in MM.

It is now possible to measure some specific recurrent chromosomal changes. However, few chromosomal abnormalities are discernible in MM patients by conventional analysis because of the weak proliferation of the tumor cells (making it difficult to obtain good metaphases). Recently, the development of techniques such as double-color interphase fluorescence *in situ* hybridization (DC-FISH) may help circumvent the non-proliferative pitfall. Additionally, we established a global real-time quantitative/reverse transcription-polymerase chain reaction (RQ/RT-PCR) technique for detecting the expression of six 14q32 chromosomal translocation-associated proto-oncogenes in marrow plasma cells from MM patients [16]. This RQ/RT-PCR technique can detect transcriptional activation of 14q32-associated proto-oncogenes, is easy to perform on clinical samples, and may be more efficient and cost-effective than interphase FISH [16].

We have now extended our analysis to a consideration of the prognostic value of determining the levels of expression of 14q32-associated proto-oncogenes such as *CCND1*, *FGFR3* and *c-MAF* in 123 consecutive patients with newly-diagnosed MM. The goal of the present study was to investigate whether elevated expression of particular oncogenes is associated with prognosis and treatment efficacy in MM and to compare the global RQ/RT-PCR technique and interphase FISH in terms of clinical usefulness.

2. Materials and methods

2.1. Patients and control subjects

This study included 121 symptomatic MM and 2 plasma cell leukemia (PCL) patients, diagnosed between 1996 and 2010 at five different hospitals in Japan. Patients with other plasma cell disorders such as asymptomatic myeloma (SMM, $n=8$), monoclonal gammopathy of undetermined significance (MGUS, $n=17$), two solitary plasmacytoma, two Castleman's disease, and two primary amyloidosis were included as controls. The diagnosis and classification of MM and other plasma cell neoplasms was according to the criteria proposed by the International Myeloma Working Group (IMWG) [17]. We took the clinical characteristics at the initial diagnosis of MM/PCL for our analyses. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients prior to bone marrow sampling, in accordance with the Declaration of Helsinki.

2.2. Quantification of 14q32 chromosomal translocation-associated proto-oncogene CCND1, FGFR3 and c-MAF mRNAs

Plasma cells were purified from mononuclear cells obtained from 1 to 2 mL of bone marrow aspirate by positive selection using anti-CD138 antibody-coated beads and an automatic magnetic cell sorting system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. *CCND1*, *FGFR3* and *c-MAF* mRNA levels in purified plasma cells were determined by modified global RQ/RT-PCR as described in the Supplemental Methods. The copy number ratio of each proto-oncogene was calculated by dividing its expression level by that of *β-ACTIN*, with a ratio $\geq 10^{-2}$ being defined as positive, as in our previous study [16].

2.3. Conventional Giemsa (G)-banded karyotyping and Fluorescence in situ hybridization (FISH)

Conventional G-banded karyotyping and FISH for detecting chromosomal translocations t(11;14), t(4;14) and t(14;16) were performed by SRL Co., Ltd (Tokyo Japan) as described in the Supplemental Methods.

2.4. Statistical analysis

CCND1 mRNA levels were compared in patients with or without t(11;14) using the Mann–Whitney *U* test. Survival analyses were performed by the Kaplan–Meier method, and survival curves were compared using the log-rank and Breslow–Gehan–Wilcoxon tests. For survival analysis, the baseline was taken as the date of bone marrow collection (initial diagnosis of MM/PCL), and all patients who were still alive were censored at the date of last follow-up. Hazard ratios for overall survival (OS) and/or progression-free survival (PFS) in MM/PCL cases with or without ASCT or treatment with novel drugs in *CCND1*$^+$, *FGFR3*$^+$/*c-MAF*$^+$ and negative for all three proto-oncogenes (triple negative) groups were calculated by the Cox proportional hazards model. Univariate and multivariate analyses were performed with the Cox proportional hazard model. Multivariate analyses used variables selected by stepwise regression from any variables that were shown to be significant by univariate analysis. The clinical characteristics of *CCND1*$^+$, *FGFR3*$^+$, *c-MAF*$^+$ and triple-negative MM/PCL patients were compared by Chi-square testing. Data were analyzed with the aid of StatView software (SAS Institute, Version 5.0, Cary, NC). $P<0.05$ was considered significant.

3. Results

A total of 123 MM/PCL patients was studied, including 36 undergoing ASCT and 59 treated with novel drugs such as bortezomib, thalidomide and lenalidomide. There were 53 males and 70 females with an age range of 34–93 years (median age, 68 years). Their characteristics are summarized in Table 1.

3.1. CCND1, FGFR3 and c-MAF mRNA expression in MM/PCL patients

CCND1, *FGFR3* and *c-MAF* were expressed in 44 (36%), 28 (23%) and 16 (13%) of the 123 patients, respectively (Fig. 1). None of these three proto-oncogenes was expressed in 42 patients (34%), whereas in 7, both *FGFR3* and *c-MAF* were positive (Fig. 1). Expression of *CCND1* precluded expression of *FGFR3* and/or *c-MAF* in these patients, as previously reported [16]. *CCND1* expression was also detected in 2 of 8 SMM, 3 of 17 MGUS, and one of the two primary amyloidosis cases. It was not detected in the solitary plasmacytoma and Castleman's disease patients. *FGFR3* was expressed in 1 of 8 SMM and 1 of 17 MGUS cases, but not in solitary plasmacytoma, Castleman's disease or primary amyloidosis. Finally, c-*MAF* was not detected in any patients with these other plasma cell disorders.

3.2. Associations between CCND1, FGFR3 and c-MAF mRNA expression and 14q32 chromosomal translocations detected by FISH

The presence or absence of t(11;14) was evaluated by FISH in 31 of the 44 *CCND1*$^+$ MM/PCL patients. Thirteen (42%) had t(11;14), 12 (39%) had polysomy 11, and the other 6 (19%) were normal. Patients with *CCND1* expression and carrying t(11;14) had higher levels of *CCND1* mRNA than those with polysomy 11 or normal status (Mean ± 1SD of the copy-number ratio of *CCND1*: 124.4 ± 89.3 versus 10.6 ± 21.0, $P<0.0001$). Of the 28 *FGFR3*$^+$ MM/PCL patients, 17 were evaluated for t(4;14) by FISH; all were found to harbor t(4;14). Finally, of the 16 *c-MAF*$^+$ MM/PCL patients, 9 were evaluated for t(14;16). Five patients (56%) who were *c-MAF*$^+$/*FGFR3*$^-$ had t(14;16), whereas the remaining four had t(4;14) but not t(14;16) (Fig. 1).

Table 1
Patients' characteristics.

Total number	123
Age (year), median (range)	68 (34–93)
Sex (male/female)	53/70
M-protein	
IgG	63 (51.2%)
IgA	24 (19.5%)
BJP	27 (22.0%)
IgD	6 (4.9%)
Non secretory	3 (2.4%)
Stage (Durie–Salmon)	
I	3 (2.4%)
II	27 (22.0%)
III	93 (75.6%)
WBC ($\times 10^3/\mu L$), median (range)	5.0 (1.3–24.5)
Hb (g/dL), median (range)	9.2 (4.0–14.5)
PLT ($\times 10^3/\mu L$), median (range)	190.0 (43.0–558.0)
Ca (mg/dL), median (range)	9.9 (8.4–16.8)
TP (g/dL), median (range)	9.1 (4.5–14.3)
Albumin (g/dL), median (range)	3.5 (1.9–5.1)
Creatinine (mg/dL), median (range)	0.8 (0.3–12.7)

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