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FLUORESCENCE IN SITU HYBRIDIZATION DETECTION OF CYTOGENETIC ABNORMALITIES AND PROGNOSIS IN MULTIPLE MYELOMA

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Abstract: We evaluated the prognosis of patients with newly diagnosed multiple myeloma (MM) and attempted to find a suitable treatment strategy for them. Interphase fluorescence *in situ* hybridization (FISH) detection was performed on 57 patients with MM. The following probes: IgH, p53, 1q21, RB1, and D13S319 specific for the rearrangements of 14q32, 17p13, 1q21 and 13q14 were used. Fluorescent hybridization signals were observed using an Olympus BX60 epifluorescence microscope equipped with filters for detecting fluoroisothiocyanate (FITC), Texas red, and 4'-6-Diamidino-2-phenylindole (DAPI). Triple color clone-specific images were captured using a Quips XL genetic workstation. The mortalities in patients with moderate prognosis (66.7%) and poor prognosis (50%) were significantly higher compared with that in patients with good prognosis (15%, $P<0.05$). All the patients in good and moderate prognosis groups achieved complete remission (CR)/very good partial remission (VGPR)/partial remission (PR), whereas only half of the cases in the poor prognosis group reached this level. Patients supported by autologous hematopoietic stem-cell transplantation presented CR/PR and long survival. For those with poor prognosis, a proper therapeutic regimen and timely transplantation, especially tandem transplantation, was necessary due to the rapid progression and complications.

Key words: Multiple myeloma; interphase fluorescence in situ hybridization detection; cytogenetic abnormalities; prognosis; autologous hematopoietic stem-cell transplantation

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INTRODUCTION

Multiple myeloma (MM) is a cancer which is manifested by an accumulation of abnormal plasma cells in the bone marrow (Bataille and Harousseau, 1997){Bataille, 1997 #1286}. The overall annual incidence of multiple myeloma is higher in old people (Kyle et al., 2004). It is reported that the incidence has increased slightly over a 56-year period from 1945 to 2001, with this increase being restricted to males (Turesson et al., 1984). Actually, the disease is characterized by a high degree of clinical heterogeneity, with a median survival ranging from a few months to more than 10 years (Barlogie et al., 1989; Kyle, 1994). Currently, studies have demonstrated that plasma cell disorders are associated with recurrent chromosomal abnormalities (Desikan et al., 2000; Zojer et al., 2000).

Fluorescence in situ hybridization (FISH), a cytogenetic technique developed in the early 1980s, can be used to indentify specific chromosomal aberrations (Drach et al., 1995). FISH has been widely used for clinical outcome and prognostic assessment in MM (Langer-Safer et al., 1982; Flactif et al., 1995). Compared with the conventional cytogenetics analysis, FISH shows a rapid detection and a more precise incidence of complex chromosome abnormalities (Tabernero et al., 1996). Metaphase cytogenetic abnormalities of chromosome 13 and 11q, especially the deletions of chromosome 13, have been found to be associated with poor clinical outcome and prognosis in patients with MM (Dewald et al., 1985). Adverse prognostic factors of MM are t(4;14) (p16;q32), t(14;16) (q32;q23), -17p13,-13q14. Patients with t(11;14) display a good prognosis and a long survival, whereas those with neither t(4;14) nor t(11;14) show an median prognosis (Tricot et al., 1995). Although many studies focus on demonstrating the relationship between prognosis and cytogenetic abnormalities, its mechanism has still not been fully elucidated.

In this paper, we have extended FISH analysis to evaluate the prognosis in 57 patients with newly diagnosed MM and to find a suitable treatment strategy for patients with MM in the future. We anticipate this work will improve our understanding of the prognostic impact of cytogenetic abnormalities and individualized treatment strategy.

SUBJECTS AND METHODS

Subjects

All patients gave their informed consent prior to their inclusion in the study, and all human studies were approved by the Third Military Medical University Ethics Committee and performed in accordance with the ethical standards. Fifty-seven subjects received consecutive diagnoses of MM between March 2010 and December 2012. Cases were selected from the Daping Hospital, Third Military Medical University, including 35 men and 22 women. The average age was 62.60 years, ranging from 45 to 83 years. According to the Durie and Salmon staging system, 4 patients presented symptomatic stage I; 3, stage IIA; 1, stage IIB; 30, stage IIIA; and 19, stage IIIB. The distribution of the immunoglobulin isotypes was immunoglobulin A kappa (IgA/kappa) in 5 patients, IgA/lambda in 6 patients, IgG/kappa in 20 patients, IgG/lambda in 10 patients, IgM/kappa in 1 patient, and light chains in 8 patients, whereas 7 patients did not produce any monoclonal component (nonsecretory MM).

FISH analysis

Interphase FISH analysis was performed on 57 patients with MM. Briefly, slides were treated as follows: the slides were dehydrated in an ice-cold ethanol (in 70% ethanol for 2 min, 85% for 2 min, and 100% for 2 min) then air-dried. The cellular DNA was extracted from the marrow and then denatured in 70% formamide/2×standard sodium citrate (SSC) for 5 min, respectively, at 76°C in a Coplin jar. Ten microliters of probe cocktail (4:1 mixture of buffer solution and reference probes) was added to each slide, cover slips added, and sealed with rubber cement. The probes IgH, p53, 1q21, RB1, and D13S319 were specific for the rearrangements of 14q32, 17p13, 1q21 and 13q14, respectively. Slides were placed in a humidified chamber and incubated at 42°C. After 16 h, the cover slip was removed, and slides were washed in 50% formamide (two times), 2×standard sodium citrate (SSC) and NP-40 for 5 min, respectively, at 48°C, and in 70% ethanol for 3 min. The slides were then air-dried in the dark and baked for 10 min at 48°C. Fluorescent hybridization signals were observed using an Olympus BX60 epifluorescence

microscope equipped with appropriate filters for detecting fluoroisothiocyanate (FITC), Texas red, and 4'-6-Diamidino-2-phenylindole (DAPI, Sigma, USA). The triple-color clone-specific images were captured using a Quips XL genetic workstation (Vysis). In the normal interphase cells, probe 1q21 presented 2 red signals; D13S319, 2 red signals; probe RB1, presented 2 green signals; p53, 2 green signals; whereas IGH presented 2 yellow signals. The percentage of cells with abnormal signals more than 5% was considered positive.

Chemotherapy

All the patients received chemotherapy of vincristine (0.4mg/d, d1-4), adriamycin (9mg/d, d5-8), and dexamethasone (10mg-40mg/d,d1-4,9-12,17-20) (VAD regimen). Most of the patients received these as front-line therapy. Patients with good prognosis were treated with thalidomide (200mg/d) when they had achieved a complete remission (CR) or very good partial remission (VGPR) after the chemotherapy with VAD; those with poor prognosis received autologous hematopoietic stem-cell transplantation when they entered a partial remission (PR), and were then treated with interferon or thalidomide; patients with a moderate prognosis were treated with thalidomide after 2 to 4 courses of VAD or received autologous hematopoietic stem-cell transplantation when they had achieved a CR or PR with VAD. Finally, only 6 patients received autologous hematopoietic stem-cell transplantation.

Statistical analysis

Statistical evaluation was performed with the Fisher exact and $\chi 2$. Medium-survival was calculated from the transplantation date by the Kaplan-Meier method. A p-value less than 0.05 was considered statistically significant.

Table 1. Main biological characteristics of the 57 patients; cases were classified as FISH-positive and FISH-negative.

		FISH-positive	FISH-negative	P
	Mortality	11(45.8%)	4(12.1%)	<0.01
	Medium-survival time	11.0 months	23.5 months	0.782
Cause of death	Infection	3 (27.3%)	3 (75.0%)	0.323
	Basic diseases	7 (63.6%)	1 (25.0%)	
	Infection rate at diagnosis	7 (29.2%)	3 (9.1%)	0.106
Immunoglobulin isotypes	IgA/Kappa	1 (20.0%)	4 (80.0%)	0.468
	IgA/Lambda	1 (16.7%)	5 (83.3%)	
	IgG/Kappa	8 (40.0%)	12 (60.0%)	
	IgG/Lambda	6 (60.0%)	4 (40.0%)	
	IgM/Kappa	0 (0.0%)	1 (100.0%)	
	LAM light chains	5 (62.5%)	3 (37.5%)	
	Nonsecretory MM	3 (42.9%)	4 (57.1%)	
Courses	1 course	8 (33.3%)	12 (40.0%)	0.753
	2-3 courses	3 (12.5%)	5 (16.7%)	
	4 courses	13 (54.2%)	13 (43.3%)	
Therapeutic effect	PR	5 (41.7%)	8 (53.3%)	0.188
	CR/VGPR	4 (33.3%)	7 (46.7%)	
	NR	3(25.0%)	0 (0%)	

RESULTS

Biological characteristics of FISH-positive and negative cases

A total of 24 cases were classified as FISH-positive. The median survival time was shorter for FISH-positive patients (11 months) compared with the FISH-negative ones (23.5 months, Table 1). The mortality of FISH-positive cases was 45.8%, which was significantly higher than that of FISH-negative ones (12.1%, P<0.05). Deaths were mainly caused by basic diseases in patients in the FISH-positive group (63.6%), whereas it was caused by infection in the other group (75.0%). It was found that the infection rate at diagnosis in FISH-positive cases was 29.2%, higher than that in the negative ones (9.1%). We also demonstrated that the FISH-negative population completely achieved CR/VGPR/PR. However, 3 cases with no remission (25.0%) were found in FISH-positive patients. As for immunoglobulin isotypes, there was no difference between the two groups nor correlation with genetic abnormalities (P>0.05).

Comparison of patients with different prognosis

Patients were divided into three groups according to the results of FISH analysis: a good prognosis group (40 cases), moderate prognosis group (3 cases) and poor prognosis group (14 cases). FISH-negative and t(11;14) translocation were regarded as good prognosis; RB1 deletion or D13S319 deletion were regarded as moderate prognosis; whereas p53 deletion, 1q21 amplification, IGH (translocation t(4;14) t(14;16)) or multiple abnormalities were regarded as poor prognosis. Patients with good prognosis were treated with thalidomide in the plateau phase after chemotherapy.

As shown in Table 2, there was no difference of the infection rate at diagnosis and during chemotherapy among the three groups (P>0.05). The mortalities in patients with moderate prognosis (66.7%) and poor prognosis (50%) were

Table 2. Comparison of the biological characteristics in patients with good, moderate and poor prognosis after FISH detection.

		Good prognosis group (n, %)	**Moderate prognosis group** (n, %)	**Poor prognosis group** (n, %)	**P**
	Infection rate (first diagnosis)	5 (12.5%)	1 (33.3%)	4(28.6%)	0.213
	Mortality	6 (15%)	2 (66.7%)	7 (50%)	0.01
	Medium-survival time	12.0 months	34 months	17 months	0.397
	Infection	5 (83.3%)	0 (0.0%)	1 (14.3%)	
Cause of death	Basic diseases	1 (16.7%)	2 (100.0%)	5 (71.4%)	0.001
	Loss to follow-up	0 (0.0%)	0 (0.0%)	1 (14.3%)	
	1 course	13 (35.1%)	2 (66.7%)	5 (35.5)	
Courses	2-3 courses	5 (13.5%)	0 (0.0%)	3 (21.4%)	0.817
	4 courses	19 (51.4%)	1 (33.3%)	6 (42.9%)	
	PR	11 (55.0%)	0 (0.0%)	2 (33.3%)	
Therapeutic effect	CR/VGPR	9 (45.0%)	1 (100.0%)	1 (16.7%)	0.013
	NR	0 (0%)	0 (0.0%)	3 (50.0%)	

FISH-negative and t(11;14) translocation were regarded as good prognosis (40 cases); RB1 deletion or D13S319 deletion were regarded as moderate prognosis (3 cases); whereas p53 deletion, 1q21 amplification, IGH (translocation t (4;14) t (14;16)) or multiple abnormalities were regarded as poor prognosis (14 cases).

significantly higher compared with that in patients with good prognosis (15%, P<0.05) (Fig. 1). Death was mostly caused by infection in the good prognosis group (83.3%), while the basic diseases rate in the moderate and poor prognosis groups was 100.0% and 71.4%, respectively. Patients in the good and moderate prognosis groups achieved CR/VGPR/PR, whereas only half of those in the poor prognosis group reached this level. The difference of therapeutic effect on the patients of the three groups was significant (P<0.05). A total of 6 patients received autologous hematopoietic stem-cell transplantation and achieved either CR (3 patients, 50%), or PR (3 patients, 50%). Among them, 4 cases who received tandem transplantation all survived, whereas 1 of the other 2 cases who received single transplantation finally died.

DISCUSSION

In this study, the prognosis factors of MM have been assessed with FISH analysis. Patients in FISH-negative and FISH-positive groups with good prognosis (t(11;14) translocation) exhibited a significantly lower mortality compared to those with moderate and poor prognosis. The mortality of patients with moderate or poor prognosis is mainly due to the diseases. The median-survival period of FISH-negative cases was longer than that of FISH-positive cases, indicating a better prognosis for the FISH-negative cases. Multiple genetic abnormalities, which mean a higher risk of mortality, are more common than single abnormality in FISH-positive cases.

The impact of the main prognostic parameters, such as chromosomal rearrangement and deletion, has also been investigated in previous studies. Translocation t(11;14) was had an incidence of 15-20% by FISH, which is associated with longer survival time compared with other chromosomal abnormalities (Avet-Loiseau et al., 1999; Konigsberg et al., 2000). This indicates that t(11;14) is associated with good prognosis of MM (Fonseca et al., 2002). Although it is only detectable in 5% of MM patients by conventional cytogenetics, the results confirm that t(11;14) means a good prognosis (Sawyer et al., 1995; Fonseca et al., 1998). Besides, the subtype immunoglobulin A (IgA) myeloma and a low beta2-microglobulin level (<4g/L) tend to have a favorable prognostic impact (Gahrton et al., 1995).

Patients with t(4;14) transplantation, 1q21 amplification and p53 deletions present a poor prognosis after high-dose chemotherapy in our study. The t(4;14) transplantation is associated with a shorter event-free survival (EFS) time and overall survival time, indicating a poor prognosis (Wiesmann et al., 1975). The incidence is about 10%, lower than that of t(11;14) (Avet-Loiseau et al., 2002). Nevertheless, MM with t(4;14) transplantation recurred rapidly after high-dose therapy even though CR or VGPR were achieved in more than a half of the cases. Furthermore, t(4;14) translocation is closely related to chromosome13

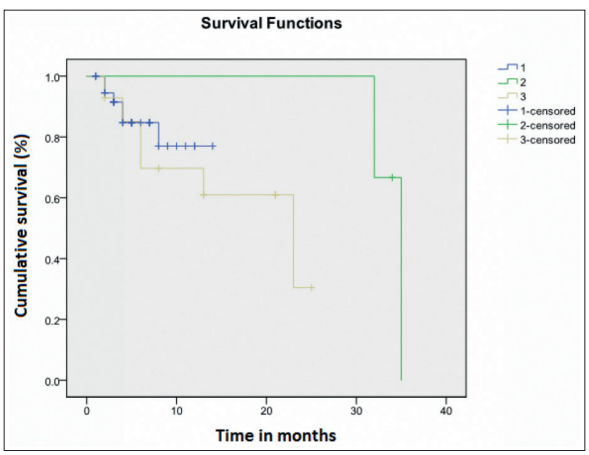

Fig. 1. Kaplan-Meier curves of survival for patients with different prognoses. 1 indicates a good prognosis; 2 – moderate prognosis; 3 – poor prognosis; Log Rank (Mantel-Cox) $\chi 2=2.831$.

abnormalities, of which the partial or complete deletions resulted in poor prognosis (Tricot et al., 1995; Moreau et al., 2002). Amplification of 1q21 in MM is associated with poor survival, which can be reflected by increased expression of the CKS1B gene and proteolysis of the cyclin-dependent kinase inhibitor p27 Kip1.The expression of CKS1B detected by FISH is closely correlated with DNA copy number in a subset of 197 cases (Shaughnessy, 2005). Moreover, a gain of 1q21 copy number and over-expression of CKS1B gene is found to be associated with poor-risk cytogenetic categories such as t(4;14) and chromosome 13 deletion, resulting in a reduced survival (Fonseca et al., 2006). The p53 deletions were associated with higher serum calcium and creatinine levels rather than β2-microglobulin, C-reactive protein, or immunoglobulin isotype (Chang et al., 2005). Unlike 1q21, p53deletions are not associated with 13q deletions or translocations of t(11;14) or t(4;14). It was seen that a p53 deletion is associated with shorter progression-free and survival time compared with those without a deletion, both from the time of diagnosis (Drach et al., 1998). Besides, poor prognosis is also associated with the high proliferative index, which is an indicator of short survival in MM.

We also found that the infection rate is higher in FISH-positive cases than in the negative cases, suggesting a high risk for autologous hematopoietic cell transplantation. Generally, only patients that are FISH-positive in the plateau phase at diagnosis have a long survival due to its non-progression, although they are non-responders (Blade et al., 1986; Joshua et al., 1991). Rapid progression of MM and short overall survival is always associated with genetic abnormalities. Fortunately, patients with MM, who have received autologous hematopoietic stem-cell transplantation present CR (50%) or PR (50%), and usually have a long overall survival. The results are in accordance with previous studies. Patients who have entered CR after the transplant have a longer progression-free and overall survival rate than those who present PR or fail to respond (Gahrton et al., 1995). The occurrence of grade III to IV graft-versus-host disease (GVHD) after transplantation of MM is correlated with poor prognosis and short survival (Ferrara et al., 2009). However, there is no definite correlation between genetic abnormalities and immunoglobulin isotypes. We have not detected the relationship between dysfunction of the kidney and genetic abnormalities in the present study.

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Authors' contributions: X. Zhou participated in the design of this study, and they both performed the statistical analysis. J. Zhao carried out the study, together with Z. Li, collected important background information, and drafted the manuscript. J. Wang conceived of this study, and participated in the design and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest: The authors declare no conflict of interest.

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