Exp Oncol 2007
29, 2, 116–120

Experimental ONCOLOGY

MOLECULAR CYTOGENETIC ABERRATIONS IN PATIENTS WITH MULTIPLE MYELOMA STUDIED BY INTERPHASE FLUORESCENCE *IN SITU* HYBRIDIZATION

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Background: **Multiple myeloma (MM) is an incurable hematological disorder characterized by the accumulation of malignant plasma cells within the bone marrow (BM). The clinical heterogeneity of MM is dictated by the cytogenetic aberrations present in the clonal plasma cells (PCs). Cytogenetic studies in MM are hampered by the hypoproliferative nature of plasma cells in MM. Therefore, fluorescence *in situ* hybridization (FISH) analysis combined with magnetic-activated cell sorting (MACS) is an attractive alternative for evaluation of numerical and structural chromosomal changes in MM.** ***Methods:*** **Interphase FISH studies with three different specific probes for the regions containing 13q14.3 (D13S319), 14q32 (IGHC/IGHV) and 1q12(CEP1) were performed in 48 MM patients. Interphase FISH studies with LSI IGH/CCND1, LSI IGH/FGFR3, and LSI IGH/MAF probes were used to detect t(11;14)(q13;q32), t(4;14)(p16;q32), and t(14;16)(q32;q23) in patients with 14q32 rearrangement.** ***Results:*** **Molecular cytogenetic aberrations were found in 40 (83.3%) of the 48 MM patients. 13 patients (27.1%) simultaneously had 13q deletion/monosomy 13 [del(13q14)], illegitimate IGH rearrangement and chromosome 1 abnormality. Del(13q14) was detected in 21 cases (43.7%), and illegitimate IGH rearrangements in 29 (60.4%) including 6 with t(11;14) and 5 with t(4;14). None of 9 patients with illegitimate IGH rearrangements and without t(11;14) or t(4;14) we detected had t(14;16) (q32;q23). 24 of the 48 MM patients (50%) had chromosome 1 abnormalities. Among 21 patients with del(13q14), 15 patients had Amp1q12;16 had IgH rearrangements. Whereas, among 27 cases without del(13q14), 8 had Amp1q12; 13 had IgH rearrangements. There was a strong association between del(13q14) and Amp1q12($\chi^2 = 8.26$, $p < 0.01$), and between del(13q14) and IgH rearrangement($\chi^2 = 3.88$, $p < 0.05$).** ***Conclusion:*** **13q deletion/monosomy 13, IGH rearrangement and chromosome 1 abnormality are frequent in MM. They are not randomly distributed, but strongly interconnected. Interphase FISH technique combined with MACS using CD138-specific antibody is a highly sensitive technique at detecting molecular cytogenetic aberrations in MM.**

Key Words: **fluorescence *in situ* hybridization, multiple myeloma, cytogenetic abnormality.**

Multiple myeloma (MM) is a terminally differentiated clonal B-cell neoplasm characterized by the accumulation of malignant plasma cells (PCs) within the bone marrow (BM). Its prognosis is highly variable, with survival ranging from a few days to more than 10 years [1, 2]. A median survival of about 3 years was obtained with conventional chemotherapy. Despite improvements in the clinical management of patients in the past decade, especially with the use of high-dose therapy followed by autologous stem cell transplantation [3] and with the use of new drugs such as thalidomide, lenalidomide and proteasome inhibitors [4–6], MM remains incurable. Therefore, it appears essential, at diagnosis, to recognize clinical or biological parameters predicting the outcome and identifying patients for whom an aggressive therapy would be indicated. Molecular cytogenetic studies have revealed that, to a great extent, the clinical heterogeneity of MM is dictated by the cytogenetic aberrations present in the clonal plasma cells. Karyotypic deletion of chromosome 13, hypodiploidy, and molecular cytogenetics are specific independent prognostic factors for accurate risk stratification in MM [7].

Received: May 9, 2007.
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Abbreviations used: BM – bone marrow; FISH – fluorescence *in situ* hybridization; MACS – magnetic-activated cell sorting; MM – multiple myeloma; PCs – plasma cells.

Although there are many reports that cytogenetic changes are associated with prognosis, cytogenetic studies in MM are hampered by the hypoproliferative nature of clonal PCs in MM. Abnormal karyotypes have been reported only in 30–45% of *de novo* cases, and 35–60% in previously treated and relapsing patients [8]. Fluorescence *in situ* hybridization (FISH) overcomes the limitations of standard cytogenetics and allows for the detection of numerical and structural chromosomal abnormalities in both metaphase spreads and interphase nuclei. Thus, FISH is an attractive alternative for evaluation of chromosomal aberrations of MM. In contrast with the incidence of abnormal karyotypes detected in MM with conventional cytogenetics, studies using FISH techniques have identified chromosome changes in BM PCs in more than 80% of the patients [9, 10]. Since PCs are low in BM samples of patients with MM, we enriched $CD138^+$ myeloma cells by magnetic-activated cell sorting (MACS) to improve the sensitivity of the interphase FISH method.

Here we investigated the most prevalent genetic changes in patients with MM. We performed interphase FISH using 6 probes that have been reportedly related to MM and studied BM samples from 48 newly diagnosed patients. We suggested that the detection of at least these three genetic changes, 13q14 deletion, illegitimate IGH rearrangements, and Amp1q12, would be helpful for patients with MM.

MATERIALS AND METHODS

Patients and BM samples. We studied 48 newly diagnosed and untreated MM patients. BM samples of patients with MM were obtained at diagnosis and under informed consent, and enriched for mononuclear cells using the Ficoll-gradient centrifugation method. Myeloma cells were enriched by MACS using the CD138-specific monoclonal antibody B-B4. $CD138^+$ cell suspensions fixed in methanol/acetic acid (3 : I) and stored at –20 ˚C.

Interphase fluorescence in situ hybridization. Slides were treated with 100 µg/ml RNAse for 30 min at 37 ˚C followed by 2 × SSC washing for 5 min × 2 and treated with 0.005% pepsin for 5 min at 37 ˚C, then washed twice for 5 min each in phosphate buffered saline (PBS) and dehydrated in increasing concentrations of ethanol (70, 85 and 100%) at room temperature for 1 min in each solution. The slides were denatured in a 70% formamide solution at 72 ˚C for 2 min, dehydrated in an ethanol series and air-dried. Probes (3 µl) were mixed well with hybridization buffer (5 µl) and denatured at 72 ˚C for 5 min. Probes were applied immediately to slides and hybridized at 37 ˚C overnight. After hybridization, slides were washed at 72 ˚C for 2 min in 0.4 × SSC/0.3% NP-40 and in 2 × SSC/0. 1% NP-40 for 1 min at room temperature. Slides were then air-dried and mounted using 10 µl of 4',6'-diamidino-2-phenylindole (DAPI II) (Vysis, Downers Grove, USA) counterstain for 1 h.

To detect a deletion on the long arm of chromosome 13, we used D13S319 SpectrumGreen probe. To determine the translocations involving IGH, we used the LSI IGH/IGHV dual-color, break-apart rearrangement probe; the LSI IGH/CCND1 dual-color, dual-fusion translocation probe; the LSI IGH/FGFR3 dual-color, dual-fusion translocation probe; the LSI IGH/MAF dual-color, dual-fusion translocation probe. To detect amplifications of 1q, we used the CEP 1 SpectrumOrange Probe at 1q12. All probes purchased from Vysis, USA.

Fluorescent images were captured with epifluorescence microscope (Leica DRMA2, Germany) equipped with CCD camera (AI company, UK), and using appropriate filters. Five hundred nuclei were analyzed for each probe. Chromosome 13 deletions were identified with only one signal in interphase cells using D13S319 probe. Rearrangements of the 14q32 region were determined by means of a dual-color FISH assay. Our strategy was based on identifying the split and translocation of these sequences on interphase nuclei. We first looked for illegitimate IgH rearrangements with separate signals using 14q32 (IGHC/IGHV) probe, which mapping at the centromeric and telomeric borders of the IgH locus were labeled with SpectrumGreen and SpectrumOrange, respectively. Then using LSI IGH/CCND1, LSI IGH/FGFR3 and LSI IGH/MAF probes, we detected fused signals in patients with 14q32 rearrangements. Amplifications of 1q were identified with more than two signals in interphase cells.

BM cells samples of 8 cytogenetically normal persons were used as normal controls. The cutoff levels for positive values for each probe in I-FISH, which were set at the mean of normal controls plus three standard deviations, were as follows: del(13q14), 10%; IgH rearrangements (IGHC/IGHV), 8.9%; t(11;14), 9.1%; t(4;14), 5%; t(14;16) 6.5% and Amp1q12, 5%.

Statistical analysis. χ^2 or Fisher's exact tests were used for between-group comparison of the discrete variables.

RESULTS

Characteristics of the patients. 48 newly diagnosed and untreated MM patients were studied. There were 33 males and 15 females (the male to female ratio was 2.2 to 1), with a median age of 65 (range 45~77) years. Out of 48 patients, the types of M-protein were IgGκ in 21, IgGλ in 11, IgAκ in 9, IgAλ in 5, and only kappa light chain in 2 patients. According to Durie and Salmon staging [11], 9 were stage I, 16 were stage II, and 23 were stage III. According to International Staging System [12], 22 were stage I, 16 were stage II, and 10 were stage III.

Interphase FISH studies. The patients with at least one of the three frequent molecular cytogenetic aberrations, del(13q14), IGH rearrangement, and Amp1q12 were 40 (83.3%) of the 48 MM patients. Of these 48 patients, 13 (27.1%) had all three abnormalities. 21 (43.7%) showed evidence of del(13q14). The median number of PCs with deletions was 88% (range 27~94%). Rearrangements involving 14q32 region were the most common structural abnormalities, found in 29 (60.4%) patients and 6 (20.7%) of these corresponded to a t(11;14)(q13;q32). Another partner 4p16 was identified in 5 (17.2%) cases. Of 9 patients with IGH rearrangement and without t(11;14) or t(4;14) we detected, none had t(14;16)(q32;q23). 24 of the 48 MM patients (50%) had abnormalities in chromosome 1 : 1 with 1 copy of 1q12, and 23 with at least 3 copies of 1q12 (Amp1q12). Among 21 patients with del(13q14), 15 had Amp1q12, and 16 had IgH rearrangement. Whereas, among 27 cases without del(13q14), 8 had Amp1q12, and 13 had IgH rearrangement. There was a strong association between del(13q14) and Amp1q12 ($\chi^2 = 8.26$, $p < 0.01$), and between del(13q14) and IgH rearrangement ($\chi^2 = 3.88$, $p < 0.05$).

The clinical data and FISH results of 48 patients with MM were presented in Table.

Correlation of these three frequent molecular cytogenetic aberrations with Durie and Salmon staging, International Staging System and type of paraprotein. There was no correlation between del(13q14), illegitimate IGH rearrangement, or Amp1q12 and Durie and Salmon staging, International Staging System, or type of paraprotein ($p > 0.05$).

DISCUSSION

MM cells are characterized by high genetic instability, resulting in a complex set of numerical and structural chromosomal abnormalities [13]. The detection of genetic changes is important, not only because of their association with clinical prognosis, but also because they can be used as measurable targets for response to treatment. The sensitivity of detection of genetic changes depends on the methods used. Owing to low ratio of PCs in BM samples and the low prolifera-

Table. The clinical data and FISH results of 48 patients with MM

No	Age (years)	age	D – S*	ISS	Isotype	D13S319$^{\diamond}$	14q32# (IGHC/IGHV)	IGH/CCND1	IGH/FGFR3	IGH/MAF	CEP1**
1	F	50	I	I	IgAκ	no	R	yes	no		2
2	M	56	I	I	κ	no	R	yes	no		2
3	M	67	II	I	IgAκ	89% yes	R	yes	no		3
4	M	69	III	III	IgAλ	no	R	yes	no		2
5	M	72	III	I	IgGκ	no	R	yes	no		2
6	F	67	III	II	IgAλ	no	R	yes	no		2
7	M	65	II	I	IgGκ	64% yes	R	no	yes		3
8	M	66	II	I	IgGκ	89% yes	R	no	yes		3
9	F	56	I	II	IgGκ	no	R	no	yes		2
10	M	59	II	II	IgGλ	no	R	no	yes		2
11	M	65	III	III	IgGκ	92% yes	R	no	yes		3
12	F	48	I	I	IgAλ	no	G	no	no		2
13	M	55	I	I	IgGκ	no	G	no	no		2
14	M	65	II	I	IgGκ	no	G	no	no		2
15	F	59	II	I	IgGκ	74% yes	G	no	no		3
16	M	45	III	I	IgGλ	no	G	no	no		2
17	M	75	III	I	IgAκ	no	G	no	no		3
18	F	65	III	I	IgGκ	no	G	no	no		3
19	M	68	I	II	IgGκ	no	G	no	no		3
20	M	71	II	II	IgAκ	no	G	no	no		2
21	F	65	II	II	IgGκ	93% yes	G	no	no		2
22	M	63	III	II	IgGκ	no	G	no	no		1
23	F	64	III	II	IgGκ	no	G	no	no		2
24	M	66	III	II	IgGκ	no	G	no	no		2
25	M	70	III	II	IgGλ	no	G	no	no		3–4
26	M	75	III	II	IgGλ	27% yes	G	no	no		2
27	M	72	I	III	IgGλ	56% yes	G	no	no		3
28	M	63	III	III	IgGκ	no	G	no	no		2
29	M	50	III	III	IgAκ	no	G	no	no		3
30	F	61	III	III	IgGκ	88% yes	G	no	no		2
31	F	62	I	I	IgGλ	78% yes	R	no	no		3
32	M	58	I	I	IgAλ	82% yes	R	no	no	no	3–4
33	M	51	II	I	IgAκ	no	R	no	no	no	3
34	F	58	II	I	IgGκ	70% yes	R	no	no		2
35	F	76	II	I	IgAλ	93% yes	R	no	no	no	3
36	F	66	III	I	IgAκ	no	R	no	no		3
37	F	57	III	I	κ	28% yes	R	no	no		2
38	M	52	III	I	IgGλ	88% yes	R	no	no		3
39	M	68	III	I	IgGλ	94% yes	R	no	no	no	3
40	M	70	II	II	IgAκ	no	R	no	no		2
41	M	74	II	II	IgGκ	no	R	no	no		2
42	F	71	II	II	IgGκ	64% yes	R	no	no	no	3
43	M	77	III	II	IgGκ	65% yes	R	no	no		4
44	M	73	III	II	IgGλ	88% yes	R	no	no	no	3
45	M	69	II	III	IgGλ	90% yes	R	no	no		3
46	M	67	II	III	IgGλ	94% yes	R	no	no	no	2
47	M	58	III	III	IgGκ	no	R	no	no	no	2
48	M	68	III	III	IgAκ	no	R	no	no	no	4

Notes: *D – S: Durie and Salmon staging; $^{\diamond}$percentage of interphase nuclei with one signal and interpretation of findings where "no" indicates absence of deletion, "yes" indicates a deletion is present; #R: 14q32 rearrangement; G: "germ"(without 14q32 rearrangement); **the number of signals of chromosome 1q12.

tive activity of PCs, it is difficult to detect cytogenetic changes by conventional R-banding methods [14]. We enriched CD138^{+} myeloma cells by MACS to improve the sensitivity of the interphase FISH method.

Recent studies based on molecular cytogenetic methods have shown that virtually all MM patients have chromosomal abnormalities in their plasma cells [15]. Common genetic changes include 13q deletion/monosomy 13, IGH rearrangement, chromosome 1 abnormality, hyperdiploidy, hypodiploidy, 17p13 deletion, 11q deletion, t(11;14), t(4;14), and trisomy 12 [16–18]. In this study, we have studied three prevalent genetic changes: 13q deletion/monosomy 13, IGH rearrangement and Amp1q12 in 48 patients with MM by I-FISH and detected these aberrations in 83.3% of MM patients.

Deletion of 13q/monosomy 13 is common in MM. Deletions of 13q14 have been detected in 30–50% of MM patient samples by interphase FISH studies, which have been seen as a powerful adverse prognostic factor in MM patients treated with high-dose chemotherapy and stem cell support [19, 20]. In our study, FISH analysis of the 13q14 region was performed on immuno-magnetically selected plasma cells. We detected del(13q14) in 21 (43.7%) of the 48 MM patients with D13S319 probe. The prevalence of the del(13q14) is similar to that most other investigators reported using interphase FISH. However, it is lower than that reported by Fiserova[13] [13] using interphase FISH on purified PCs. PCs that scored positive with this deletion ranging from 27~94% are similar to that reported by Chang (ranging from 11~85%) [21]. We analyzed concurrently the correlation between del(13q14) and Durie and Salmon staging, International Staging System,or type of paraprotein. However, there was no association between them.

Chromosomal abnormalities of 14q32 are the most frequent chromosomal abnormalities, which have been observed in about 75% of patients with a plasma cell malignancy and have been associated in the oncogenesis of MM [18]. Five recurrent chromosomal partners (oncogenes) are involved in IgH translocations in MM: 4p16 (MMSET and usually FGFR3), 6p21 (cyclin D3), 11q13 (cyclin D1), 16q23 (c-MAF), and 20q11 (MAFB). Together, the combined prevalence of these five IgH translocation partners is about 40% in MM, with approximately 15% 4p16, 3% 6p21, 15% 11q13, 5% 16q23, and 2% 20q11 [22, 23]. t(4;14) and t(14;16) are poor prognosis factors [24]. In our series, IGH rearrangements were found in 29 of 48 (60.4%) MM patients with different partner chromosomes: 11q13 (CCND1) (6/29, 20.7%), 4 p16(FGFR3)(5/29,17.2%) and other partners(18/29,62.1%). In our study, we found that there was no correlation between IGH rearrangement and Durie and Salmon staging, International Staging System, or type of paraprotein.

14q32 translocations and del(13q14) are not randomly distributed [25]. Avet-Loiseau et al. [26] defined 4 major genetic categories of patients according to the correlations between them: (1) patients lacking any 14q32 abnormality (25%) and generally also lacking del(13q14); (2) patients presenting either t(4;14) or t(14;16), almost always associated with a del(13q14) (15% of patients); (3) patients with other 14q32 abnormalities and presenting del(13q14) (25%); and (4) patients with other 14q32 abnormalities but not presenting del(13q14) (35%). The strong correlation might be the basis for a novel genetic classification of MM because this genetic stratification is highly associated with immunological status and clinical presentation and with some major prognostic factors and supports different models for MM oncogenesis. In our study, of 48 patients with MM, the number of the 4 major genetic categories was 19 (39.6%), 5 (10.4%), 13 (27.1%), and 11 (22.9%), respectively. Among 19 without illegitimate IGH rearrangements, only 5 harbored del(13q14). However, among 29 with illegitimate IGH rearrangements, 16 had del(13q14). Therefore, our study demonstrates that the 2 most frequent cytogenetic abnormalities, 14q32 translocations and 13q deletions, are strongly interconnected. We then analyzed

the incidence of del(13q14) in each 14q32 category of MM. 60% (3/5) with t(4;14) displayed del(13q14).This percentage was dramatically higher than that observed in t(11;14) MM patients, of which only 16.7% (1/6) harbored del(13q14) concurrently. However, there was no significant difference between del(13q14) incidence in t(4;14) MM patients and the incidence observed in the overall population (60% versus 41.9%, $p > 0.05$).

Chromosome 1 instability is also common structural abnormality, and plays an important role in the pathogenesis of MM. Chromosome 1 aberrations are frequently described, the short arm being preferentially involved in deletions and the long arm in gains. It was reported that abnormalities of chromosome 1p and 1q were found in 36% and 40% of patients with an abnormal karyotype [27]. J.D. Shaughnessy et al [28] performed microarray analysis on tumor cells from 532 newly diagnosed patients with MM. They found 70 genes, 30% mapping to chromosome 1, were linked to early disease-related death. Importantly, most up-regulated genes mapped to chromosome 1q, and down-regulated genes mapped to chromosome 1p, and concluded that altered transcriptional regulation of genes mapping to chromosome 1 may contribute to disease progression. In our study, we choose CEP 1 SpectrumOrange probe to detect the chromosome 1q aberration in 48 patients with MM. 47.9% of MM had amplification of chromosome 1q. Amp1q12 was not significantly associated with clinical staging and the types of paraprotein. It is reported that gain of 1q is also associated with t(4;14) and chromosome 13 deletion but not t(11;14). We found that Amp1q12 was significantly associated with del(13q14), but not with t(11;14) or t(4;14).

In summary, our study illustrates that 13q deletion/monosomy 13, IGH rearrangement and chromosome 1 abnormality are frequent in MM. They are not randomly distributed, but strongly interconnected. The correlation of them with the clinical prognosis should be studied further. Interphase FISH technique combined with MACS using CD138-specific antibody is a highly sensitive technique at detecting molecular cytogenetic aberrations and should be used in the routine evaluation of MM.

ACKNOWLEDGEMENT

This study was supported by the Foundation of Social Development of Jiangsu Province (BS2006071) and 135 Foundation of Jiangsu Province (RC2002044, WK200210/135XY0201).

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МОЛЕКУЛЯРНЫЕ ЦИТОГЕНЕТИЧЕСКИЕ АБЕРРАЦИИ У БОЛЬНЫХ МНОЖЕСТВЕННОЙ МИЕЛОМОЙ, ИЗУЧЕННЫЕ МЕТОДОМ ИНТЕРФАЗНОЙ ФЛУОРЕСЦЕНТНОЙ ГИБРИДИЗАЦИИ *IN SITU*

Обоснование: **множественная миелома (ММ) — неизлечимое гематологическое заболевание, характеризирующееся накоплением злокачественных плазматических клеток в костном мозге (КМ). Клиническая гетерогенность ММ определяется цитогенетическими аберрациями, присутствующими в клоне плазматических клеток (ПК). Цитогенетические исследования ММ осложнены гипопролиферативными особенностями ПК. В связи с этим флуоресцентная гибридизация *in situ* (FISH) в комбинации с сортировкой клеток, активированных магнитными полями (MACS) представляется достойной альтернативой методам оценки точечных и структурных изменений хромосом при ММ.** ***Методы:*** **интерфазные исследования методом FISH с использованием трех различных специфических зондов для участков, содержащих 13q14.3 (D13S319), 14q32 (IGHC/IGHV) и 1q12(CEP1), проводили у 48 больных с ММ. Интерфазные исследования методом FISH с использованием зондов LSI IGH/CCND1, LSI IGH/FGFR3 и LSI IGH/MAF применяли для детекции t(11;14)(q13;q32), t(4;14)(p16;q32), и t(14;16)(q32;q23) у пациентов с перестройкой 14q32.** ***Результаты:*** **молекулярные цитогенетические аберрации выявляли у 40 (83,3%) из 48 больных с ММ. У 13 пациентов (27,1%) одновременно определены 13q делеция/моносомия 13 [del(13q14)], аномальная перестройка IGH и аномалия хромосомы 1. Del(13q14) детектировали в 21 случае (43,7%), а аномальные перестройки IGH — в 29 (60,4%), в том числе у 6 пациентов с t(11;14) и 5 с t(4;14). Ни у одного из 9 больных с аномальными перестройками IGH и без t(11;14) или t(4;14) не выявляли транслокацию t(14;16) (q32;q23). У 24 из 48 пациентов с ММ (50%) определяли аномалии хромосомы 1. В группе из 21 больных с del(13q14) в 15 случаях имелись перестройки IgH Amp1q12;16. В то же время из 27 случаев без del(13q14) у 8 содержались Amp1q12; в 13 случаях отмечали перестройки IgH. Выявлена взаимосвязь между del(13q14) и Amp1q12($\chi^2 = 8,26$, $p < 0,01$) и между del(13q14) и перестройками IgH ($\chi^2 = 3,88$, $p < 0,05$).** ***Выводы:*** **13q делецию/моносомию 13, перестройку IGH и аномалию хромосомы 1 часто отмечают при ММ, причем их распределение не случайно и тесно взаимосвязано. Интерфазный анализ FISH в комбинации с MACS с использованием CD138-специфичных антител является высокочувствительным методом детекции молекулярных цитогенетических аберраций при ММ.**

Ключевые слова: **флуоресцентная гибридизация *in situ*, множественная миелома, цитогенетическая аномалия.**