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Evaluation of chromosomal abnormalities by cIg-FISH and association with proliferative and apoptotic indexes in multiple myeloma

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Abstract

Eighty-six newly diagnosed multiple myeloma (MM) patients from a public hospital of São Paulo (Brazil) were evaluated by cIg-FISH for the presence of del(13)(q14), t(4;14)(p16.3;q32) and del(17)(p13). These abnormalities were observed in 46.5, 9.3, and 7.0% of the patients, respectively. In order to identify the possible role of del(13)(q14) in the physiopathology of MM, we investigated the association between this abnormality and the proliferative and apoptotic indexes of plasma cells. When cases demonstrating t(4;14)(p16.3;q32) and del(17)(p13) were excluded from the analysis, we observed a trend towards a positive correlation between the proportion of cells carrying del(13)(q14) and plasma cell proliferation, determined by Ki-67 expression (r = 0.23, P = 0.06). On the other hand, no correlation between the proportion of cells carrying del(13)(q14) and apoptosis, determined by annexin-V staining, was detected (r = 0.05, P = 0.69). In general, patients carrying del(13)(q14) did not have lower survival than patients without del(13)(q14) (P = 0.15), but patients with more than 80% of cells carrying del(13)(q14) showed a lower overall survival (P = 0.033). These results suggest that, when del(13)(q14) is observed in a high proportion of malignant cells, it may have a role in determining MM prognosis. Another finding was a statistically significant lower overall survival of patients with t(4;14)(p16.3;q32) (P = 0.026). In the present study, almost half the patients with t(4;14)(p16.3;q32) died just after diagnosis, before starting treatment. This fact suggests that, in São Paulo, there may be even more patients with this chromosomal abnormality, but they probably die before being diagnosed due to unfavorable socioeconomic conditions. This could explain the low prevalence of this chromosomal abnormality observed in the present study.

Key words: Multiple myeloma; Cell proliferation; Apoptosis; Prognosis; Fluorescence *in situ* hybridization

Introduction

Despite recent progress in the management of multiple myeloma (MM), this neoplasm remains incurable and is characterized by a heterogeneous prognosis. The variable prognosis of the disease involves interaction between intrinsic features of the disease biology such as chromosomal abnormalities and host factors. Del(13)(q14) is detected in almost 50% of cases and is associated with lower survival only when other high-risk genetic features such as del(17)(p13) and t(4;14)(p16.3;q32) are present (1,2). However, this does not mean that del(13)(q14) has no biological importance since data suggest that this abnormality is a prerequisite for clonal expansion (1,2).

The aim of the present study was to determine the prevalence of the most relevant chromosomal abnormalities and their impact on prognosis in a group of MM patients from a public hospital in São Paulo. Although these abnormalities have been studied extensively, there are few studies focusing on their prevalence in developing countries (3-5). We also investigated the association between del(13)(q14) and the proliferative and apoptotic indexes of bone marrow plasma cells (PC) in order to study the possible role of this abnormality in the biology of the disease.

Patients and Methods

Patients

Bone marrow aspirates from 92 recently diagnosed

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MM patients were obtained with a heparin-coated syringe. All patients were treated at Divisão de Hematologia, Hospital das Clínicas, Universidade de São Paulo, and were diagnosed consecutively between February 2007 and April 2011. The Institutional Ethics Review Board approved the study (protocol No. 016/06) and written informed consent was obtained from all patients allowing the specimen to be used for research purposes.

Patients received conventional dose induction chemotherapy consisting of a melphalan- or dexamethasone-based ± thalidomide regimen (6). Fourteen patients received high-dose chemotherapy followed by autologous peripheral blood stem-cell support (ASCT) (6). The median follow-up was 37.3 months (0-54.8 months).

Fluorescence *in situ* hybridization (FISH) studies

Cytospin slides were prepared after enrichment of mononuclear cells, fixed in 95% ethanol for 5 min at room temperature and stored at -20°C for future use. We used cIg-FISH with light chain-specific immunofluorescent detection of clonal PC (7). Del(13)(q14) was determined with a retinoblastoma gene-1 LSI RB1 probe (Vysis, USA), del(17)(p13) with an LSI p53 probe (Vysis) combined with a chromosome 17 centromeric probe (Vysis), and t(4;14)(p16.3;q32) with an LSI IGH/FGFR3 probe (Vysis). Two independent observers read blindly and independently each slide, counting a total of 200 nuclei per sample. The cut-off values (fusion probe = 10%, numerical abnormalities = 20%) adopted were in accordance with the recommendations of the European Myeloma Network FISH workshop (8).

Flow cytometry

Monoclonal antibodies (MoAb) anti-CD138-PE (DAKO, Denmark), anti-CD38-APC (eBioscience, USA) and anti-CD45-PC5 (Immunotech-Beckman Coulter, France) were used to identify bone marrow PC. To detect proliferative cells, membrane cells were permeabilized with saponin and cells were stained with the anti-Ki-67 MoAb (Ki-67 FITC, Pharmingen, USA) (9). The Annexin-V-FITC kit (DAKO, The Netherlands) was used to detect phosphatidylserine expression on the surface of apoptotic cells after enrichment of mononuclear cells using the Ficoll-gradient centrifugation method. All acquisitions were performed using a FACSCalibur flow cytometer (BD Biosciences, USA) and the CellQuest program (BD Biosciences) and at least 2 x 10^3 PC/tube were recorded.

Statistical analysis

Differences among groups were determined using the Fisher exact test for categorical variables. Differences among groups were determined using the Kruskal-Wallis test for continuous variables. Correlations between continuous variables were investigated using the nonparametric Spearman test.

Overall survival (OS) was calculated from the time of diagnosis to death from any cause or last contact using the Kaplan-Meier method. Event-free survival (EFS) was calculated from the time of diagnosis to progression, loss of response or death from any cause, or last contact, using the Kaplan-Meier method. The survival curves were compared by the log-rank test. All P values were 2-tailed and the alpha error was defined as 5%. The statistical analyses were performed using the Stata Statistical Software, Release 11 (StataCorp LP, USA).

Results

Characteristics of patients at diagnosis

The median age of the 92 patients was 63.5 years (range = 36-93); 51.2% were males.

Sixty-four percent of patients had an IgG paraprotein, 20.2% had an IgA paraprotein, 12.3% had light chains only (kappa = 5.6% and lambda = 6.7%), and 3.4% were non-secretory. Fifty percent of patients were classified as International Staging System (ISS) (10) stage III, 36.6% as stage II, and 13.4% as stage I. The clinical and laboratory features of the patients are summarized in Table 1.

Prevalence of chromosomal abnormalities

The chromosomal abnormalities t(4;14)(p16.3;q32), del(17)(p13) and del(13)(q14) were studied in 86 patients and were detected in 8 (9.3%), 6 (7%), and 40 (46.5%) of them, respectively. There were no significant differences in age or gender between the different groups. The median age of patients with t(4;14)(p16.3;q32), del(17)(p13), and with only del(13)(q14) was 59 (range = 54-73), 57.5 (range = 43-76), and 65 (range = 36-79), respectively. Forty patients with a median age of 63.5 years (range = 36-93) did not have any of the abnormalities evaluated in this study. In 6 of 92 patients, it was not possible to study the different chromosomal abnormalities due to hybridization failure.

In del(13)(q14)-positive cases, the median number of PC with this abnormality was 75% (range = 28-98%). In t(4;14)(p16.3;q32)-positive cases, the median number of PC with this abnormality was 84% (range = 76-94%), and 6 of these patients also had del(13)(q14). In del(17)(p13)-positive cases, the median number of PC with this abnormality was 35% (range = 21-76%) and 2 of these patients also had del(13)(q14).

The majority of patients (84.8%) were treated just with conventional doses of chemotherapy. Only 14 patients received ASCT: 8 of them with at least one of the abnormalities studied, and 6 of them without any of these chromosomal alterations. The characteristics of patients according to their chromosomal abnormalities are described in Table 1.

Del(13)(q14) versus PC proliferation and apoptosis

Ki-67 MoAb staining was performed in 78 patients. The

percentage of Ki-67-positive PC ranged from 0 to 26% (median: 3.2%). Annexin-V staining was carried out in 69 cases and the percentage of annexin-V-positive PC ranged from 0 to 55% (median: 8.1%). The percentage of Ki-67 expression and annexin-V labeling in PC according to clinical and laboratory characteristics is described in Table 2.

When the correlation between the percentage of PC carrying del(13)(q14) and proliferation was calculated, a weak correlation was found between Ki-67 expression and the proportion of cells carrying del(13)(q14) (r = 0.22, P = 0.058). This finding persisted even when all cases with additional t(4;14)(p16.3;q32) and del(17)(p13) were excluded in order to examine only the effect of del(13)(q14) on Ki-67 expression (r = 0.23, P = 0.06).

A cut-off of 8% was used for Ki-67 expression (11,12). Based on the ROC curve, we determined that the best threshold for the percentage of PC with del(13)(q14) that was associated with Ki-67 above 8% was greater than 75%. Indeed, whereas 54.5% of the cases with del(13)(q14) in at least 80% of their PC showed Ki-67 expression above 8%, only 4% of patients carrying del(13)(q14) in less than 80% of their PC showed Ki-67 expression above 8% (P < 0.001).

No correlation was detected between del(13)(q14) and annexin-V staining (r = 0.05, P = 0.69).

Due to the small number of cases positive for t(4;14) (p16.3;q32) (N = 8) or del(17)(p13) (N = 6) in our sample, no attempt was made to establish a correlation between proliferative and apoptotic indexes and the proportion of cells with these abnormalities.

Survival analysis

The overall survival from diagnosis was analyzed, and a statistically significant difference was observed between patients with t(4;14)(p16.3;q32) and all other patients (estimated 3-year OS of 25 *vs* 62.2%, respectively, P =

Table 1. Characteristics of patients according to chromosomal abnormalities.

	All patients (N = 92)	Normal [normal 13q14 and 17p13, and absence of t(4;14)] (N = 40)	del(13)(q14) [normal 17p13 and absence of t(4;14)] (N = 32)	t(4;14) (N = 8)	del(17)(p13) (N = 6)
Age (years)					
Median (range)	63.5 (36-93)	63.5 (36-93)	65 (36-79)	59 (54-73)	57.5 (43-76)
ISS					
I/II	50%	57.1%	46.4%	28.6%	66.7%
III	50%	42.9%	53.6%	71.4%	33.3%
Hemoglobin					
Median (range), g/dL	9.0 (4.4-16.1)	9.2 (5.6-13.4)	8.35 (4.4-16.1)	7.8 (6.8-12.7)	11.2 (9.1-13.0)
<10.0 g/dL	64%	60%	68.8%	87.5%	33.3%
Creatinine					
Median (range), mg/dL	1.2 (0.38-15.6)	1.2 (0.56-12.2)	1.3 (0.38-15.6)	1.6 (0.81-5.2)	1.2 (0.7-2.4)
≥2.0 mg/dL	29.1%	25%	34.4%	37.5%	16.7%
Calcium					
Median (range), mg/dL	9.9 (7.7-15.6)	9.9 (7.7-15.1)	10.0 (8.7-13.5)	12.2 (8.9-15.6)	9.5 (8.8-11.3)
≥10.5 mg/dL	41.6%	38.9%	42.9%	62.5%	20%
Albumin					
Median (range), mg/dL	3.6 (1.7-5.1)	3.7 (2.3-4.6)	3.5 (1.7-4.8)	3.4 (2.6-3.8)	3.6 (3.2-5.1)
<3.5 g/dL	43%	32.5%	50%	62.5%	50%
β_2-microglobulin					
Median (range), µg/mL	5.5 (1.8-87)	4.9 (1.8-23)	6 (1.9-87)	10.5 (2.5-18.7)	4.3 (3-7.6)
≥3.5 µg/mL	79%	77.1%	82.1%	85.7%	66.7%
Lactate dehydrogenase					
Median (range), U/L	338 (135-966)	320 (135-966)	346 (192-698)	408 (161-599)	313 (180-433)
Above normal (>480 U/L)	12.5%	7.7%	17.9%	28.6%	0%
Reactive C protein					
Median (range), mg/L	10.9 (0.36-414)	12.2 (1.3-260)	10.6 (0.88-118)	36.3 (1.02-101)	3.9 (0.57-304)
Above normal (>3.0 mg/L)	74.2%	83.3%	66.7%	83.3%	50%

It was not possible to study the chromosomal abnormalities in 6 of 92 patients due to hybridization failure. ISS = International Staging System (10). There were no statistical differences among groups in continuous variables (P > 0.05, Kruskal-Wallis test).

0.026). Del(17)(p13) was not associated with a lower estimated 3-year OS (P = 0.27). When cases with t(4;14)(p16.3;q32) and del(17)(p13) were excluded from the analysis, del(13)(q14) was also not associated with a lower estimated 3-year OS (P = 0.15; Figure 1A).

As cases with at least 80% of cells affected by del(13)(q14) showed a higher proliferative index, we compared the OS between patients carrying this abnormality in more and in less than 80% of PC. We observed that patients with more than 80% of cells affected by del(13)(q14) had a shorter median OS than patients with less than 80% of cells affected by this abnormality (estimated 3-year OS of 27.8 *vs* 67.6%, respectively, P = 0.015). When cases with t(4;14)(p16.3;q32) and del(17)(p13) were excluded from the analysis, this difference still remained (estimated 3-year OS 32.4 *vs* 69.1%, respectively, P = 0.033; Figure 1B). These patients were divided into three groups: group 1 [del(13)(q14) in less than 80% of PC]; group 2 [del(13)(q14) in more than 80% of PC and Ki-67 expression below 8%], and group 3 [del(13)(q14) in more than 80% of PC and Ki-67 above 8%]. Only group 3 was associated with lower OS (estimated 3-year OS: group 1 = 66.2%, group 2 = 50%, and group 3 = 0%, P = 0.01).

The estimated 3-year EFS from diagnosis was similar in cases with t(4;14)(p16.3;q32), del(17)(p13), del(13)(q14), and none of the abnormalities studied (P = 0.32). Although del(13)(q14) in more than 80% PC was also not associated with lower EFS (P = 0.72), the expression of Ki-67 above 8% was associated with it (P = 0.015).

Table 2. Association between characteristics of patients and Ki-67 expression and annexin-V labeling.

	Ki-67	Annexin-V
ISS		
I/II	2.7% (0-26.4%)	8.3% (0.31-35.8%)
III	3.2% (0-24.7%)	7.3% (0.12-55.0%)
Hemoglobin		
≥10.0 g/dL	2.5% (0.4%-20.9%)	6.7% (0.1-35.8%)
<10.0 g/dL	3.6% (0-26.4%)	9.5% (0.2-55.0%)
Creatinine		
<2.0 mg/dL	3.2% (0-26.4%)	8.1% (0.1-55.0%)
≥2.0 mg/dL	3.1% (0-18.9%)	8.7% (0.7-49.5%)
Calcium		
<10.5 mg/dL	3.1% (0-20.9%)	9.8% (0.1-49.5%)
≥10.5 mg/dL	3.6% (0.4-26.4%)	6.7% (0.7-27.7%)
Albumin		
≥3.5 g/dL	2.7% (0-26.4%)	10.0% (0.1-55.0%)
<3.5 g/dL	3.7% (0-24.7%)	5.8% (0.2-49.5%)
β_2-microglobulin		
<3.5 µg/mL	2.2% (0.8-26.4%)	13.1% (0.8-35.8%)
≥3.5 µg/mL	3.2% (0-24.7%)	7.6% (0.1-55.0%)
Lactate dehydrogenase		
≤480 U/L	2.8% (0-24.7%)	7.6% (0.1-55.0%)
>480 U/L	5.0% (0.4-26.4%)*	21.5% (0.3-29.4%)
Reactive C protein		
≤3.0 mg/L	2.3% (0-6.5%)	8% (1-32.1%)
>3.0 mg/L	4.6% (0-26.4%)*	6.4% (0.1-55.0%)

Data are reported as median with range in parentheses. ISS = International Staging System (10). *P < 0.05, Ki-67 expression of above normal data compared to normal data (Kruskal-Wallis test).

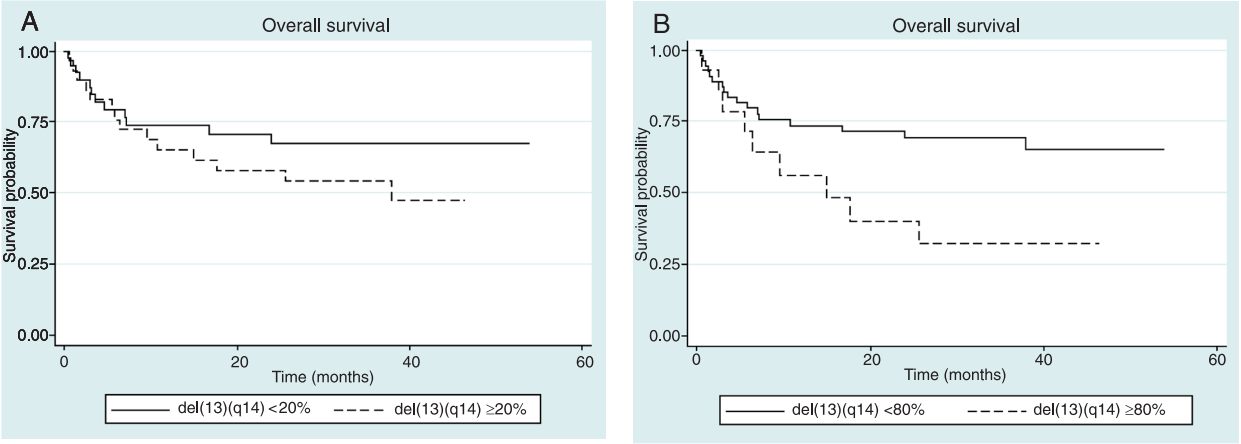

Figure 1. *A*, Overall survival (OS) in patients without del(13)(q14) (N = 40) and with del(13)(q14) (N = 32) and not t(4;14)(p16.3;q32) nor del(17p13) (P = 0.15, log-rank test). *B*, OS in patients harboring del(13)(q14) in less (N = 58) and in more (N = 14) than 80% of their plasma cells and not t(4;14)(p16.3;q32) or del(17)(p13) (P = 0.033, log-rank test).

Discussion

The prevalence of del(13)(q14) and del(17)(p13) was similar to that observed in studies carried out in other countries. However, t(4;14)(p16.3;q32) was observed in a lower percentage of patients than in other studies (1). In the present study, t(4;14)(p16.3;q32) was associated with a very dismal prognosis, with almost half the patients dying immediately after diagnosis, before starting treatment. This suggests that, in São Paulo and perhaps in Brazil, there may be even more patients with this chromosomal abnormality, but they may die before being diagnosed due to unfavorable socioeconomic conditions, leading to under-detection of this chromosomal abnormality in our population.

We also observed that del(13)(q14) was associated with a poorer outcome when present in more than 80% of myeloma cells. Only 15.4 and 18.9% of patients with del(13)(q14) in more than and in less than 80% of PC, respectively, received ASCT (data not shown). Therefore, we could not detect any significant difference in therapy administered to the two groups that would justify this difference.

Our results suggest that, in the group of patients evaluated in this study, the proportion of cells with del(13)(q14) was more important for determining prognosis than the raw presence of this abnormality in myeloma cells. Since the late nineties, the presence of del(13)(q14) in myeloma cells has been associated with a poor prognosis (13,14), although Avet-Loiseau et al. (15) and Gutierrez et al. (16) showed that most of the prognostic power of this abnormality was related to its frequent association with t(4;14)(p16.3;q32) and del(17)(p13). Despite the limited value of del(13)(q14) as a sole abnormality in the determination of prognosis, we decided to evaluate its association with the proliferative and apoptotic indexes of plasma cells in order to unveil if this abnormality has any importance in the biology of the disease.

While Gastinne et al. (11) did not detect an association between del(13)(q14) and increased cell proliferation, Fonseca et al. (14) and Zojer et al. (13) reported a positive relationship between del(13)(q14) and higher growth fraction (determined by Ki-67 staining or S-phase). However, these studies did not report the status of other important cytogenetic abnormalities such as del(17)(p13), t(4;14)(p16.3;q32) or t(14;16)(q32;q23). A recent study showed an association of higher PC proliferative index with del(13)(q14) but not with other chromosomal abnormalities (17).

Gastinne et al. (11) and Alexandrakis et al. (12) previously described a cut-off of 8% for Ki-67 expression for prognosis in MM. Our study showed that there is an association between del(13)(q14) in more than 80% of PC and a higher Ki-67 expression (>8%), and that this association persisted even when patients with t(4;14)(p16.3;q32) and del(17)(p13) were excluded from the analysis. Also, the lower OS in the group of patients with more than 80% of PC affected by del(13)(q14) was observed only in cases with high Ki-67 expression. Thus, probably the cause of this unfavorable prognosis is not merely the presence of del(13)(q14) in PC, but also their higher proliferative index.

One hypothesis is that del(13)(q14) may be involved in the allelic loss of a tumor suppressor gene. As suggested by Fonseca et al. (14), additional mechanisms could lead to inactivation of the second allele, resulting in a complete loss of normal function of the affected gene, selecting these cells to become more proliferative. Consequently, the cells with del(13)(q14) would preferentially accumulate. On the other hand, when the majority of PC are affected by del(13)(q14), but the cause is not a proliferative advantage of these cells, the prognosis is not unfavorable. As far as we know, correlation between del(13)(q14) and apoptosis has not been studied before and our results suggest that del(13)(q14) is not important for cell apoptosis control. Other genetic mechanisms may be more relevant for this biologic feature than 13q14 status.

Another finding of the present study was that patients with del(17)(p13) did not have an unfavorable prognosis. This may be due to the low number of patients studied or to the low proportion of cells with this abnormality in the affected patients (median = 35%, range = 21-76%). These findings strengthen those described by Avet-Loiseau et al. (15,18), who suggested that cases with more than 74% of plasma cells affected by del(13)(q14) or with more than 60% of plasma cells affected by del(17)(p13) were characterized by a worse outcome than patients harboring these abnormalities in a smaller proportion of cells.

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