Multiple Myeloma

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Interstitial deletions at the long arm of chromosome 13 may be as common as monosomies in multiple myeloma. A genotypic study

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Background and Objectives. Deletions at the long arm of chromosome 13, mostly at the q14, and monosomy of chromosome 13 are described to be common in multiple myeloma (MM). 13q- has been associated with an adverse outcome and it has been proposed as one of the most important prognostic factors for MM patients. Deletions of 13q14 are rare in monoclonal gammopathy of undetermined significance (MGUS) and are thus believed to be associated with the development of the full myeloma phenotype.

Design and Methods. A genotyping analysis on purified neoplastic plasma cells was performed on 14 consecutive MM cases to determine the minimally deleted region at chromosome 13. Freshly obtained bone marrow was analyzed by flow cytometry in order to establish the percentage of plasma cell infiltration ($CD38^+BB4^+CD56^{+/-}CD19^-$). Neoplastic enrichment was carried out using BB4 coated immunomagnetic beads. This method allowed us to monitor the enrichment process. DNA obtained from the enriched neoplastic population and DNA from the clean fraction was amplified using combination sets of chromosomes 12 and 13. Amplimers were run on acrylamide gels, analyzed by automatic fluorescence quantification, and their size determined using the software programs Genescan and Genotyper.

Results. In 11 patients electropherograms were suggestive of loss of heterozygosity (LOH) for polymorphic markers located at the long arm of chromosome 13. Four patients showed monosomy and 7 had interstitial deletions in the telomeric region. LOH was not evidenced at chromosome 12 in any sample. The minimal region with deletion was defined by the markers D13S159 (13q32.2, centromeric) and D13S1267 (13q32.3, telomeric). A gene with a potentially pathogenic role may be located in this very small region. Three patients did not show LOH at chromosome 13 by genotypic analysis; however, in one of these, proximal deletion at the long arm of chromosome 13 (13q2.1-2.2) was demonstrated by comparative genomic hybridization (CGH).

Interpretation and Conclusions. These findings suggest that interstitial deletions of the long arm of chromosome 13 may be more common than previously recognized. The methodologic approach reported in this work may simplify LOH analysis in MM patients. The potential uses of genotyping analysis in risk stratification remain to be investigated.

Key words: multiple myeloma; genetics; chromosome 13; loss of heterozygosity.

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Tumorigenesis is a multistep process involving a series of acquired genetic changes, including activation of proto-oncogenes and inactivation of tumor suppressor genes. Multiple myeloma (MM) is a malignancy of clonal plasma cells with a wide variability in clinical features and survival times among patients.[1] Chromosomal aberrations that are present in nearly 90% of patients with MM have been extensively studied and seem to play an important pathogenic role.[2-10] Fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) studies performed on MM patients have revealed that chromosome 13 deletions are very common and probably mark the transformation from monoclonal gammopathy of unknown significance (MGUS) to overt MM. Chromosome 13 deletions confer a defined biological behavior and are associated with an adverse outcome. Earlier studies suggest that this cytogenetic lesion involves large segments or the entire long arm. However, small interstitial deletions have also been described.[11-20] Such cases may be particularly interesting in defining the minimal critical region of loss in MM and may simplify the loss of heterozygosity (LOH) search.

In this work we performed an LOH analysis in an enriched neoplastic plasma cell population.[21] The aims were to investigate the status of the long arm of chromosome 13 and to delimitate the region where a putative(s) tumor suppressor gene may be located.

Design and Methods

Patients' material and CD138+ cell enrichment

Fourteen consecutive patients with MM, diagnosed and treated in the Clinical Hematology Unit of Hospital de la Santa Creu i Sant Pau between September 1999 and March 2000, were included in the analysis. The patients´ clinical characteristics are shown in Table 1. There were 7 males and 7 females with a median (range) age of 62 (44–75) years. The majority of patients were diagnosed in advanced stages and alternating chemotherapy cycles coupled with autologous stem cell transplantation as consolidation therapy in first response were administered in 4 of these cases. An aliquot of the diagnostic bone marrow aspirate was used for the immunophenotypic and separation procedures. Bone marrow infiltration by plasma cells ranged from 23% to 95% when assessed by morphologic methods. Antigen expression was analyzed using triple combinations of the following monoclonal antibodies (MoAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and the phycoerythrin-cyanine 5 (PE/Cy 5) fluorochrome tandem. The MoAbs used in the study were: CD38 FITC AT13/5 clone and CD19 PE-CY5 HD37 from Dako, Glostrup, DK; CD56 PE MY31 clone from Becton-Dickinson, San José, CA, USA; and CD138 PE (BB4) IQP-142R from Immunoquality, Groningen, The Netherlands (CD38/CD56/CD19 and CD38/CD138/CD19). Cases of MM were positive for CD138,CD38,CD56 and negative for CD19. By flow cytometry bone marrow infiltration ranged from 2% to 19.3%. Bone marrow cell aspirates were layered on Lymphoprep (Nycomed Pharma, Oslo, Norway). Cells were then labeled with CD138 microbeads and purified using the miniMACS immunomagnetic system (Miltenyi Biotec, Bergisch Gladbach, Germany).[22] Enriched fractions were assessed for purity using the same triple combinations employed just before the enrichment process. Samples were processed as soon as possible in order to minimize the CD138 antigen loss associated with the process of apoptosis.[23] DNA was purified by means of the Qiamp tissue kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer (Table 2).

Table 1. Clinical data.

UPN	Sex	Age (years)	Clin. stage (Durie-Salmon classification)	M component	Therapy	Outcome, follow up
#1	M	71	IA	Non-secretory	MP/PDN	Died from PD,27 mo
#2	M	58	IIIA	IgGκ	VBCMP/VBAD + BMT	CR, +24 mo
#3	F	52	IIIB	Non-secretory	VBCMP/VBAD + ASCT	Died from PD,23 mo
#4	M	74	IIA	IgGκ	MP/PDN	Died from PD,8 mo
#5	M	44	IIIB	IgGκ	VBCMP/VBAD + ASCT	PD, +28 mo
#6	M	55	IIA	IgGκ	VBCMP/VBAD + ASCT	PR, +25 mo
#7	M	57	IIA	IgAGκ	No	Died from PD,12 mo
#8	F	53	IIA	IgGκ	No	Stable disease, +25 mo
#9	F	68	IIIA	IgGκ	VBCMP/VBAD+ MP/PDN	Died from PD 15 mo
#10	F	63	IA	IgGκ	No	Stable disease, +28 mo
#11	F	75	IIA	IgGκ	MP/PDN	Died from PD 2 mo
#12	F	71	IIA	IgAGκ	MP/PDN	Stable disease, +24 mo
#13	M	60	IA	IgGκ	No	Stable disease
#14	F	66	IIIA	IgGκ	VBCMP/VBAD + ASCT	Died from PD,4 mo

UPN: unique patient number; M: male; F: female; MP/PDN: melphalan/prednisone; VBCMP/VBAD: vincristine, carmustine, cyclophosphamide, melphalan and prednisone/vincristine, carmustine, adriamycin and dexamethasone; BMT: allogeneic bone marrow transplantation; ASCT: autologous stem cell transplantation; CR: complete remission; PR: partial remission; PD: progressive disease.

Table 2. Tumor cell enrichment parameters.

	BM infiltration: morphology	BM infiltration: flow cytometry	Enrichment	DNA obtained
UPN 1	55%	3.66%	93.4%	11.6 mg
UPN 2	95%	3.14%	39.4%	1.4 mg
UPN 3	23%	19.3%	97.4%	8.75 mg
UPN 4	32%	10%	97%	17.75 mg
UPN 5	90%	2.94%	93.5%	6.09 mg
UPN 6	56%	8%	58.25%	38.2 mg
UPN 7	31%	6%	87.16%	23.5 mg
UPN 8	35%	3%	98.6%	4.45 mg
UPN 9	45%	11%	94.1%	20.2 mg
UPN 10	30%	11.4%	99.2%	26.1 mg
UPN 11	77%	14.7%	99.1%	11.8 mg
UPN 12	55%	13.8%	80.24%	51.4 mg
UPN 13	23%	2%	97.5%	14.99 mg
UPN 14	80%	5%	56.2%	23.67 mg

Table 3. Polymorphic markers.

Chromosome 12	*Chromosome 13*
D12S352	D13S175
D12S99	D13S217
D12S336	D13S171
D12S364	D13S218
D12S310	D13S153
D12S1617	D13S170
D12S345	D13S265
D12S85	D13S159
D12S368	D13S1267
D12S83	D13S174
D12S326	D13S158
D12S351	D13S173
D12S346	D13S1265
D12S78	D13S285
D12S79	
D12S86	
D12S324	
D12S1659	
D12S1723	

Comparative genomic hybridization

In three cases (cases #1, 9 and 11) CGH was performed. Tumoral and normal genomic DNA was labeled by nick translation using FITC and Texas Red, respectively (Vysis nick translation kit). Probes were checked in a 1% agarose gel to obtain fragments between 300 and 3,000 base pairs. A mixture of 300 ng test DNA, 100 ng reference DNA and 10 μg Cot-1 DNA was hybridized with normal metaphase target slides (Vysis CGH kit). Thereafter, DAPI II was applied and metaphase images were captured using a fluorescence microscope (Leica DMRB) through a CCD camera (Photometrics Sensys) and a filter system specific for DAPI, Texas Red and FITC. The ratios of the FITC/Texas Red intensities were calculated along the chromosomes using the Quips Vysis software. Loss and gain thresholds were 0.80 and 1.20, respectively. Chromosomal copy number changes at 1p32-pter, 19 and 22 chromosomal terminal bands were not included in the analysis as a described technical limitation.

Polymerase chain reaction and GeneScan analysis

Aliquots of PCR products (1-2 μL) corresponding to polymorphic markers encompassing chromosomes 12 and 13 (Table 3) were mixed with loading buffer (2 μL formamide, 0.5 μL EDTA) and 0.5 μL of the internal size standard (Genescan-500) were included for precise determination of the length of the amplimers. After denaturation for 4 min at 94º C, the products were separated on polyacrylamide gels and analyzed by automatic fluorescence quantification and size determination using the Abiprism 377 and software programs Genescan and Genotyper (Applied Biosystems, Foster City, CA, USA).

For two markers (D13S 174 and D13S1267) an alternative protocol was performed: the amplified products were transferred to a nylon membrane (Hybond-N+; Amersham, Buckinghamshire, UK) and hybridized with a modified 5´ (CA)n probe using the ECL labeling system (Amersham).

Determination of LOH and microsatellite instability (MSI)

LOH and MSI analyses were performed for each patient on paired samples corresponding to the neoplastic rich fraction and the clean fraction as determined by flow cytometric methods.

All samples in which 2 distinct alleles of similar intensity were present in the normal DNA were considered to be informative (I) and samples showing only one peak were considered homozygous for the tested microsatellite and scored as non-informative (NI). LOH was scored as positive when a clear reduction in signal intensity was detected in 1 of the alleles (single height peak reduction). MSI may be revealed as extra microsatellite alleles in tumor DNA compared with matched normal DNA. MSI is caused by inactivating mutations in genes of the DNA mismatch repair system (MMR genes).

Results

Enrichment process

There is a marked discordance between the infiltration demonstrated by morphologic methods and those observed using flow cytometry (Table 2). To circumvent this limitation, we purified neoplastic plasma cells by means of the BB4 (CD138) antibody, which is expressed by most non-apoptotic plasma cells. This simple procedure allowed us to obtain neoplastic rich populations and enough DNA to repeat the LOH analysis. No arbitrary primed amplification was employed in order to increase available material.[24,25] In 11 samples the enrichment process obtained neoplastic rich fractions above 80% (Figure 1). In the remaining three patients the isolation percentages were 39.4%, 58.25% and 56.2%. These low figures were attributed to the apoptotic process which caused a marked BB4 loss.[23]

LOH analysis

In 11/14 patients a LOH was detected using polymorphic markers at chromosome 13 (Figures 2 and

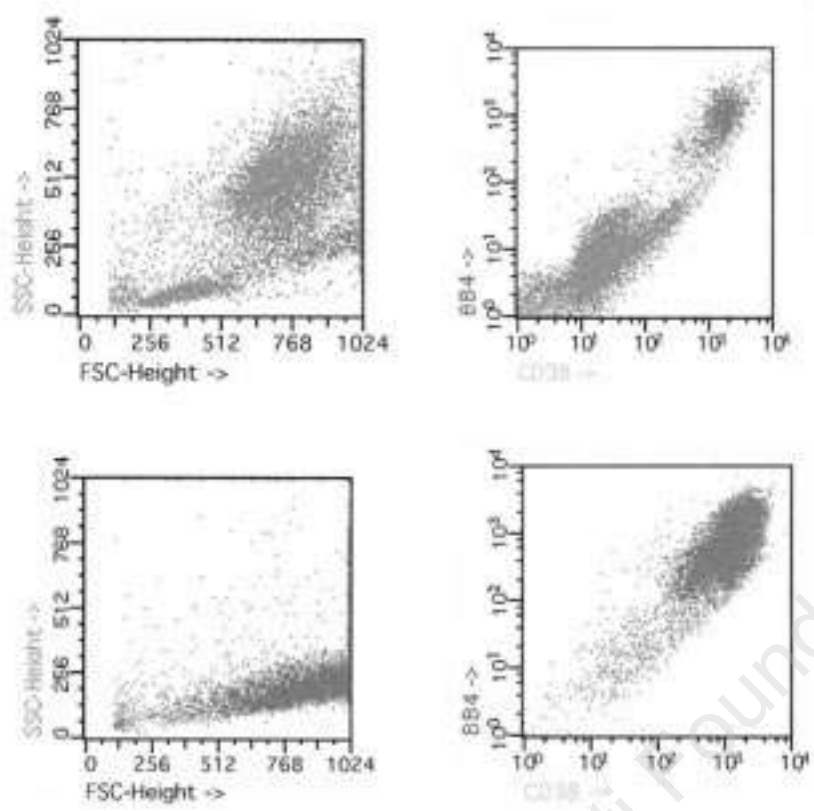


Figure 1. Flow cytograms obtained before (top) and after (bottom) the enrichment process. Neoplastic plasma cells are depicted in pink and the normal cells in light blue.

Table 4. Molecular refinement of the long arm of chromosome 13 in MM.

Patient	*D13S173*	*D13S158*	*D13S174*	*D13S1267*	*D13S159*	*D13S265*	*D13S153*	*D13S171*
UPN 1	I	I	I	I	I	NI	NI	NI
UPN 2	I	I	LOH	LOH	NI	NI	I	I
UPN 3	LOH	LOH	LOH	NI	NI	LOH	LOH	LOH
UPN 4	NI	LOH	LOH	NI	I	NI	NI	I
UPN 5	I	I	NI	LOH	I	NI	NI	I
UPN 6	I	I	I	NI	LOH	NI	NI	NI
UPN 7	NI	I	I	I	I	NI	I	I
UPN 8	NI	NI	LOH	LOH	I	I	I	NI
UPN 9	NI	LOH	LOH	NI	LOH	LOH	NI	LOH
UPN 10	I	LOH	LOH	LOH	I	NI	I	NI
UPN 11	LOH	LOH	NI	NI	LOH	LOH	NI	LOH
UPN 12	NI	LOH	NI	NI	LOH	LOH	LOH	LOH
UPN 13	I	NI	I	NI	I	I	I	I
UPN 14	I	LOH	NI	NI	LOH	I	I	I

I:informative allele; NI/NI:non-informative allele; LOH:loss of heterozygosity.

3). In three patients (cases #1, 7 and 13) we were not able to detect such allelic losses. Nevertheless, in patient #1, CGH data disclosed an interstitial deletion encompassing the proximal segment of the long arm of chromosome 13 (Figure 4). In this region polymorphic markers were all non-informative.

In four cases (patients #3, 9, 11 and 12) LOH analysis was indicative of chromosome 13 monosomy. In two of these cases in which CGH analysis was performed results obtained using this technique were in line with these findings. In addition, other cytogenetic lesions were identified (*data not*

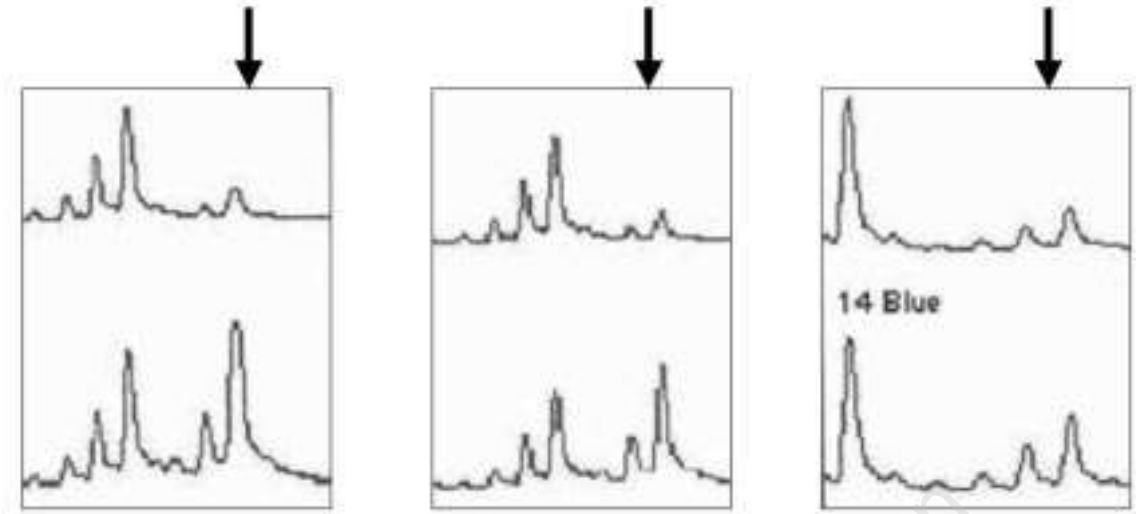
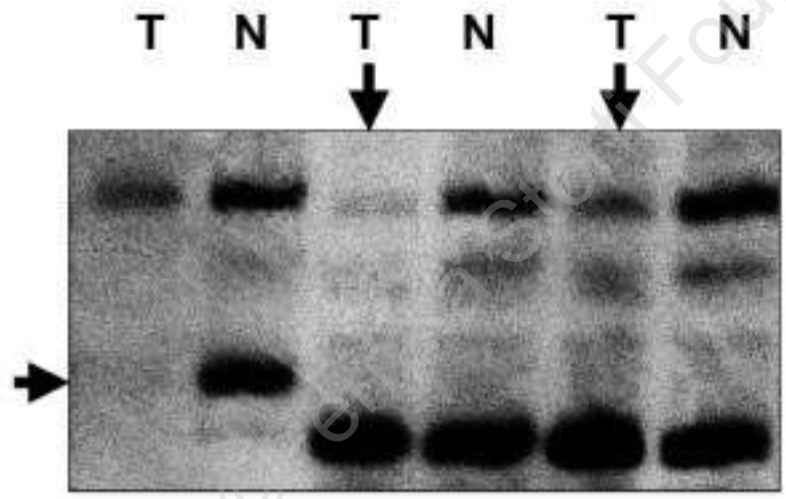


Figure 2. Representative LOH in MM. Left and middle: D13S159 marker. Right: D13S158 marker.



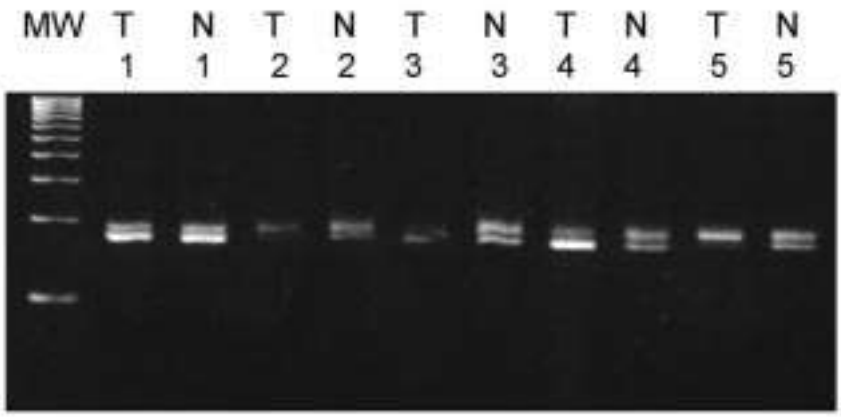


Figure 3. LOH at the D13S174 (top) and D13S1267 (bottom) loci. Top: LOH at the D13S174 was detected in tumoral (T) samples from UPN 2, 3 and 4. Bottom: lanes 2 (UPN2), lanes 3 (UPN5), lanes 4 (UPN8) and lanes 5 (UPN10) showed LOH at the D13S1267 locus whereas lane 1 (UPN1) was informative without LOH. T: tumoral sample; N: normal sample (plasma-cell poor fraction); MW: molecular weight marker.

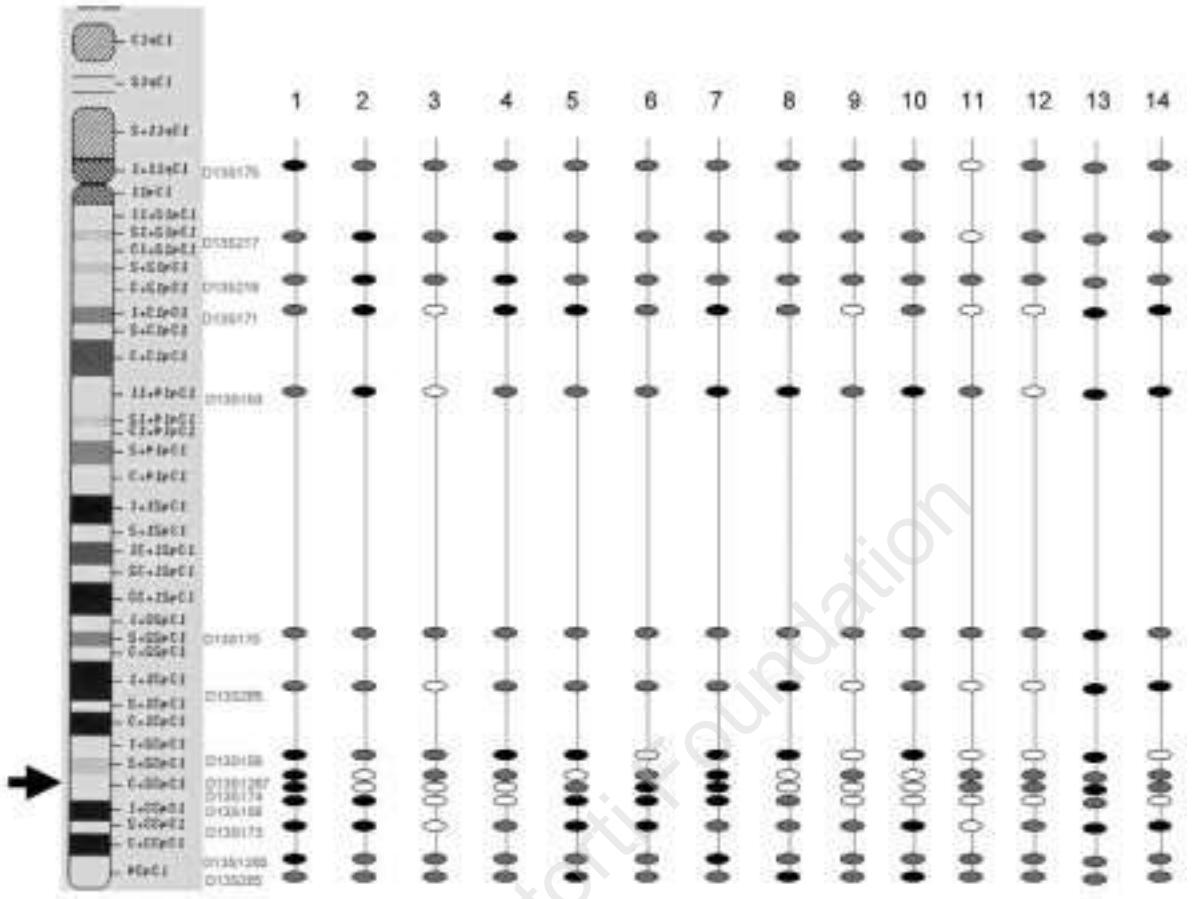


Figure 5. LOH analysis in MM. Arrow: minimally deleted region. Black: infomative allele. Gray: non-informative allele. White: loss of heterozygosity (LOH).

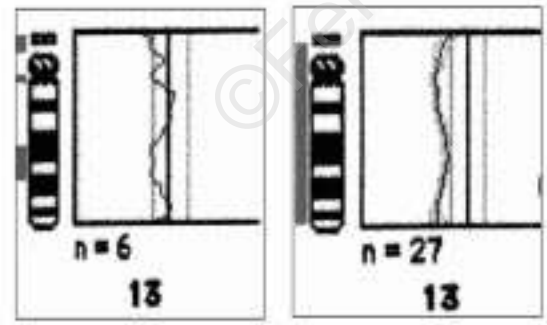


Figure 4. Partial CGH results corresponding to patients 1 (left) and 3 (right) showing an interstitial deletion at 13q21->q22 (left) and a loss of the whole chromosome 13 (right).

shown). In the remaining 7 cases an interstitial deletion was detected. This figure contrasts sharply with previous cytogenetic data by which most patients were assigned as having chromosome 13 monosomy. Molecular delineation of the region of allelic loss is shown in Table 4. The narrowest region of LOH was defined by the markers D13S159 (13q32.2, centromeric) and D13S1267 (13q32.3, telomeric) (Figure 5). LOH may be detected using simple agarose (Metaphor®, Cambrex, North Brunswick, NJ, USA) electrophoresis (Figure 3).

In this region a few genes (PCCA, ZIC 2 and ZIC 5 genes, EBI2, KIAA1058, SLC15A1) and some other coding sequences without known functions have been mapped (Genome database, NCBI). The presence of inactivating mutations in a small gene (EBI2) located inside the critical region was assessed by SSCP analysis. This study gave negative results in all the samples included in the study (*data not shown*).

No allelic loss was identified in any of the markers corresponding to chromosome 12, suggesting

that deletions in this chromosome do not contribute to MM pathogenesis. Chromosome 12 was normal in three patients for whom CGH study was available (cases #1, 3 and 11). MSI was also absent in all the polymorphic markers tested.

Discussion

Deletions at the long arm of chromosome 13 are recurrent cytogenetic abnormalities which seem to participate in the origins of several neoplastic disorders. 13q deletions have been reported in chronic lymphocytic leukemia,[26] acute myeloid leukemia,[27,28] acute lymphoblastic leukemia,[29] multiple myeloma[11-20] and breast cancer.[30] Despite an extensive search for genes located in this area that could be responsible for the neoplastic phenotype in the aforementioned hematologic disorders, they remain elusive.

CGH studies have revealed multiple gains and losses of DNA in neoplastic plasma cells. Among these changes, 13q loss is the most commonly reported (occurring in up to 80% of the cases of plasma cell leukemia).[19] Fonseca *et al.*[17] suggested that the 13q deletion is a good marker of a MM/MGUS syndrome with a particular propensity to acquire the t(4;14)(p16.3;q32) chromosomal translocation.

One of the major problems when initiating a search for LOH is sample procurement. MM has two major advantages to overcome this problem: it is a fluid neoplasm and tumoral cells display a characteristic phenotype that renders them easily distinguishable from their normal counterparts.[31] Furthermore, the strategy used in this study enabled us to control the efficiency of the enrichment, which is very important at the time of allele evaluation. Genotype analysis using polymorphic markers that cover chromosome 13 should perhaps be employed to analyze 13 q deletions in MM patients and this technique may provide complementary information to the cytogenetic approach. The results obtained in the present study suggest that there are two regions with a possible pathogenic role in MM patients. The first region is located at 13q21 (case 1) whereas a second region more frequently involved in MM development is localized at 13q32.2-32.3. Both regions would be affected in cases showing monosomy at chromosome 13 (four cases in our study).

The small number of cases analyzed does not allow us to establish survival or biological differences between the groups with and without deletions at the telomeric region of 13q.

Additonal studies will clarify whether these interstitial deletions are associated with mutations in a putative tumor suppressor gene or whether the functional haploinsufficiency resulting from this telomeric defect participates in the origins of MM.

Contributions and Acknowledgments

JFN: conception, design and experimental work. AL, MB, MJC: molecular techniques; JU: plasma cell enrichment; SC, AA: cytogenetics; GS, VS, MB: discussion; AS: clinical information.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received April 15, 2002; accepted June 18, 2002.

What is already known on this topic

Cytogenetic studies have shown that 13q- and monosomy 13 can be found in several patients with multiple myeloma, and that these abnormalities might be associated with an adverse outcome.

What this study adds

Genotyping analysis on purified neoplastic plasma cells suggests that interstitial deletions of the long arm of chromosome 13 may be more common than previously recognized.

Potential implications for clinical practice

Larger prospective studies are required in order to establish whether evaluation of the above cytogenetic abnormalities has any diagnostic/prognostic significance.

Mario Cazzola, Editor-in-Chief