Integrative genomic analysis reveals distinct transcriptional and genetic features associated with chromosome 13 deletion in multiple myeloma

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ABSTRACT

Background and Objectives

The chromosome 13 deletion (Δ^{13}) is one of the most frequent chromosomal alterations in multiple myeloma (MM). Δ^{13} is associated with an unfavorable prognosis, although there is increasing agreement that its prognostic relevance must be related to the ploidy status and the presence of different chromosomal translocations. The aim of this study was to provide a comprehensive analysis of the transcriptional features of Δ^{13} in MM.

Design and Methods

Highly purified plasma cells from 80 newly diagnosed MM patients were characterized by means of fluorescence *in situ* hybridization (FISH) and high-density oligonucleotide microarray for gene expression profiling and chromosomal alterations.

Results

We identified 67 differentially expressed genes in the patients with and without the chromosome 13 deletion, all of which were downregulated in the cases with Δ^{13}: 44 mapped along the whole chromosome 13, seven on chromosome 11 and three on chromosome 19. Functional analyses of the selected genes indicated their involvement in protein biosynthesis, ubiquitination and transcriptional regulation. An integrative genomic approach based on regional analyses of the gene expression data identified distinct chromosomal regions whose global expression modulation could differentiate Δ^{13}-positive cases, in particular the upregulation of 1q21-1q42 and the downregulation of 19p and almost the entire chromosome 11. FISH analyses confirmed the close relationship between Δ^{13}-positivity and the presence of extra copies of 1q21-1q42 ($p=6\times10^{-4}$) or the absence of chromosome 11 and 19 trisomy ($p=5\times10^{-4}$).

Interpretation and Conclusions

Our results indicate that distinct types of chromosomal aberrations are closely related to the transcriptional profiles of Δ^{13}-positive cases, suggesting that the contribution of Δ^{13} to the malignancy should be considered together with associated abnormalities.

Key words: multiple myeloma, chromosome 13 deletion, gene expression profiling, genome wide profiling, integrative genomics.

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Multiple myeloma (MM) is a fatal B-cell malignancy characterized by a heterogeneous clinical course and profound genomic instability. Together with immunoglobulin-heavy chain (*IGH*) translocations at 14q32, hyperdiploidy and chromosome 1q gain, chromosome 13 deletion (Δ^{13}) is one of the most frequent chromosomal alterations: it has been detected in 15-20% of patients by means of conventional karyotype analysis (an underestimate mainly due to the limited mitotic activity of the neoplastic plasma cells), and in as many as 50% of the patients by fluorescence *in situ* hybridization (FISH) analysis.[1] The chromosome 13 abnormalities involve complete monosomy in most MM patients;[2,3] the minimal common deleted region of approximately 350 Kb has been identified at 13q14 (1.5 Mb telomerically to the *RB1* oncosuppressor gene).[4]

Several authors have reported that chromosome 13 abnormalities indicate an unfavorable prognosis in MM,[5-8] and various studies have shown that chromosome 13 deletions are early events and represent an adverse prognostic factor in the premalignant condition of monoclonal gammopathies of undetermined significance (MGUS).[2,9,10] Shaughnessy *et al.*[11] found that the presence of Δ^{13} and other hypodiploid cytogenetic abnormalities were associated with a poorer prognosis in patients treated with intensive chemotherapy and autologous bone marrow transplantation, and distinguished four groups of patients on the basis of combined FISH, conventional cytogenetic and gene expression profiling analyses. However, despite recent advances in identifying the transcriptional features of subgroups of MM patients,[12-17] no comprehensive analysis of the transcriptional profiles associated with Δ^{13} in MM patients at diagnosis has been published.

The results of functional genomics studies suggest a close relationship between genomic structural abnormalities (i.e. deletions or amplifications) and expression imbalances, and have identified co-ordinated transcriptional profiles of physically contiguous genes.[18-20] In addition, recent genotyping studies have found that copy numbers have a considerable influence on gene expression patterns, and have shown that organizing gene-expression data by genomic mapping location and scanning for regions containing statistically modulated gene expression signals can lead to the detection of chromosomal amplifications and deletions.[21,22]

Taken together, these considerations prompted us to investigate the transcriptional profiles associated with Δ^{13} and develop a statistical model for identifying globally modulated chromosome regions associated with Δ^{13}-positive or Δ^{13}-negative patients.

Design and Methods

Patients and samples preparation

Bone marrow specimens from four normal donors and pathological samples from 90 untreated MM patients (51 males; median age 64 years, range 39-85; 50 described in previous reports)[12,14,23] were obtained during standard diagnostic procedures after the subjects had given their informed consent. Fifty-six patients had an IgG protein monoclonal component, 18 IgA, two IgG/IgA, and one IgD protein; 49 patients had the light chain κ, and the κ/λ ratio was 1.3. The patients were diagnosed and clinically staged according to previously described criteria:[24] 30 patients were in stage IA, 34 in stage IIA/B and 26 in stage IIIA/B. No conventional cytogenetic (G-banding) analyses were available.

Plasma cells were purified from the bone marrow samples using CD138 immunomagnetic microbeads (MidiMACS® system, Miltenyi Biotec, Auburn, CA, USA) as previously described.[14,23] The purity of the selected plasma cell populations was assessed by means of morphology and flow cytometry, and was > 90% in all cases.

Gene expression profiling

Total RNA was extracted and purified, and biotin-labeled cRNA was synthesized as previously described.[14] In accordance with the Affymetrix protocols, 15 μg of fragmented cRNA were hybridized on HG-U133A Probe Arrays (Affymetrix Inc., Santa Clara, CA, USA), and the oligonucleotide arrays were scanned using an Agilent GeneArray Scanner G2500A (Agilent Technologies, Waldbronn, Germany). The quality reports for the scanned arrays were as follows; scaling factor: median 1.066, range 0.468-2.459; percentage of present genes: median 38.85, range 27.7-46.5; 3'/5' actin ratio: median 1.23, range: 0.83-2.27; 3'/5' GAPDH ratio: median 1.075, range 0.75-2.40.

Microarray data analysis

The probe level data were converted to expression values using the Bioconductor function for the **r**obust **m**ulti-**a**rray **a**verage (RMA) procedure,[25] in which perfect match intensities are background adjusted, *quantile-quantile* normalized, and log_2-transformed. The detection calls were calculated using the default parameters of the Affymetrix MAS 5.0 software package. Data with absent calls in all of the arrays were filtered out; no filtering procedure was applied to the intensity levels. The overexpression of *CCND1*, *CCND2* and *CCND3* genes was calculated as previously described.[12] Unsupervised analyses were applied to a subset of genes whose average change in expression levels varied at least 2-fold from the mean across the whole panel. In order to perform the hierarchical agglomerative clustering of the selected probe lists, Pearson's correlation coefficient and

average-linkage were used as distance and linkage methods in DNA-Chip Analyzer (dChip) software,[26,27] as previously described.[14]

The differentially expressed genes discriminating Δ^{13}-positive and Δ^{13}-negative classes were identified using **s**ignificant **a**nalysis of **m**icroarrays (SAM) software (Excel front-end publicly available at *http://www-stat.stanford.edu/~tibs/SAM/index.html*).[28] The cut-off for significance was determined by tuning the Δ parameter on the false discovery rate (median FDR=0% and 90th percentile FDR=0%) and controlling the q-value for the gene list. The selected probe list was visualized by means of dChip software. The chromosomal regions with modulated gene expression signals were identified using a non-parametric model-free statistical method called locally adaptive statistical procedure (LAP).[29] For the purposes of this study, the intensity levels were generated from CEL files using RMA and, after annotation, the probe sets without any chromosomal location information were filtered out, as were those on chromosomes X and Y. The differential expressions between the Δ^{13}-positive and Δ^{13}-negative groups were calculated using the regularized t-statistic d_i and led to 11613 unique ID. The null statistic was defined by means of 100000 permutations of the statistic values d_i (i.e. by randomly assigning the scores to the 11613 loci) and then, for each permutation, smoothed over the chromosomal coordinate. Finally, differentially expressed chromosomal regions were identified using the *q*-value calculated from the distribution of empirical *p*-values.

The data discussed in this article have been deposited in National Center for Biotechnology Information's Gene expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series, accession number GSE6365.

Real-time quantitative polymerase chain reaction (RT-Q-PCR)

One microgram of total cellular RNA from purified plasma cell populations was reverse transcribed to cDNA using random hexamer primers. RT-Q-PCR was performed in triplicate using an ABI PRISM 7700 Sequence Detector (Perkin Elmer, Foster City, CA, USA). The results were expressed using the comparative Ct method ($2^{-\Delta\Delta Ct}$), according to manufacturer's manual (Applied Biosystems. Relative Quantification of Gene Expression. ABI PRISM 7700 Sequence Detection Systems. User Bulletin #2, PE Applied Biosystems, 1997). The average Ct value for *RB1* was normalized with respect to the average Ct value for *GAPDH* in order to yield the ΔCt. The ΔCt value obtained from JJN3 human myeloma cell line (chosen as the calibrator) was then subtracted from the average ΔCt value for each patient, to obtain $\Delta\Delta$Ct. Human *RB1* and endogenous control *GAPDH* were analyzed using, respectively, assay on-demand and pre-developed assay reagents (PDAR, Applied Biosystems, Foster, CA, USA).

Fluorescence in situ hybridization (FISH)

The FISH procedure and specific probes for the detection of *IGH* translocations, the 13q14 deletion and chromosome 11 polysomy have been previously described.[12,23] Additional copies of chromosome 19 were investigated using two BAC clones located ~2 Mb from the pericentromeric regions 19q13.11 (CTD-2632B17) and 19p13.11 (CTD-3149D2). The gain of 1q21 and 1q42 regions was analyzed by means of two BAC clones covering the *BCL9* gene (CTD-2555I11) and *ARF1* gene (RP11-155G12), which map, respectively, to 1q21.1 and 1q42.13. Hyperdiploid status was determined according to the criteria recently proposed by a major investigational group in MM,[30] based on the FISH investigation of chromosomes 5, 9 and 15. The following probes were used: the 9 and 15 α satellite probes (*kindly provided by M. Rocchi, University of Bari, Italy*) and a specific selected BAC probe (CTD-2530B8) containing the STS marker D5S630 mapping at 5p15.31. Two co-hybridizations steps were performed in each case. All of the clones were selected by browsing the UCSC Genome Database (*http://genome.ucsc.edu/*).

Results

Correlation between Δ^{13} status and the main genetic lesions in MM

Eighty of the 90 samples included in our microarray database were characterized by FISH for the presence of the chromosome 13q14 deletion leading to the identification of 43 (53.8%) positive patients. The correlations with the main chromosomal aberrations associated with MM are shown in Table 1. Hyperdiploidy was observed in 28 (43%) of the 65 tumors for which material for FISH was available: 12 out of 36 Δ^{13}-positive patients and 16 out of 29 Δ^{13}-negative patients. Although we found a similar frequency to that reported in the literature[31] and identified a greater prevalence of hyperdiploid patients in the Δ^{13}-negative group, our data did not show a statistically significant correlation between Δ^{13}-positive cases and ploidy status, as had been previously reported.[30,32] Chromosome 1q amplification was identified by FISH in 37 (53%) of 70 patients for whom material was available; the close correlation between Δ^{13}-positivity and additional chromosome 1 material (28/39 vs 9/31 patients; $p=6\times10^{-4}$) indicated that the 1q gains can be considered specifically associated with Δ^{13}-positivity (*see below*).

Finally, the 80 patients investigated for Δ^{13} status were stratified according to the recently proposed translocation/cyclin D (TC) molecular classification, which takes into account the presence of the main *IGH* chromosomal translocations and the expression of *cyclin D* genes, whose deregulation represents a common event in MM. Following the criteria reported by Hideshima *et al.* and

Table 1. Molecular characterization of the Δ^{13}-positive and Δ^{13}-negative patients.

Δ^{13}-positive samples						Δ^{13}-negative samples					
Patients	*TC*	*HD*	*+11*	*+19*	*+1q21 +1q42*	*Patients*	*TC*	*HD*	*+11*	*+19*	*+1q21 +1q42*
MM-019	1	–	+	–	+	MM-015	1	–	+	+	+
MM-037	1	–	–	–	–	MM-026	1	nd	–	–	–
MM-052	1	–	–	+	+	MM-031	1	–	+	+	+
MM-070	1	–	–	–	–	MM-032	1	–	+	–	–
MM-100	1	–	–	–	–	MM-054	1	nd	+	nd	nd
MM-115	1	–	+	–	+	MM-055	1	–	–	–	–
MM-159	1	–	+	–	–	MM-111	1	–	–	–	–
MM-038	2	nd	–	+	–	MM-119	1	nd	–	nd	nd
MM-027	3	+	–	+	+	MM-126	1	nd	+	nd	nd
MM-036	3	–	+	–	+	MM-128	1	–	+	–	–
MM-040	3	+	–	+	+	MM-140	1	–	–	–	–
MM-047	3	+	–	+	+	MM-014	2	+	+	+	–
MM-048	3	+	+	+	+	MM-030	2	+	+	+	–
MM-072	3	nd	–	–	+	MM-034	2	+	–	+	–
MM-078	3	–	+	+	–	MM-035	2	nd	+	+	–
MM-082	3	+	–	+	+	MM-039	2	+	+	+	–
MM-094	3	–	–	–	+	MM-043	2	–	+	+	+
MM-101	3	nd	+	nd	nd	MM-049	2	+	+	+	–
MM-103	3	nd	+	+	nd	MM-056	2	nd	+	nd	nd
MM-107	3	nd	–	+	nd	MM-077	2	+	+	+	–
MM-114	3	+	–	+	+	MM-079	2	+	+	+	–
MM-117	3	–	–	+	–	MM-121	2	+	+	+	–
MM-129	3	+	–	+	+	MM-131	2	–	+	+	–
MM-160	3	+	nd	+	–	MM-143	2	+	+	+	–
MM-161	3	+	–	+	+	MM-146	2	+	–	+	+
MM-167	3	–	–	–	+	MM-151	2	+	+	+	–
MM-021	4	–	–	–	+	MM-152	2	+	+	+	–
MM-042	4	+	–	+	+	MM-016	3	+	+	+	+
MM-063	4	nd	–	–	–	MM-050	3	nd	–	nd	nd
MM-067	4	–	–	–	+	MM-092	3	–	–	+	+
MM-074	4	+	–	+	+	MM-106	3	–	–	+	–
MM-083	4	–	–	–	+	MM-148	3	+	–	+	+
MM-087	4	–	–	–	+	MM-149	3	+	+	+	–
MM-089	4	nd	–	–	nd	MM-150	3	+	–	+	+
MM-104	4	–	–	–	+	MM-153	3	–	+	+	+
MM-109	4	–	–	–	–	MM-066	4	–	–	–	–
MM-113	4	+	–	–	–	MM-004	5	nd	nd	nd	nd
MM-123	4	–	–	+	+						
MM-133	4	–	–	–	+						
MM-158	4	–	–	–	+						
MM-025	5	–	+	+	+						
MM-069	5	–	+	+	+						
MM-154	5	–	–	–	+						

nd: not determined; TC: translocation/cyclin classification; HD: hyperdiploidy.

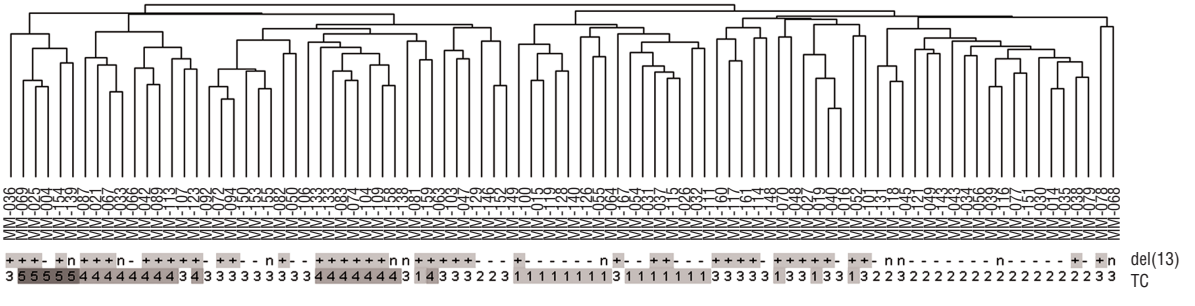

Figure 1. Unsupervised analysis of the samples from the 90 MM patients. Unsupervised analysis of gene expression profiles in purified CD138$^+$ plasma cell samples from 90 MM cases. The dendrogram was generated using a hierarchical clustering algorithm based on the average-linkage method and Pearson's correlation. The samples are grouped on the basis of the expression levels of the 546 most variable genes. Information about chromosome 13 deletion (+=presence of Δ^{13}; n=data not available) and TC classes is included alongside the MM patients' progressive numbers.

recently investigated by us,[12,33] the patients were stratified in five groups: TC1, characterized by the t(11;14) or t(6;14) translocation, with consequent overexpression of *CCND1* or *CCND3*, and a non-hyperdiploid status; TC2, showing low to moderate levels of the *CCND1* gene in the absence of any primary *IGH* translocation, and a hyperdiploid status; TC3, including tumors that do not fall into any of the other groups, most of which expressed *CCND2*; TC4, showing high *CCND2* levels and the presence of the t(4;14) translocation; and TC5, expressing the highest levels of *CCND2* in association with either the t(14;16) or t(14;20) translocation. It is worth noting that 16 out of 17 TC2 patients did not show any chromosome 13 abnormalities (94%, $p<10^{-4}$), whereas 14 out of 15 TC4 patients, who had the t(4;14) translocation, were included in the Δ^{13}-positive group (93%, $p<10^{-4}$) (Table 1). These data confirm our previous observations concerning the distribution of Δ^{13}-positivity within TC classes.[12]

Δ^{13}-positive patients showed downregulation of genes mainly located on chromosome 13

An unsupervised analysis using the hierarchical clustering algorithm was made in order to determine whether the clustering of the gene expression profiles of the MM samples was associated with the presence of Δ^{13}. The 90 MM cases described by the 546 most variable genes throughout the database (i.e. genes with at least a two-fold average change in expression from the mean across the whole panel), generated the dendrogram shown in Figure 1. The clustering algorithm split the samples into two major groups of 49 and 41 specimens, with the first containing 26/43 Δ^{13}-positive patients (60%, $p=5.9\times10^{-4}$) and the second 27/37 Δ^{13}-negative patients (73%, $p=2.9\times10^{-4}$). However, despite the significant correlation of the two major subgroups with the prevalence of Δ^{13}, the dendrogram was mainly driven by the presence of the principal *IGH* translocations and TC stratification, as previously described.[12,14]

Using SAM on the 80 samples for which FISH Δ^{13} characterization was available, we performed a supervised analysis in order to identify the transcriptional fingerprints characterizing Δ^{13}-positive and Δ^{13}-negative groups, and found 87 differentially expressed transcripts (specific for 67 genes), all of which were downregulated in the Δ^{13}-positive group (Figure 2): in particular, 44 were localized along the whole chromosome 13 (evenly distributed from 13q11 to 13q34), seven on chromosome 11, and three on chromosome 19 and 14q (*see Supplemental data 1 for the complete list*). The selected genes included a relatively large class of transcripts involved in protein biosynthesis encoding for ribosomal protein related to large (L21, L22, L24, L31, L36a, LP2) and small ribosome subunits (S2, S29, and the mitochondrial S31), together with *EIF3S7*, *FAU* and *DHPS* genes involved in translational machinery; notably, only two of these (L21 and S31) mapped to chromosome 13q. We also identified genes involved in transcription regulation (*SAP18, POLR1D, GTF3A, ELF1, GTF2F2, MED4, PHF11, MYCBP2*), DNA repair (*PARP4, HMGB1, PSCP1, ERCC5*), chromatin assembly and cell-cycle (*HSMPP8, RFP2, CDC16, CUL4A*), ubiquitin-dependent protein catabolism (*RNF6, C13orf22, UCHL3*), protein transport (*DNAJC15, KPNA3, RANBP5*), signaling (*FNBP4, AKAP11, STK24, ARHGEF7, GMFG*), cytoskeleton components (*TUBGCP3, PDLIM1*), and RNA metabolism (*NUFIP1, METTL3*). In particular, we identified the presence of the putative tumor suppressor genes *RFP2* and *RNF6* (mapping to 13q14.3 and q12.2, respectively), and *GLTSCR2*, located at 19q13.3. The supervised analysis did not identify the *RB1* tumor suppressor gene located at 13q14.2 as being differentially expressed between Δ^{13}-positive and Δ^{13}-negative cases, although a downregulation (average 1.8- fold change) was observed in del^{13}-positive (*data not shown*). However, the transcript could be identified in a SAM analysis made under less stringent conditions (FDR <1%) of those with a higher q-value (0.4711, over the 280th position in the list; *data not shown*). *RB1* expression levels were validated by means of a RT-Q-PCR analysis in a panel containing 15 Δ^{13}-positive and 14 Δ^{13}-negative samples from our dataset; this analysis showed a significant downregulation of *RB1* transcript in the Δ^{13}-positive patients ($p=4.205\times10^{-4}$ in a Wilcoxon's exact rank test; Figure 3).

Transcriptional regional analysis identified globally modulated regions in Δ^{13}-positive patients

The gene expression data from the Δ^{13}-positive and Δ^{13}-negative samples were also analyzed with respect to the physical localization of the genes in the genome in order to verify whether the deletion of chromosome 13 is reflected in chromosomal regions with transcriptional imbalances. To assess the correspondence between the differentially expressed chromosomal regions and Δ^{13}-positivity, the gene expression signals were analyzed using a non-parametric model-free statistical method (LAP). The LAP procedure allowed the identification (at a q-value=0) of 1063 differentially expressed genes located on chromosomes 1, 3, 11, 13, 14, and 19 (Figure 4). It is worth noting that almost the entire chromosome 13 was globally downregulated in the Δ^{13}-positive group. Additionally, downregulation was observed in the regions 19p13.3-p13.2 (absolute positions: 447,489-10,689,754 base pairs), 14q24.1-q24.3 (69,862,537-72,672,931 bp), 3p22.3-p21.1 (35,696,119-47,032,925 bp), 11p15.5-p15.3 (192,923-10,830,920 bp), 11q12.2-q14.3 (60,414,779-93,157,095 bp) and 11q22.3-q23.3 (102,318,933-117,735,511 bp). Finally, the LAP analysis revealed upregulation of chromosome 1 regions spanning 1q21-q22 and 1q31.3-q42.3 (155,393,568-237,264,911 bp).

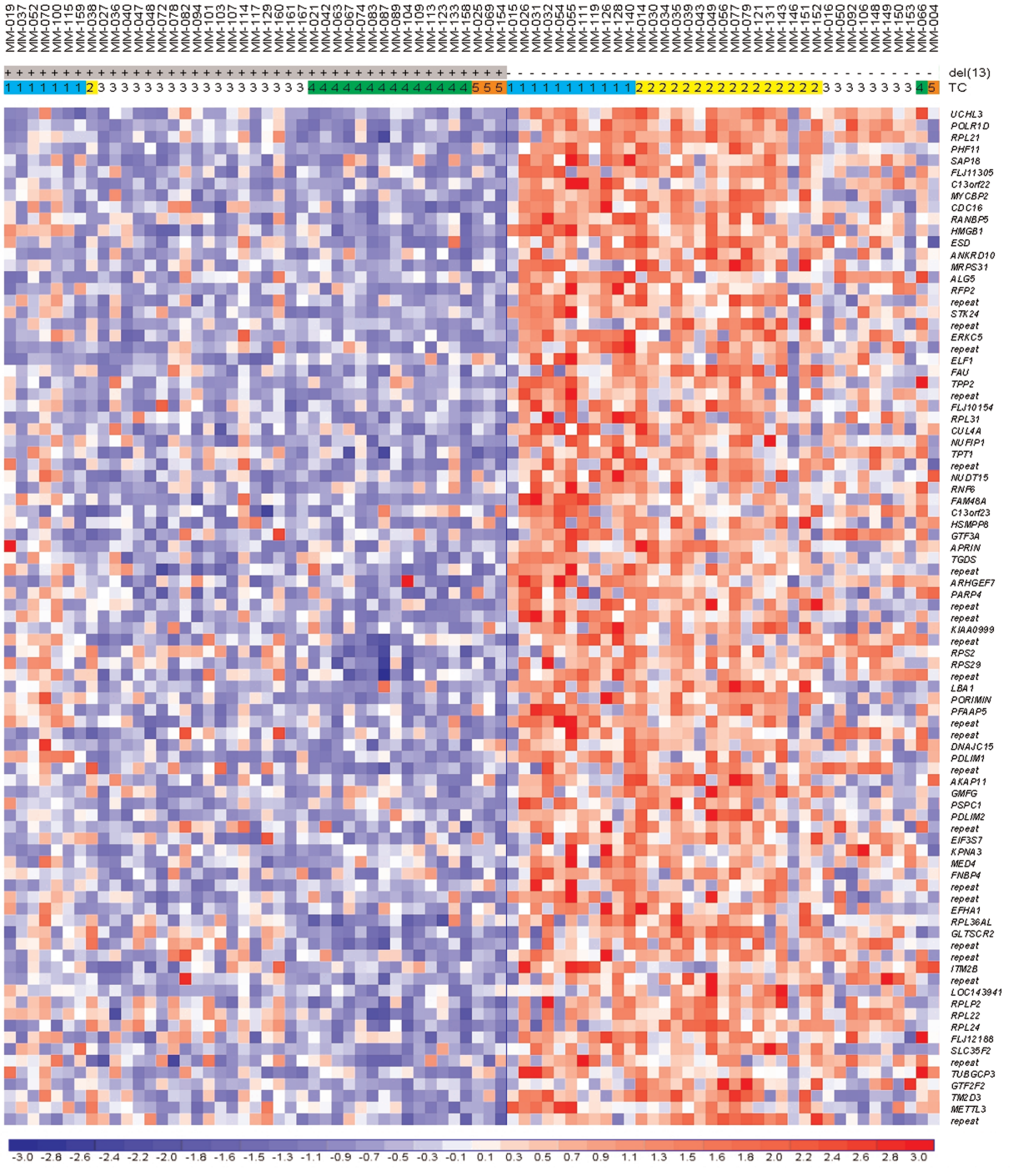

Figure 2. Supervised analysis of samples from Δ^{13}-positive vs Δ^{13}-negative MM patients. Expression profiles of the 80 MM samples (Δ^{13} status assessed by FISH) for the 87 probe sets selected by SAM analysis. The color scale bar represents the relative gene expression changes normalized by the standard deviation. Information about chromosome 13 deletion and TC class stratification is included alongside the MM patients' progressive numbers.

FISH analyses revealed a correlation of gene expression levels and chromosome copy number in Δ^{13}-positive and Δ^{13}-negative patients

Following the identification of globally modulated regions in Δ^{13}-positive tumors, FISH analyses were made in order to correlate the transcriptional profiles with their respective chromosome copy number in Δ^{13}-positive and Δ^{13}-negative patients. Chromosomes 1, 11 and 19 were investigated (Table 1), and extra copies of chromosome 11 were found in 33 (42%) of the 78 patients for whom material was available. There was a close correlation (p=5×10^{-4}) between Δ^{13}-positivity and the absence of

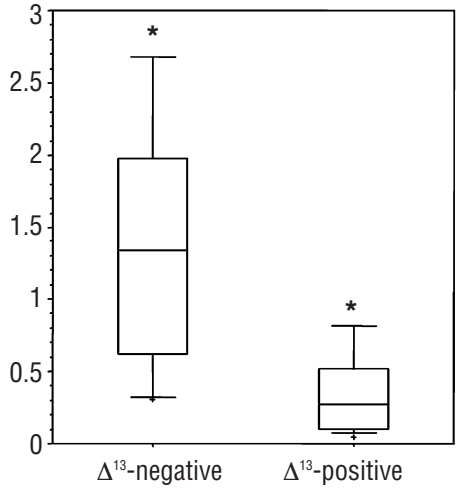

Figure 3. RT-Q-PCR analysis of *RB1*. Box plot of *RB1* mRNA expression levels obtained by means of RT-Q-PCR analysis in Δ13–negative and Δ13–positive patients.

additional copies of chromosome 11, as well as a valid correlation (p=0.015) between Δ13-positivity and the absence of extra copies of chromosome 19, which was found in 44 (60%) of the 73 patients for whom material was available. Finally, the two well-defined regions of chromosome 1q identified as upregulated by chromosomal regional analysis were analyzed using locus-specific probes and showed extra signals of both the 1q21 and 1q42 regions in 37 (53%) of 70 cases analyzed; a different number of extra copies was detected in only three cases (MM-069, MM-016 and MM-148). The results revealed a close correlation between Δ13-positivity and additional chromosome 1 material (28/39 patients vs 9/31 patients; p=6×10^{-4}), thus indicating that the extra copies of 1q21 and 1q42 regions can be considered specifically associated with Δ13-positivity in MM.

Discussion

Although Δ13 has frequently been identified as a hallmark in MM, many aspects concerning its biological consequences remain to be investigated; in particular, there is still a need for a comprehensive transcriptional analysis of Δ13 in MM patients at diagnosis. The aim of this study was to provide new insights into the molecular characterization of Δ13 in MM.

Our results confirm that Δ13 is rarely observed as a sole genetic abnormality in MM, a finding that had already suggested that the negative prognostic value of Δ13 should not be considered *per se* but in relation to its frequent association with other adverse prognostic factors, such as non-hyperdiploidy or the presence of t(4;14) and t(14;16)/t(14;20) chromosomal translocations.[34,35] Our analyses support this argument by showing that the clustering based on transcriptional profiling of MM samples was driven more by the distribution of the TC groups than by the presence of Δ13. They also further extend the evidence that Δ13 is randomly distributed among TC1 and TC3 patients, absent in TC2 patients, and strictly associated with patients in groups TC4 and TC5.[12] Moreover, an overall survival analysis of the patients in our dataset did not reveal any significant difference between Δ13-positive and Δ13-negative groups, although the relatively small number of cases, the treatment heterogeneity and the rather short follow-up do not allow any definite conclusions to be drawn (*Supplemental data 2*).

Previous data on the expression profiles associated with Δ13 are quite limited. Shaughnessy *et al.*[11] reported the results of a supervised analysis of Δ13-positive versus Δ13-negative patients in the context of patient stratification based on the presence/absence of cytogenetic alterations, and in relation to high-dose conventional therapy followed by autologous bone marrow transplantation. They found 35 differentially expressed genes, 32 of which (including *RB1*) mapped to chromosome 13, with only one (*IGFR1* localized on chromosome 12q) being upregulated in Δ13-positive patients. Our conventional supervised analyses on MM patients at diagnosis were made using stringent criteria and confirmed that Δ13-positivity leads to greater haploinsufficiency of specific chromosome 13 genes, as 44 of the 67 downregulated genes were localized on chromosome 13. It is worth noting that functional genomic annotation analyses of the selected genes indicate that these play important roles in basic cell biological processes. We identified *RFP2* and *RFN6*, which encode for RING finger proteins, as putative tumor suppressor genes located on chromosome 13. *RFP2* (also known as *LEU5*) maps within the minimally 13q deleted region close to D13S272, and shares significant homology with the *BRCA1* tumor suppressor gene;[36] *RFN6* maps to 13q12 and has been found to be deleted and mutated in esophageal squamous cell carcinomas. A third putative tumor suppressor gene, *GLTSCR*, localized on 19q13.3 and involved in the phosphorylation and stability of the tumor suppressor gene *PTEN*, is frequently associated with allelic loss in human diffuse gliomas. It is worth noting that the previously described *RFP2*, together with five of the other genes included in our list (*C13orf23, MRPS31, AKAP11, GTF2F2* and *NUDT15*), have recently been described by Carrasco *et al.*[37] as candidate genes at 13q14 showing significantly reduced expression in a subgroup of MM patients with Δ13, as assessed by array comparative genomic hybridization analyses.

As regards the putative tumor suppressor gene *RB1*, the published gene expression data are rather controversial: the earlier observation by Shaughnessy *et al.*,[11] showing *RB1* as a gene discriminating Δ13-positive from Δ13-negative patients has not been confirmed by more recent studies.[37,38] In our panel, RB1 was not identified as downregulated in Δ13-positive patients when the stringent criteria of SAM analysis were applied; however, differential expres-

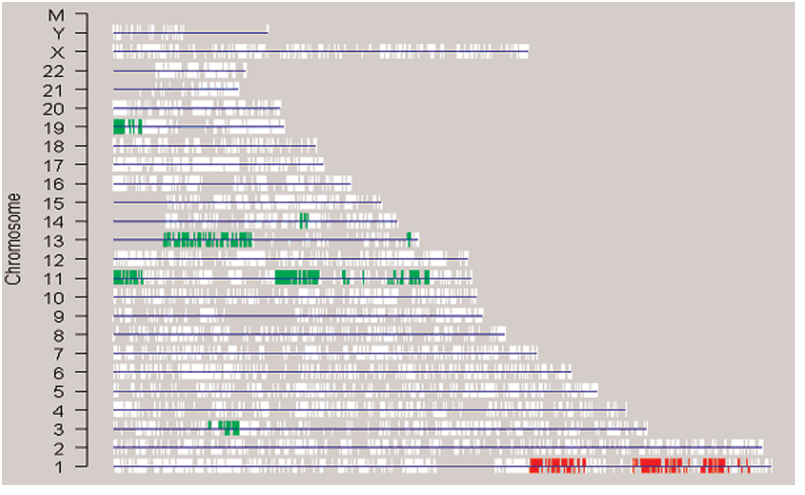

Figure 4. Regional analysis of samples from Δ^{13}-positive vs Δ^{13}-negative patients. Whole genome plot of the differentially expressed regions at *q*-value=0 in the Δ^{13}-positive and Δ^{13}-negative patients. The vertical axis represents the progressive chromosome number; the horizontal axis (blue lines) shows the progressive absolute position of the probes represented on the HG-U133A array for each chromosome. The white bars indicate the exact chromosomal locations, and the colored perpendicular lines the locations and up- (red) or down-regulation (green) of the 1063 differentially expressed genes in Δ^{13}-positive patients (*see geneplotter package from Bioconductor for details*).

sion was observed if the supervised analysis was performed using less stringent criteria, and was further supported by RT-Q-PCR validation of a subset of patients. The most likely explanation is the variability of *RB1* expression level (i.e. high variance) within Δ^{13}-positive and Δ^{13}-negative groups, as indicated by both the gene expression and RT-Q-PCR data.

A large proportion of the downregulated transcripts in Δ^{13}-positive patients, which were mainly localized on chromosome 13, encode for proteins involved in the negative control of cell proliferation and the cell cycle. For example, *SAP18* encodes for a protein that interacts with SIN3, a component of histone-deacetylase complexes, and enhances SIN3-mediated transcriptional repression,[39] and *APRIN* (also known as *AS3*) is implicated in negative cell cycle regulation by androgens in epithelial cells.[40] Furthermore, the product of the *CDC16* gene is a member of the anaphase-promoting complex, which functions as a protein ubiquitin ligase that regulates the mitotic cyclin degradation system governing the exit from mitosis. It has also been shown that inactivation of the *CUL4A* gene, which is involved in a similar pathway, causes massive DNA re-replication in *C. elegans*, most probably due to failed degradation of replication-licensing factors.[41] Interestingly, together with *CDC16* and *CUL4A*, many other genes encoding proteins involved in ubiquitin-dependent catabolism, such as the putative tumor suppressor genes *RNF6* and *RFP2* described above, and the *C13orf22, UCHL3, MYCBP2* and *TPP2* genes, seem to be downregulated in Δ^{13}-positive patients. Proteolysis via the ubiquitin system plays an important role in a variety of basic cell processes, such as cell cycle regulation, growth and differentiation. Ubiquitin-mediated protein degradation involves the conjugation of multiple moieties of ubiquitin to the protein, and the degradation of the conjugated protein by the 26S proteasome complex. Because of the large pleiotropic spectrum of protein substrates, the ubiquitin-proteasome pathway has been implicated in the pathogenesis of a number of diseases, including cancer. In recent years, new and selective proteasome inhibitors have been employed as anti-tumor agents, particularly in MM.[42] In addition, various down-regulated genes in Δ^{13}-positive patients (all located on 13q) are involved in DNA repair mechanisms (*PARP4, HMGB1, PSPC1, ERCC5*), and their defects are associated with a variety of human cancers. This finding strongly suggests that haploinsufficiency of the proteins encoded by these genes may play a role in the genomic instability and more adverse outcomes of Δ^{13}-positive patients.

A significant fraction of genes downregulated in Δ^{13}-positive patients encode for molecules involved in the biosynthesis of mainly ribosomal and translational-associated proteins, but only two of the 11 genes involved in this pathway are located on chromosome 13q. We have previously reported that the highly co-ordinated expression of genes involved in the translational machinery, mainly located on chromosome 19, is a distinctive feature of MM patients belonging to the TC2 group,[12] who show an almost complete association with gains in chromosome 11 and 19 (Table 1). The presence of TC2 patients in the Δ^{13}-positive group, and the highly significant inverse correlation between the presence of Δ^{13} and the presence of extra copies of chromosome 11 and 19 may, therefore, account for the relative downregulation of the genes involved in protein biosynthesis in Δ^{13}-positive patients.

As it is known that chromosomes 11 and 19 are associated with hyperdiploidy in MM, and previous findings indicate that Δ^{13} is associated with non-hyperdiploid tumors,[34] it can be argued that the transcriptional profiles observed in Δ^{13}-positive patients may be partially related to the ploidy status. However, we did not find a significant correlation between Δ^{13}-positivity and non-hyperdiploidy, or the downregulation of genes or modulated regions located on other chromosomes involved in hyperdiploidy (such 5, 9 and 15). These findings are in accordance with the observations of a recent study showing that Δ^{13}-positivity is associated with a fraction of hyperdiploid tumors.[37] Overall, it may be suggested that the

transcriptional profile of Δ^{13}-positive cases is not directly related to the ploidy status, despite the higher prevalence of *TC2* within Δ^{13}-negative patients and/or TC4 or TC5 translocations within Δ^{13}-positive patients.

One important finding of our study is the strong association between Δ^{13} MM patients and extra copies of chromosome 1q, in particular of the 1q21 and 1q42 regions. The association with 1q21 gain, determined by conventional cytogenetics, had been previously described by Nakagawa *et al.*,[43] although the number of cases was too few to define a significant correlation between the two aberrations. More recently, a significant association was reported by Gutierrez *et al.*, who used comparative genomic hybridization in a larger panel of cases.[44] As discussed above, the chromosome 1q gain, together with Δ^{13}-positivity, has recently been considered a discriminating molecular feature of hyperdiploid patients with a shorter survival.[37] Our study extends this finding by identifying a significant correlation between 1q21 and 1q42 regions and Δ^{13} using FISH and integrative genomic approaches. In particular, the application of the novel LAP algorithm to our series revealed the presence of globally upregulated expression levels for the genes residing at 1q21-1q42 in Δ^{13}-positive patients. It is worth noting that, when the transcriptional profiles of Δ^{13}-positive and Δ^{13}-negative patients were compared with an albeit limited number of four normal donors by means of the LAP algorithm, the results suggested that the upregulation of the 1q regions (absolute position: 162,332,107-229,426,616 bp) could be considered as being specific to the Δ^{13}-positive group, as well as the expected downregulation of most of chromosome 13, whereas the analysis did not provide any evidence of modulated expression on chromosomes 11 and 19 distinguishing Δ^{13}-positive and Δ^{13}-negative groups (*data not shown*). The fact that none of the genes found to be differentially expressed in our Δ^{13}-positive and Δ^{13}-negative samples was located on the 1q arm should be considered in the light of the stringent criteria applied in the supervised analysis (FDR=0%) because, when less stringent criteria were applied (FDR<15%, 2164 transcripts), an appreciable fraction of upregulated genes located on 1q arm (about 6 %) were identified in Δ^{13}-positive patients (*data not shown*).

One intriguing aspect of Δ^{13} in MM is the possible involvement of microRNA localized in this chromosome. MicroRNA represent a growing class of small non-coding RNA that are thought to regulate gene expression; the loss or amplification of microRNA has been reported in a large variety of tumors and may affect normal cell growth and proliferation. Interestingly, two microRNA are deleted and downregulated in B-cell chronic lymphocytic leukemia with deletion and translocations at 13q14. Furthermore, it has been reported that microRNA profiles can be useful to distinguish B-cell chronic lymphocytic leukemias with different clinical outcomes.[45] Future investigation of microRNA profiles in MM may provide important and novel contributions to the understanding of the mechanisms of myelomagenesis.

In conclusion, our findings indicate that the transcriptional differences between Δ^{13}-positive and Δ^{13}-negative patients mainly involve genes located on chromosome 13, thus suggesting the existence of a strict correlation between transcriptional features and chromosomal alterations. Our data also show that the impact of Δ^{13} on the neoplastic phenotype should be considered together with concomitant abnormalities, such as the gain of chromosome 1q21-q42 region.

Author Contributions

LA: preparation and analysis of gene expression profiling data, and manuscript preparation; SB, AC: statistical analysis and manuscript preparation; SF: FISH analyses; LB, FM, FB, GLD: patients recruitment, clinical data and critical revision of the manuscript; DI: RT-Q-PCR analyses; DV: cell purification and FACS analyses; LL: study design and manuscript preparation; AN: study design and manuscript preparation. All authors approved the final version of the article to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

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