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UNIVERSITÀ DEGLI STUDI DI TORINO

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Genome-wide analysis of primary plasma cell leukemia identifies recurrent imbalances associated with changes in transcriptional profiles

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Abstract

Primary plasma cell leukemia (pPCL) is a rare, yet aggressive form of de novo plasma cell tumor, distinct from secondary PCL (sPCL) which represents a leukemic transformation of pre-existing multiple myeloma (MM). Herein, we performed a comprehensive molecular analysis of a prospective series of pPCLs by means of FISH, single nucleotide polymorphism (SNP) array and gene expression profiling (GEP). IGH@ translocations were identified in 87% of pPCL cases, with prevalence of t(11;14) (40%) and t(14;16) (30.5%), whereas the most frequent numerical alterations involved 1p (38%), 1q (48%), 6q (29%), 8p (42%), 13q (74%), 14q (71%), 16q (53%), and 17p (35%). We identified a minimal biallelic deletion (1.5 Mb) in 8p21.2 encompassing the PPP2R2A

gene, belonging to a family of putative tumor suppressors and found to be significantly down-regulated in deleted cases. Mutations of TP53 were identified in four cases, all but one associated with a monoallelic deletion of the gene, whereas activating mutations of the BRAF oncogene occurred in one case and were absent in N- and K-RAS. To evaluate the influence of allelic imbalances in transcriptional expression we performed an integrated genomic analysis with GEP data, showing a significant dosage effect of genes involved in transcription, translation, methyltransferase activity, apoptosis as well as Wnt and NF- κ B signaling pathways. Overall, we provide a compendium of genomic alterations in a prospective series of pPCLs which may contribute to improve our understanding of the pathogenesis of this aggressive form of plasma cell dyscrasia and the mechanisms of tumor progression in MM. *Am. J. Hematol.* 2013

Introduction

Primary plasma cell leukemia (pPCL) is a rare, yet aggressive form of plasma cell tumor accounting for 1–4% of plasma cell dyscrasias. According to current accepted criteria, pPCL is defined by circulating plasma cells $>2 \times 10^9/l$ or a relative plasmacytosis $>20\%$ of peripheral blood leukocytes with evidence of monoclonal gammopathy [1]. pPCL can be distinguished from secondary PCL (sPCL) which represents the leukemic transformation of a previously diagnosed multiple myeloma (MM). As described by several authors [1, 2], pPCL shows characteristic clinical–biological features compared with MM or sPCL, such as younger age (about a decade), more frequent extramedullary disease, renal failure, higher bone marrow infiltration, and proliferative activity of the malignant clone, and less bone disease.

Genomic studies from a retrospective series of pPCLs, mainly based on fluorescence in situ hybridization (FISH) analyses, indicated a spectrum of major genetic lesions overlapping those found in MM, although higher frequency of t(11;14) and hypodiploidy have been reported [3–8]. MYC rearrangements may also occur in pPCL associated with worse clinical outcome [6, 8].

In the present report, we performed a comprehensive molecular analysis of a prospective series of pPCLs using FISH and single nucleotide polymorphism (SNP) array approaches. Moreover, since global gene expression profiles were also available, we performed an integrated genomic analysis to evaluate the influence of distinct allelic imbalances in the expression of specific genes in pPCL.

Methods

Patients

Our series included 23 newly diagnosed primary pPCL patients (Table I), enrolled in a multicenter Italian clinical trial (RV-PCL-PI-350, EudraCT 2008-003246-28), aimed at evaluating safety and anti-tumor activity of combined lenalidomide and dexamethasone as first-line treatment. The median age was 60 years (range, 44–80) and the sex ratio (M/F) was 0.92. For the purpose of the present work, we considered progression free (PFS) and overall survival (OS), defined as secondary endpoints of the study, and evaluated their association with the occurrence of genomic alterations; a detailed description of the study is beyond the scope of this work and will be provided elsewhere (Musto et al., manuscript in preparation). The median follow-up was 23 months (range, 9–32). Highly purified ($\geq 90\%$) bone marrow plasma cell (PC) samples were obtained using CD138 immunomagnetic microbeads as previously described [9].

Table I. Biological and Clinical Characteristics of the 23 PCLs Included in the Study

Patients	Age	Sex	PP type	del (13q) ^a	del (17p) ^a	1q gain	1p loss ^a	8p loss % MB ^b	t(4;14)	t(6;14)	t(11;14)	t(14,16)	t(14,20)	c-myc	TP53 mutation	N,K-RAS mutation	BRAF mutation	GEP	SNP
PCL-010	80	M	IgGλ	-	-	-	-	nd	-	nd	+	-	-	nd	nd	nd	nd	Y	N
PCL-014	46	M	BU-λ	+	+	-	+	nd	-	nd	+	-	-	nd	nd	nd	nd	Y	N
PCL-015	78	M	BU-k	+	-	+	-	93	-	-	-	+	-	nd	wt	wt	V600E	Y	Y
PCL-016	57	F	IgGκ	+	-	+	+	-	-	-	-	+	-	-	wt	wt	wt	Y	Y ^c
PCL-017	68	F	IgGκ	+	+	+	+	-	-	-	-	+	-	+	wt	wt	wt	Y	Y ^c
PCL-018	59	F	BU-k	+	+	-	-	-	-	-	+	-	-	-	wt	wt	wt	Y	Y ^c
PCL-019	67	F	IgMκ	+	+	+	+	73	-	-	-	+	-	-	R280K	wt	wt	Y	Y ^c
PCL-020	79	F	IgGλ	-	-	+	-	-	-	-	-	-	-	-	wt	wt	wt	Y	Y ^c
PCL-021	48	M	IgGλ	+	-	+	-	88/10	+	-	-	-	-	-	wt	wt	wt	Y	Y
PCL-022	50	M	IgGκ	+	+	-	+	79	-	-	+	-	-	-	nd	nd	nd	Y	N
PCL-023	60	M	IgGκ	+	+	+	+	nd	-	nd	-	-	-	nd	wt	nd	nd	Y	Y ^c
PCL-024	44	M	IgDλ	+	-	nd	nd	nd	-	nd	-	-	nd	nd	nd	nd	nd	N	N
PCL-025	64	F	IgGκ	-	-	+	+	-	-	-	-	-	nd	nd	nd	nd	nd	N	N
PCL-026	59	M	IgGκ	+	-	+	+	-	-	-	-	+	-	+	wt	wt	wt	Y	Y
PCL-027	65	M	BU-λ	-	+	-	-	100	-	-	+	-	-	-	I195T	wt	wt	Y	Y
PCL-028	57	F	BU-k	+	+	-	-	-	-	-	+	-	-	-	wt	wt	wt	Y	Y ^c
PCL-029	51	M	IgAλ	-	-	-	-	-	-	-	-	+	-	-	wt	wt	wt	Y	Y ^c
PCL-030	52	F	BU-k	+	+	-	+	69/29	-	-	-	-	+	-	R273C	wt	wt	Y	Y ^c
PCL-032	65	F	IgGκ	+	-	+	-	nd	+	-	-	-	-	nd	wt	wt	wt	Y	Y
PCL-034	59	F	IgGλ	+	-	nd	nd	nd	+	nd	-	-	-	nd	nd	nd	nd	Y	N
PCL-035	76	F	BU-k	-	-	-	-	nd	-	-	+	-	-	-	wt	wt	wt	Y	Y
PCL-036	71	M	IgGκ	+	-	-	-	nd	-	nd	+	-	-	-	wt	wt	wt	Y	Y
PCL-037	72	M	IgAλ	+	-	-	-	-	-	-	+	-	-	-	466_471 del6	nd	nd	Y	Y
postested (%)				17/23	8/23	10/21	8/21	6/15	3/23	0/17	9/23	7/23	1/21	2/15	4/17	0/15	1/15		
				73.9%	34.8%	47.6%	38.1%	40.0%	13.0%	0.0%	39.1%	30.4%	4.8%	13.3%	23.5%	0.0%	6.7%		

- A: Only monoallelic deletion.
- B: M, monoallelic deletion; B, biallelic deletion.
- C: CD138-negative counterpart available.

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PCL-014	46	M	B μ - λ	+	+	-	+	nd	-	nd	+	-	-	nd	nd	nd	nd	Y	N
PCL-015	78	M	B μ -k	+	-	+	-	93	-	nd	-	+	-	nd	wt	wt	V600E	Y	Y
PCL-016	57	F	IgGk	+	-	+	+	-	-	-	-	+	-	-	wt	wt	wt	Y	Y ^c
PCL-017	68	F	IgGk	+	+	+	+	-	-	-	-	+	-	+	wt	wt	wt	Y	Y ^c
PCL-018	59	F	B μ -k	+	+	-	-	-	-	-	-	-	-	-	wt	wt	wt	Y	Y ^c
PCL-019	67	F	IgMk	+	+	+	+	73	-	-	+	+	-	-	wt	wt	wt	Y	Y ^c
PCL-020	79	F	IgG λ	-	-	+	-	-	-	-	-	-	-	-	wt	wt	wt	Y	Y
PCL-021	48	M	IgG λ	+	+	+	-	88/10	+	-	-	-	-	-	wt	wt	wt	Y	Y
PCL-022	50	M	IgGk	+	+	+	+	79	-	-	+	-	-	-	nd	nd	nd	Y	N
PCL-023	60	M	IgGk	+	+	+	+	nd	-	nd	-	+	-	nd	wt	nd	nd	Y	Y ^c
PCL-024	44	M	IgD λ	+	-	nd	nd	nd	-	nd	-	-	nd	nd	nd	nd	nd	N	N
PCL-025	64	F	IgGk	+	-	+	+	-	-	-	-	-	nd	nd	nd	nd	nd	N	N
PCL-026	59	M	IgGk	+	+	+	+	-	-	-	-	+	-	+	wt	wt	wt	Y	Y
PCL-027	65	M	B μ - λ	-	+	-	-	100	-	-	+	-	-	-	1195T	wt	wt	Y	Y
PCL-028	57	F	B μ -k	+	-	-	-	-	-	-	+	-	-	-	wt	wt	wt	Y	Y ^c
PCL-029	51	M	IgA λ	+	-	-	-	-	-	-	+	-	-	-	wt	wt	wt	Y	Y ^c
PCL-030	52	F	B μ -k	+	+	-	+	69/29	-	-	-	+	-	-	wt	wt	wt	Y	Y ^c
PCL-032	65	F	IgGk	+	+	+	+	nd	-	-	-	-	+	nd	wt	wt	wt	Y	Y
PCL-034	59	F	IgG λ	+	-	nd	nd	nd	+	nd	-	-	-	nd	nd	nd	nd	Y	N
PCL-035	76	F	B μ -k	-	-	-	-	nd	-	-	+	-	-	-	wt	wt	wt	Y	Y
PCL-036	71	M	IgGk	+	-	-	-	nd	-	nd	+	-	-	-	wt	wt	wt	Y	Y
PCL-037	72	M	IgA λ	+	-	-	-	-	-	nd	+	-	-	-	wt	wt	nd	Y	Y
post/tested (%)				17/23 73.9%	8/23 34.8%	10/21 47.6%	8/21 38.1%	6/15 40.0%	3/23 13.0%	0/17 0.0%	9/23 39.1%	7/23 30.4%	1/21 4.8%	2/15 13.3%	466_471 del6 4/17 23.5%	0/15 0.0%	1/15 6.7%	Y	Y

^a Only monoallelic deletion.
^b M, monoallelic deletion; B, biallelic deletion.
^c CD138-negative counterpart available.

Fluorescence in situ hybridization

Pathological samples from all patients were characterized by FISH analysis for the presence of the main IGH@ chromosomal translocations and for del(13)(q14), del(17)(p13), 1p33 (CDKN2C) loss and 1q21.3 (CKS1B) gain aberrations, as previously described [10–12]. The cut-off levels considered were 10% for fusion or break-apart probes and 20% for numerical abnormalities, as recommended by the European Myeloma Network (EMN) [13]. The SNP-array data concerning deletions of 8p21.2 region were validated by previously described FISH standard protocols [11] using the chromosome 8 alpha satellite probe and the BAC clone RP11-795G8 specific for the PPP2R2A gene selected from the UCSC Genome Browser Database (<http://genome.ucsc.edu/>). The presence of mono- and biallelic deletion of the TRAF3 gene at 14q32.32 was validated by FISH using the specific BAC clone CTD-3235K2 and the fosmid G248P81572G6 as previously reported by Keats et al. [14] in MM patients.

High-density SNP-arrays

High-density SNP-arrays were performed in 17 patients (Table I). Total genomic DNA was extracted using Wizard genomic purification DNA kit (Promega), then processed and hybridized on Affymetrix GeneChip® Human Mapping 250K NspI arrays following the manufacturer's protocol (Affymetrix). Each sample was compared with a set of 48 normal Caucasian HapMap references available on the Affymetrix web site (http://www.affymetrix.com/support/technical/sample_data/500k_data.affx). Genotyping Console (GC) was used to extract raw DNA copy number (CN) and loss-of-heterozygosity (LOH) probability from CEL files according to Affymetrix guidelines. CN was then estimated using circular binary segmentation and normalized on FISH data using DNACopy and FBN packages in R software, respectively, as previously described [10, 15]. LOH was defined by applying a 1 Mb smoothing window on the GC-derived value with a probability higher than 0.95 for the detection of a monoallelic polymorphism. Furthermore, DNA CN and LOH probability estimation using CD138 negative bone marrow population as normal counterpart (i.e., CD138+ <0.5% as assessed by FACS analysis) was performed in 8 patients. The thresholds for the identification of the gained and lost genomic regions resulted as follows: inferred CNs of more than 2.18, 2.52 and 3.41 corresponded to 3, 4 and 5 or more copies, whereas CNs of less than 1.75 and 1.34 corresponded to mono- and biallelic deletion.

Gene expression profiling

Total RNA was available in 21 cases and purified using the RNeasy® Total RNA Isolation Kit (Qiagen, Valencia, CA). Preparation of DNA single-stranded sense target, hybridization to GeneChip® Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA) and scanning of the arrays (7G Scanner, Affymetrix) were performed according to the manufacturer's protocols. Log₂-transformed expression values were extracted from CEL files and normalized using NetAffx Transcript Cluster Annotations, Release 31 and robust multiarray average (RMA) procedure in Expression Console software (Affymetrix). The expression values of transcript cluster IDs specific for loci representing naturally occurring read-through transcriptions were summarized as the median value for each sample. Supervised analyses were performed using Significant Analysis of Microarrays software, version 4.0 [16] as previously described [17]. The functional annotation analysis on the selected lists was performed by means of NetAffx (<https://www.affymetrix.com/analysis/netaffx/>) and the

Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 (U.S. National Institutes of Health at <http://david.abcc.ncifcrf.gov/>) tools. Only Gene Ontology Biological Process and Molecular Function terms were selected as annotation categories and high classification stringency was set for the analysis, using the DAVID Functional Annotation Clustering option. The Annotation Clusters with an Enrichment Score (ES) >1.3 were considered significant.

To evaluate whether gene dosage effects could be identified for specific genes, the relationship between relative transcript expression levels and the inferred CNs of the corresponding locus (according to NCBI36/hg18) was measured by means of Kendall's τ correlation test (P-value <0.005). The analyses were made using conventional R software packages.

The genotyping and gene expression data have been deposited at NCBI Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO Series accession number GSE39383.

Mutation analyses

TP53 mutation analysis was performed on genomic DNA of 17 pPCLs. Oligonucleotide primers used to amplify TP53 exons 5–9 and polymerase chain reaction (PCR) protocols were downloaded from the IARC TP53 database website (http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf). The amplicons were sequenced directly with sense and anti-sense primers using the Big Dye terminator v1.1 cycle sequencing kit in ABI PRISM 310 automated sequencer (Applied Biosystems, FosterCity, CA).

Mutations of BRAF (codon 600), KRAS (codons 12, 13, 61, 146) and NRAS (codons 12, 13, 61) genes were investigated by means of pyrosequencing analysis on genomic DNA of 15 pPCLs using CE-IVD kits “Anti-EGFR MoAB response” for BRAF, KRAS, and NRAS status (Diatech Pharmacogenetics, Italy). Briefly, 50–100 ng of genomic DNA were amplified using Rotor-Gene 6000 Q (Corbett Research, Australia) and 20 μ l of each PCR product were analyzed by pyrosequencing analysis using PyroMark Q96 ID instrument and Gold Q96 reagents (Qiagen), in accordance with the manufacturer's instructions. The sequences were analyzed with PyroMark ID 1.0 software (Qiagen) according to the commercial kit handbooks.

Survival analysis

Patients were stratified into two groups according to the occurrence of the genomic aberrations under consideration. The groups were tested for association with survival using the Kaplan-Meier estimator and log-rank test, and P-values were calculated according to the standard normal asymptotic distribution. Survival analysis was conducted with the `survcomp` package in R software.

Results

We first characterized 23 pPCL patients included in our dataset by FISH for the major cytogenetic alterations (Table I). The deletion of 13q was identified in 17 patients (73.9%), whereas del(17)(p13) was found in 8 cases (34.8%). Translocations at 14q32 involving IGH@ locus were detected in the large majority of cases (20/23=87%); t(11;14) was the most frequent (9/23=39.1%), t(14;16) was identified in 7 cases (30.4%), and t(4;14) was present in 3 patients (13%). The t(14;20) was investigated in 21 cases and showed a positive result in one case (4.8%). The t(6;14) was analyzed in 17 cases, all of which found to be negative for the lesion. Alterations of chromosome 1 were analyzed in 21 patients and 1p loss was identified in 8 cases (38.1%) and 1q gain in 10

(47.6%); five patients showed the concomitant presence of both aberrations. Finally, rearrangements of c-MYC gene were detected in two samples (2/15=13.3%). Overall, a contingency analysis revealed highly significant inverse correlation between the presence of 1q gain and the occurrence of the t(11;14) (P-adjusted=0.006).

We tested whether the occurrence of any of the identified cytogenetic alterations might correlate with different outcome. In particular, we evaluated whether the prevalence of t(4;14), t(11;14), MAF-related translocations, 1p loss, 1q gain, del(13)(q14), and del(17)(p13) correlated with the secondary endpoints of the clinical trial, namely OS and PFS. However, none of the considered cytogenetic aberrations was significantly associated neither with OS nor with PFS (Supporting Information Fig. 1).

SNP-array

The genome-wide profiles were investigated by SNP-array in the 17 pPCLs for whom DNA material was available. To confirm CN and LOH estimation based on public references, CN and LOH were also estimated using DNA from CD138 negative bone marrow populations available from eight patients (Table I). With the exception of point-wise aberrant signals that were discarded from downstream analyses, all the estimated CN segments were concordant within ± 0.1 , whereas the estimated probabilities of LOH (higher than 95% versus the normal samples subset) overlapped exactly with those obtained comparing the eight tumor samples against their normal counterparts (data not shown), suggesting the accuracy of the reported results. The analysis allowed the identification of copy number alterations (CNA) in all cases, showing complete concordance with FISH data. A total of 454 aberrations were detected with a median of 20 per sample (range 6-82). Losses were more prevalent (273/454 CNAs; 60.1%), including mono- (229/454=50.4%) or biallelic deletions (44/454=9.7%) (Fig. 1 and Supporting Information Table I). Of the remaining CNAs, 165 (36.3%) were gains (3 or 4 copies), and 16 (3.5%) represented amplifications (5 or more copies). The latter were identified in 2 patients showing a near-tetraploid karyotype (PCL-020) and a complex pattern involving chromosome 16q characterized by wide regions of amplification and loss (PCL-027) (Supporting Information Table I). Supporting Information Fig. 3 and Supporting Information Table II described the major regions of occurrence of LOH identified in our series.

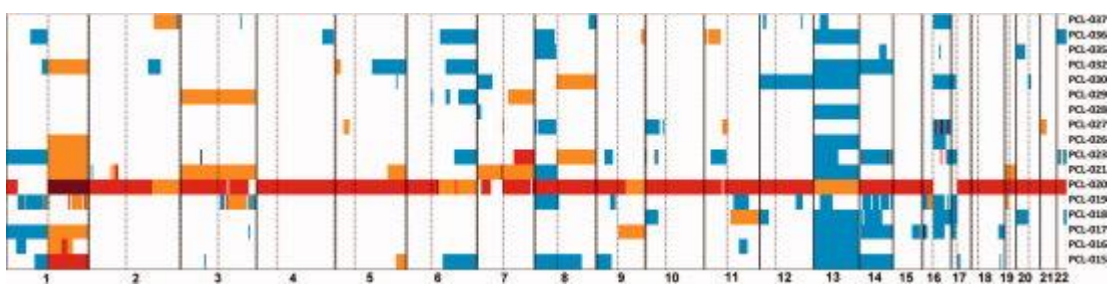


Figure 1. Heatmap of significantly altered DNA copy number regions in pPCL samples. Horizontal axis: chromosome localization. The dashed lines represent the location of the centromeres. Dark blue: biallelic deletion; light blue: loss; white: normal CN; orange: DNA gain (three copies); red: four or more copies.

Table II. Functional Annotations of the 8 Downregulated Genes in 8p Loss Patients by SAM Analysis at q-Value = 0

Gene Symbol	Gene Title	Score(d)	Fold Change	Cytoband	Gene Ontology Description
SLC25A3	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	-6.44	-1.73	12q23.1	generation of precursor metabolites and energy; transmembrane transport
METAP2	Methionyl aminopeptidase 2	-4.94	-1.73	12q22	proteolysis; protein processing
PPP2R2A	Protein phosphatase 2, regulatory subunit B, alpha	-4.9	-1.9	8p21.2	protein serine/threonine phosphatase activity; protein dephosphorylation; signal transduction
CCDC25	Coiled-coil domain containing 25	-4.47	-1.86	8p21.1	-
WRN	Werner syndrome, RecQ helicase-like	-4.41	-2.22	8p12	cell aging; telomere maintenance; regulation of growth; regulation of apoptosis; cellular response to stress; DNA synthesis during DNA repair; response to DNA damage stimulus; response to oxidative stress; response to radiation; response to UV; response to light stimulus; response to abiotic stimulus; response to UV-C; homeostatic process
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	-4.4	-2.7	8p21.3	induction of apoptosis; activation of NF-kappaB-inducing kinase activity; positive regulation of signal transduction; regulation of phosphate metabolic process
MAK16	MAK16 homolog (S. cerevisiae)	-4.2	-2.01	8p12	nucleolus
KIAA1967	KIAA1967	-4.15	-1.61	8p21.3	enzyme inhibitor activity; negative regulation of catalytic activity; regulation of transcription elongation, DNA-dependent; positive regulation of apoptosis

Since the majority of CNAs affected only a few patients, we focused our attention on chromosomal regions commonly involved in at least 5 patients (almost 30% of cases). Following this approach, chromosomes 1, 6, 8, 13, 14, 16, and 17 were the most frequently altered in our series and will be discussed in further details (Supporting Information Fig. 2A–F and Fig. 2). For each of these chromosomes the most recurrent segments of alteration were defined as minimally altered regions (MARs).

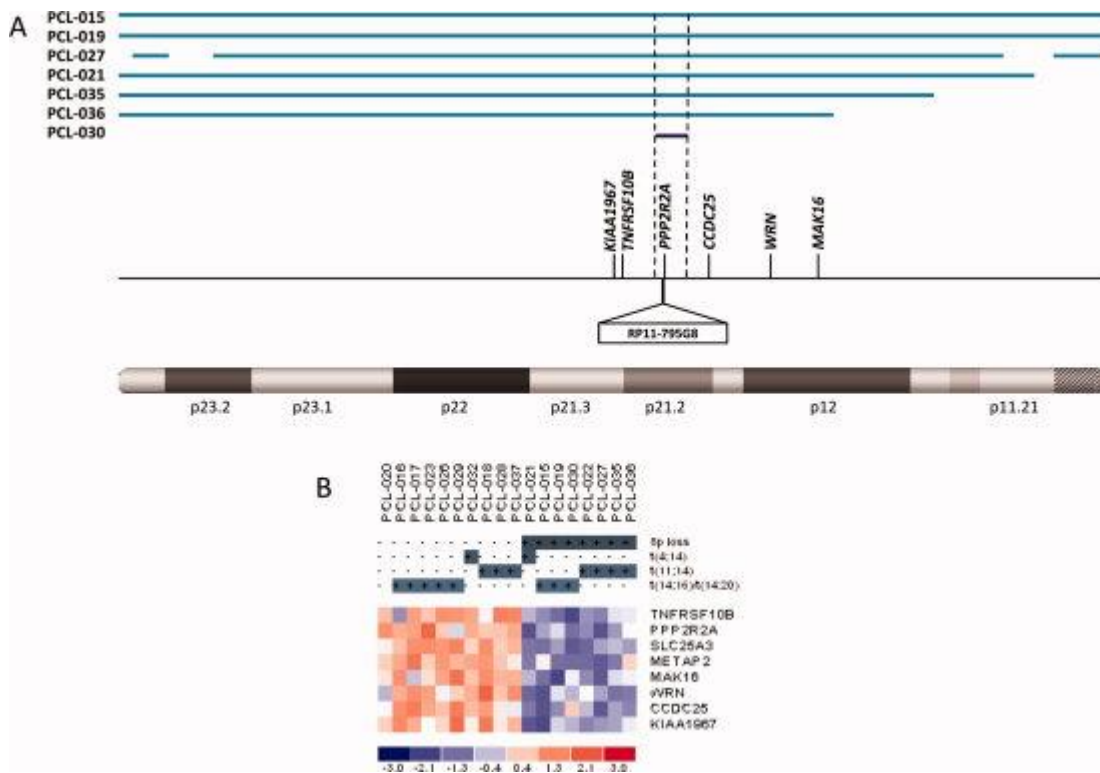


Figure 2. A: Chromosome 8p loss pattern in 7 pPCL patients detected by SNP-array. The common region of loss of 8p is indicated by dashed vertical lines. Each horizontal blue line indicates the range of loss of each pPCL patient. Light blue represents monoallelic deletion, dark blue biallelic loss. Physical position of FISH clone RP11-795G8 used to perform the 8p loss analysis and the localization of KIAA1967, TNFRSF10B,

PPP2R2A, CCDC25, WRN, and MAK16 genes are also indicated. B: Supervised analysis of 8p loss versus 8p normal pPCLs. By means of SAM supervised analysis (q-value=0), 8 genes resulted differentially expressed. Information about t(4;14), t(11;14), t(14;16)/t(14;20), and 8p loss are included alongside the patient ID (“+” = positive, “-” = negative). The color scale bar represents the relative gene expression changes normalized by the standard deviation, and the color changes in each row represent gene expression relative to the mean across the samples. The gene symbols are indicated.

Chromosome 1

As shown in Supporting Information Fig. 2A, 12 patients (70.6%) showed alterations of chromosome 1. Deletions of 1p were detected in 8 cases (47.1%), six of which had single or multiple interstitial deletions whereas two cases (PCL-017 and PCL-023) lost the whole short arm. Three MARs could be identified: the 1p33-p32.3 region (2.7 Mb in size) lost in five patients and encompassing CDKN2C and part of FAF1 gene, the 1p22.1 (1.3 Mb) containing TGFBR3, MTF2, and TMED5 genes and 1p13.3-p12 (6.9 Mb) regions including the FAM46C locus deleted in 6 (35%) cases. A short (100kb) homozygous deletion involving the AGLB4 gene, was identified at 1p33 in a single case (PCL-016).

Gains of 1q were identified in 9 cases (52.9%), 4 of which showed 3 copies of the whole long arm. Four MARs were detected on 1q; the largest (6.7 Mb), mapping on 1q21.2-q23.1, included CKS1B gene, whereas the remaining regions of 0.9, 1.3, and 3.6 Mb in size, were located at 1q25.1-q25.2, 1q25.2-q25.3, and 1q32.1, respectively (Supporting Information Fig. 2A)

Chromosome 6

Deletions of 6q were found in 5/17 cases (29.4%) with a common large telomeric deletion (43.5 Mb) at 6q22.32-ter including SGK1, TNFAIP3 and SOD2 genes. One patient (PCL-029) showed two additional interstitial deletions of 4.3 and 12.1 Mb at 6q11.1-q12 and 6q16.1-q21, respectively (see Supporting Information Fig. 2B).

Chromosome 8

Extensive deletions of the short arm of chromosome 8 were detected in 6 (41.2%) cases, whereas an additional case (PCL-030) showed a homozygous deletion at 8p21.2 defining a putative MAR of 1.5 Mb containing the PPP2R2A gene (Fig. 2A). This gene codes for a regulatory subunit of the protein phosphatase 2 (PP2A), implicated in the negative control of cell growth and division [18].

Several authors have suggested that deletion of the 8p represents a potential pathogenetic lesion in MM [19–21], although not yet fully elucidated. In our study, we investigated further the involvement of the 8p21.2 in pPCL; in particular, we performed FISH using the BAC clone (RP11-795G8) specific for the PPP2R2A gene. Hybridizations were performed in 15 patients for whom material was available, 13 of which analyzed by SNP-array. FISH analyses confirmed SNP data and copy number assignment in all patients; furthermore one case (PCL-022), not analyzed by SNP-array, showed a monoallelic deletion. Interestingly, FISH analysis revealed that case PCL-021, for whom SNP analysis indicated monoallelic deletion, had a subclone of approximately 10% of cells harboring the biallelic deletion of the 8p21.2, a finding not identified by microarray analysis, in all likelihood due to the low percentage of altered cells. Although this percentage is lower than that (20%) proposed by EMN criteria to define gains or losses positivity, we believe that it could be a reliable result based on the identification of two signals specific for the centromeric 8 alpha satellite

probe in all the nuclei presenting the homozygous deletion (Table I). Overall, the putative MAR at 8p21.2, containing the PPP2R2A gene, was lost in 8/19 (42%) patients. Of the six cases with monoallelic deletion investigated by SNP-arrays, five had LOH overlapping the deleted regions. PCL-036 lacked LOH where the inferred CN indicated allelic loss, a finding suggestive of the occurrence of subclones (Supporting Information Fig. 3 and Supporting Information Table II).

We investigated whether the 8p deletion could be associated with a specific transcriptional profile and/or the occurrence of a gene dosage effect. A supervised analysis was performed comparing the expression profiles of the 8p deleted (8) and wild-type (10) pPCL patients for whom FISH or SNP-array data concerning the alteration were available. Eight genes were found significantly (q -value=0) down-regulated in pPCLs with 8p loss, two of which mapped to 12q and six to 8p (4 in 8p21.3-8p21.1 and 2 in 8p12) (Fig. 2B). Among them we evidenced, in addition to PPP2R2A gene, the KIAA1967 gene involved in positive regulation of apoptosis; the TNFRSF10B gene, a member of the TNF-receptor superfamily, inducing pro-apoptotic signals; the WRN gene, a DNA helicase acting in DNA repair, replication and telomere maintenance; and the CCDC25 gene with unknown function, found deleted in hepatocellular carcinoma patients associated with poor outcome [22] (Table II).

Chromosome 13

As shown in Supporting Information Fig. 2C, deletion of 13q was the most frequent alteration (13/17=76.5%) detected in our dataset, with most cases (9/13=69.2%) showing loss of the entire long arm. The MAR at 13q was defined in PCL-037 and showed a large interstitial deletion of 20.4 Mb located between 13q12.3-q14.3 and containing 127 genes. The region included RB1 and the two miRNA transcript miR-15a and miR-16-1; however, no significant difference in the expression of neither RB1 nor miR-15a and miR-16-1 was evidenced between deleted and wild-type cases (data not shown). A short homozygous deletion of 125 kb was identified in only one patient (PCL-017) at 13q31.3 where the GPC5 gene is located. All the cases with 13q deletion but one had LOH overlapping the deleted regions. In PCL-018 FISH analysis revealed the presence of two putative subclones, respectively involving the 31% and the 62% of the nuclei analyzed, that in all likelihood may explain the absence of LOH (Supporting Information Fig. 3 and Supporting Information Table II).

Chromosome 14

Deletions involving 14q were identified in 12 cases (70.6%), with two patients (PCL-015 and PCL-032) showing loss of the whole chromosome and nine patients having interstitial/telomeric deletions (Supporting Information Fig. 2D). Four MARs could be identified: at 14q23 (1 Mb) and 14q24.2-q24.3 (4.6 Mb) in 7 patients (41.2%); 14q31.3 (1.3 Mb) in 6 cases (35.3%); and 14q32.31-q32.32 (1.2 Mb) in 5 samples (29.4%). Interestingly, three of the five patients harboring the telomeric MAR showed a biallelic deletion of at least 180 kb involving AMN and CDC42BPB and disrupting TRAF3 genes. These findings were validated by FISH using the BAC CTD-3235K2 and the fosmid G248P81572G6 clones (data not shown). Finally, deletion of 14q32.33-qter, associated with the loss of the IGVH gene, was identified in 8 cases, 3 of which showing a homozygous deletion.

Chromosome 16

Deletions of chromosome 16q were identified in 9 cases (52.9%) (Supporting Information Fig. 2E). Interestingly, two cases (PCL-018 and PCL-030) lost the entire long arm and seven samples showed

interstitial/telomeric losses of 16q leading to the identification of four MARs: at 16q12.1 (1.2 Mb in 7/17=41.2%) encompassing the *CYLD* gene; 16q21 (3.6 Mb in 8/17=47.1%); 16q22.2-q22.3 (3.3 Mb in 7/17=41.2%) and 16q23.1-q23.2 (1.4 Mb in 7/17=41.2%) containing *WWOX*. In line with what previously reported in MM [10, 23], in our series *WWOX* expression level was significantly lower in cases with LOH (P-value=0.037); similarly, *CYLD* showed reduced expression associated with LOH, although not reaching statistical significance (P-value=0.056).

Chromosome 17

As described in Supporting Information Fig. 2F, deletions involving 17p were found in 6 patients (35%) leading to the definition of two MARs: the larger (2.6 Mb), spanning the 17p13.2-p13.1 region including the *TP53* gene, and the shorter (0.9 Mb) at 17p12.

Chromosome 17

As described in Supporting Information Fig. 2F, deletions involving 17p were found in 6 patients (35%) leading to the definition of two MARs: the larger (2.6 Mb), spanning the 17p13.2-p13.1 region including the *TP53* gene, and the shorter (0.9 Mb) at 17p12.

Mutation analyses

We tested the frequency of inactivating *TP53* mutations in 17 cases (Table I). Four patients (4/17=23.5%) showed a functionally relevant coding mutation in the DNA binding domain critical for *TP53* tumor-suppressing activity. Three of these cases were also positive for 17p deletion and showed a missense mutation at exons 6 (I195T in PCL-027) and 8 (R280K in PCL-018 and R273C in PCL-030), respectively. The remaining *TP53*-mutated patient (PCL-037) had an in-frame deletion of 6 nucleotides affecting codons 155-156 in exon 5.

Pyrosequencing analyses of 15 cases identified a *BRAF* gene mutation in one case (V600E in PCL-015 6.7%), and a wild-type pattern of both *NRAS* and *KRAS* genes in all tested cases (Table I).

Integrative analysis of CN and gene expression levels

To further evaluate the influence of CNAs in the modulation of the expression of genes located in those regions, we tested whether a direct correlation existed between the occurrence of any CN and the expression level of the genes that reside on the corresponding loci. This analysis identified 382 genes showing a strong correlation between CN imbalances and transcriptional modulation (Supporting Information Table III). Interestingly, almost half of these genes (184/382=48.2%) mapped to chromosome 1, mainly to 1q arm (142/184=77.2%). Among the remaining transcripts, a large fraction (160/382=41.9%) mapped to the previously described chromosomes, namely those showing some alteration in at least five cases. Specifically, 23 genes mapped to the 8p arm (6.0%), 25 to 13q (6.5%), 52 to 14q (13.6%), 27 to 16q (7.1%), and 31 to 17p (8.1%). In order to identify the functional categories potentially affected by gene dosage effect we used the DAVID Functional Annotation Clustering tool on the 382 gene list resulting from the integrative analysis, selecting the annotation clusters for the upregulated transcripts in the main gained chromosomal regions and for those downregulated in the major regions of loss. Seven and nine annotation clusters with a significant enrichment score (ES>1.3) were respectively identified (Supporting Information Table IV). In particular, metabolic processes concerning protein transport, translation and biosynthesis together with genes involved in methyltransferase activity resulted positively modulated. Nucleic

acid metabolic processes such as transcription and RNA splicing, protein catabolic process and apoptosis were recognized as significantly associated with the downregulated genes in deleted regions.

Discussion

Existing studies have so far demonstrated that pPCL shows the main molecular characteristics of other plasma cell dyscrasias (e.g., IGH@ translocations or 13q and 17p deletions). However, a comprehensive analysis of numeric and structural genomic aberrations of this extremely aggressive entity is still lacking. In this study, we provided a detailed genomic characterization of a representative prospective series of pPCL patients, integrating genome-wide profiling and FISH data with the corresponding transcriptional profiles and correlated the main observed aberrations with clinical outcome.

Chromosome translocations involving the 14q32 locus and the CCND1, FGFR3/MMSET, or MAFs partners have been reported occurring in the majority of primary, as well as in secondary PCLs. Herein, we identified the occurrence of IGH@ translocations in ~87% of pPCL cases; such an incidence overlaps with those reported by several authors [3–6, 8], confirming the widely accepted notion that IGH@ translocations are almost an ubiquitous event in PCL. However, the frequency of the different types of partner loci still remains controversial and this is probably related to the heterogeneous series investigated or geographical factors, as already suggested by other Authors [8]. Specifically, in our study the frequency of the t(11;14) was approximately 40%, quite similar to that reported by some authors [3, 6], but lower than that reported by others [5, 8]. Overall, the evaluation of Cyclins D in pPCL confirmed the close association with the occurrence of IGH@ translocations, being CCND1 deregulated in t(11;14) cases and CCND2 in the remaining patients harboring t(4;14), t(14;16) or t(14;20) and in one additional case without the evidence of any major IGH@ translocation. CCND3 was expressed from low to moderate levels in all cases, whereas in our dataset cases were missing with ectopic expression of CCND3 as a consequence of t(6;14), in line with the very low prevalence of t(6;14) in multiple myeloma (<1%) [24] (Supporting Information Fig. 4). As regards IGH@ translocations involving MAF gene, we found higher frequency (30.5%) than those reported in previous works, where incidence in pPCL was approximately 10–17% [3, 4] if not absent [8], and almost identical to that reported by Chiecchio et al. [6]. Finally, we found a 13% incidence of t(4;14), quite similar to an earlier report [3] but different from other studies in which it was found at higher frequency (21–25%) [4, 5] or completely absent [6, 8].

We confirmed the remarkable frequency of del13q in pPCL which is higher than that reported in MM [21]. We found this lesion in 17/23 (74%) patients; based on SNP-array data we confirmed the loss of the entire chromosome in the majority of cases, with the exception of three pPCLs showing relatively large interstitial deletions encompassing the 13q14 region.

Our study confirmed the increased frequency of TP53 deletion in comparison with that reported in MMs. Furthermore, we identified four TP53 mutated cases, three of which associated with a gene deletion. Therefore, ~13% of cases in our series showed biallelic inactivation of TP53, similarly to that reported by other Authors [6, 8]. Furthermore, SNP-array data showed the loss of a large part of 17p in all but one case tested; this finding is reminiscent of what was reported in CLL by our previous study [25] suggesting that other important tumor suppressor genes located in this region may contribute to the tumor development and expansion in pPCL.

In the general context of chromosomal numerical aberrations in pPCL, our study provides novel data regarding the involvement of other regions known to be affected in MM. Overall, the incidence

of 1p loss and 1q gain was ~40 and 50%, respectively, which is only slightly higher than that reported in MM by us and several other groups [7, 10, 12, 26]. Notably, SNP-array analysis pointed out the involvement of specific loci such as the CDKN2C loss at 1p and the CKS1B gain at 1q, which have been strongly suggested as putative targets in MM [21, 27–31], although in our dataset a slight correlation (P-value=0.04) between DNA CN and the expression levels has been found only for CKS1B (data not shown). Chromosome 16q has been shown to be one of the most involved regions in MM. Walker et al. reported losses in ~35% of cases in their representative prospective series of MM [21]. Overall, 16q loss involving whole or interstitial deletions, occurred in ~50% of cases in our series. Interestingly, two different MARs identified encompassed the CYLD and WWOX genes, thought to be specific targets of the lesion [23].

We did not find a different incidence of 6q and 14q losses compared to previous reports in MM [21]. In particular, our study identified 14q loss involving whole or part of the chromosome in ~70% of cases, and confirmed the occurrence of biallelic losses of the TRAF3 gene in 60% of pPCL cases (3/5) showing loss of 14q32.31-q32.32 region.

Another finding of our study was the involvement of the 8p loss in approximately 40% of pPCL, a frequency slightly lower than that referred by Chiecchio et al. (55.5%) in their series of 10 pPCLs [6] but higher than that recently reported by Walker et al. in MM (25%) [21]. We identified the presence of a MAR of ~1.5 Mb region in 8p21.2 which is centered on the PPP2R2A locus and also comprises NEFM, NEFL, DOCK5, GNRH1, KCTD9, CDCA2, and EBF2 genes. According to a recent evidence reported in MM [21], the PPP2R2A transcript was found significantly down-regulated in our pPCLs with 8p loss. In line with previous findings [6], we also demonstrated that in two cases this region was homozygously lost in a fraction of malignant cells, suggesting that PPP2R2A could be the major target of this lesion. However, the correlation between transcriptional and genomic data, as well as the transcriptional patterns associated with 8p deleted cases, showed that this lesion is associated with a significant gene dosage effect involving a large number of genes in addition to PPP2R2A. Concerning the clinical impact, our data indicated that the 8p loss is not associated with survival in our cohort of pPCLs (Supporting Information Fig. 1); this finding is in agreement with what showed by Walker et al. in MM [21] but not with Sutlu et al., who reported the 8p21 deletion as an independent prognostic factor affecting both PFS and OS in MM patients receiving autologous stem cell transplantation [20]. Therefore, the clinical relevance of 8p loss in plasma cell dyscrasias needs to be further investigated in larger series of cases.

A striking result of our study is the absence of N- and K-RAS activating mutation in pPCLs, which is in contrast with the general frequency of RAS gene mutation either in MM as reported by us [32], or in pPCL as reported either by Bezieau et al. [33] (50%) or Tiedemann et al. [8] (27%) in their series including a similar number of cases. We do not have a clear explanation for this discrepancy; however, as suggested by Tiedemann et al. who reported a very high frequency of the t(11;14) and absolute absence of MAF translocation in their series, this finding could also be related to regional variations in leukemogenesis in the context of plasma cell dyscrasias.

Interestingly, we were able to demonstrate the occurrence of a BRAF activating mutation in one pPCL case. As a part of the RAS/RAF/MAPK signaling pathways, BRAF has an important role in many processes regulating cell growth and differentiation. Mutations of BRAF, mostly V600E, have been frequently found in many common types of human cancers and recently it has been found as the hallmark of hairy-cell leukemia [34]. BRAF mutations have been recently identified by whole genome sequencing in MM and reported to occur in ~4% of cases [35]. Our finding in pPCLs confirms this frequency, suggesting that some pPCL patients might putatively benefit of specific treatments with BRAF inhibitors.

Finally, the functional clustering of differentially expressed genes mapping within altered CN regions identified potentially deregulated genes involved in metabolic processes fundamental in cancer development. In particular, we found genes involved in intracellular protein transport, such as COPA and ARF1, which play a role in vesicle-mediated transport from the endoplasmic reticulum to the Golgi region, or RABIF1 related to the ubiquitous key regulators of membrane-vesicle transport Rab GTPases. Furthermore, several transcripts in gained (PSMB4, PSMD4, UCHL5) and deleted (PPP2CB, PPP2R5C, PSMC6, PSMD7) chromosomal regions were associated with a proteasomal ubiquitin-dependent protein catabolic process. In addition, some genes are implicated in several other pathways relevant to myeloma biology, such as the Wnt (DVL2, HDAC1, PPP2R5C, PPP2CB, PPP2R5E, GNG5 and MYST3) and NF- κ B (SLC16A1, GCLM, BCL10, ZCCHC11, GPR89A, IL6R, ARHGEF2, CASP4, NDIFP2, YY1, TRAF3 and VPS53) pathways. Interestingly, the ARHGEF2 gene, in addition to its capability to induce NF- κ B transactivation, promotes tumorigenesis by inhibiting apoptosis and enhancing proliferation via RhoA and cyclin D1 upregulation [36]. Notably, several genes, such as TRAF3, PSDM4, YY1, UCHL5, PARP1, and HDAC1, are associated with response to Bortezomib treatment; among them, PSDM4 and PARP1 have been previously reported to be up-regulated in MM patients with poor outcome [37, 38].

Overall, our study provides a compendium of genomic alterations in pPCL. Although the differences between our genomic data and those previously published could result from random variations due to the small number of samples analyzed, to the best of our knowledge this represents the first study involving a prospective series of patients. Our results may provide an important contribution to the understanding of the pathogenesis of this particular form of plasma cell dyscrasias and could be of potential value to better elucidate the mechanisms of tumor progression in MM.

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