Overexpression of *PDZK1* within the 1q12-q22 Amplicon Is Likely To Be Associated with Drug-Resistance Phenotype in Multiple Myeloma

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We investigated DNA copy number aberrations in 37 cell lines derived from multiple myelomas (MMs) using comparative genomic hybridization, and 11 (29.7%) showed high-level gain indicative of gene amplification at 1q12-q22. A corresponding transcriptional mapping using oligonucleotide arrays extracted three up-regulated genes (*IRTA2*, *PDZK1*, and *ST00A6*) within the smallest region of overlapping in amplifications. Among them *PDZK1* showed amplification and consequent overexpression in the MM cell lines. Amplification of *PDZK1* was observed in primary cases of MM as well. MM cell lines with amplification of *PDZK1* exhibited the resistance to melphalan-, cis-platin-, and vincristin-induced cell death compared with MM cell lines without its amplifications. Furthermore, down-regulation of *PDZK1* with an anti-sense oligonucleotide sensitized a cell line KMS-11 to melphalan, cis-platin, and vincristin. Taken together, our results indicate that *PDZK1* is likely to be one of targets for 1q12-q22 amplification in MM and may be associated with the malignant phenotype, including drug resistance, in this type of tumor. (Am J Pathol 2004, 165:71–81)

Multiple myeloma (MM) is a B-cell malignancy that is characterized by the accumulation of plasma cells with a

low-proliferation index and an extended life span within the bone marrow. Despite recent improvements in event-free and long-term survival resulting from autologous stem cell transplantation, MM remains a disease with a wide variability of clinical futures, responses to treatment, and survival times among the patients.¹

It was observed that virtually all MM patients are chromosomally abnormal.^{2–8} The primary translocations occur as early and perhaps initiating events during the pathogenesis of MM; and the clustering of rearrangement breakpoints are detected at 14q32, 16q11, and 22q11.⁸ However, these translocations are not sufficient for the malignant progression of this disease,^{9,10} and the accumulation of additional genetic alterations affecting tumor-related genes, including proto-oncogenes and tumor suppressor genes, are necessary for the emergence of a fully malignant phenotype. Indeed, karyotype analysis and recent fluorescence *in situ* hybridization (FISH) analysis indicate that the disease progression of MM correlates with the appearance of secondary nonrandom chromosomal aberrations.^{7,11}

1q rearrangement is one of secondary chromosomal changes associated with malignant phenotype in hematopoietic tumors, such as MM and non-Hodgkin's lymphoma.^{11,12} Previous comparative genomic hybridization (CGH) studies have revealed the gain or amplification of 1q, consistently involving 1q21 in MM.^{13–15} Gains of 1q can occur as duplications of translocated unbalanced derivative chromosomes, isochromosomes, or jumping translocations.¹⁶ In various other tumors, moreover, gain or amplification affecting ~1q has been reported to appear more frequent in aggressive tumors with malignant potential and resistance to chemotherapy.^{17–19} In addition, until now, it has been reported as known oncogenes that *BCL9*, *MCL1*, *ARNT*, *AF1q*, *JTB*, *MUC1*, *IRTA1*, *IRTA2*, *FCGR2B*, and *PBX1* were up-regulated by the

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juxtaposition with immunoglobulin (*IG*) genes or the chromosomal gain involving 1q rearrangement.^{20–30} Although these circumstances suggested that the region around 1q21 harbors one or more target genes associated with malignant phenotype of MM and other tumors, the critical genetic region and the essential target genes for chromosomal gain involving 1q rearrangement in MM remains obscure.

In the study reported here, we examined 37 MM cell lines by CGH to explore genomic alterations that might affect progression of this disease. Among losses and gains involving several chromosomal regions, we detected high-level gain (HLG) in 1q12-q22 in 29.7% of MM cell lines and defined the smallest region of overlap (SRO) by FISH. Through a corresponding transcriptional mapping using oligonucleotide arrays, *PDZK1* (the PDZ domain containing 1) emerged as a candidate target within the SRO. Further, *in vitro* studies using MM cell lines suggested that *PDZK1* might be associated with the resistance to chemotherapeutic reagents in MM.

Materials and Methods

Cell Lines and Primary Samples of MM

All of the 37 human MM cell lines were established from primary samples (see Table 1) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Primary MM samples from 29 independent patients were provided by the Kawasaki Medical School Hospital, with written consent from each patient in the formed style and after approval by the local ethics committee. Primary samples were composed of 4.7 to 87.0% (mean, 48.9%) tumor cells.

CGH Analysis

CGH was performed using directly fluorochrome-conjugated DNA as described by Kallioniemi and colleagues,³¹ with minor modification.³² Briefly, tumor DNAs and normal DNAs were labeled, respectively, with Spectrum Green-dUTP and Spectrum Orange-dUTP (Vysis, Chicago, IL) by nick translation. Labeled tumor and normal DNAs, together with 10 μ g of Cot-1 DNA, were denatured and hybridized to normal male metaphase chromosome spreads. The slides were washed and counterstained with 4',6'-diamidino-2-phenylindole. Shifts in CGH profiles were rated as gains and losses if they reached at least the 1.2 and 0.8 thresholds, respectively, as described elsewhere.³² Overrepresentations were considered to be HLGs, when the fluorescence ratio exceeded 1.5, as described elsewhere.³²

FISH Analysis

Metaphase chromosome slides were prepared and used in FISH experiments in the manner described previously.³³ The location of each bacterial artificial chromosome (BAC) within the region of interest was compiled from information archived (on April 2003) by the National Cen-

ter for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and confirmed or modified according to the results of FISH using normal metaphase chromosomes. Probes were labeled with biotin-16- or digoxigenin-11-dUTP by nick translation (Roche Diagnostics, Tokyo, Japan). Chromosomal *in situ* suppression hybridization and fluorescent detection of hybridization signals were performed as described elsewhere.³³ The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes.

Preparation of Labeled Probes and Hybridization on Oligonucleotide Arrays

AceGene (Human oligo chip 30K; Hitachi Software Engineering Co., Ltd., Kanagawa, Japan) containing 30,000 genes was used for mRNA expression profiling. We obtained information for each gene on the chips from the NCBI database, and the experiments for the oligonucleotide microarray were performed according to the manufacturer's instruction (<http://dnasis.hskbio.hitachi-sk.co.jp/acegene/>). In brief, cDNA probes labeled with aminoallyl-dUTP (Ambion Inc., Austin, TX) were synthesized using Oligo(dT)_{12–18} primer and SuperScript II (Invitrogen, Carlsbad, CA), and were purified using the QIAquick PCR purification kit (Qiagen K K., Tokyo, Japan). The labeled test and reference cDNA probes were coupled with Cy3- and Cy5-monoreactive dye (Amersham Biosciences Inc., Tokyo, Japan), respectively, and were purified using microbio-spin columns (Bio-Rad Laboratories Inc., Tokyo, Japan). AceGene arrays were hybridized with the labeled test and reference cDNA probes (cDNA synthesized from 50 μ g of total RNA/Chip) for 16 hours at 42°C. After being washed with 2 $\times$ standard saline citrate and 0.1% sodium dodecyl sulfate for 5 minutes at 30°C, 2 $\times$ standard saline citrate for 5 minutes at 30°C, and 1 $\times$ standard saline citrate for 5 minutes at 30°C, the hybridized chips were scanned using GenePix 4000B (Axon Instruments Inc., Foster City, CA), and the digitized image data were processed using version 4.1 GenePixPro software (Axon Instruments Inc.).

Separate images were acquired for Cy3 and Cy5. Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background was computed for each spot equal to the median pixel intensity in a square of 40 pixels in width and height centered on the spot center, excluding all pixels within any defined spots. Net signal was determined by subtraction of this local background from the average intensity for each spot. Spots deemed unsuitable for accurate quantification because of array artifacts were manually flagged and excluded from further analysis. Signal intensities between the two fluorescent images were normalized by the averaged values for blank spots. This effectively defined the signal intensity-weighted spot for the internal controls of housekeeping genes on each array to have a Cy3/Cy5 ratio of 1.0.

Semiquantitative Expression Analysis of Eight Candidate Targets within 1q21-q22 Amplification

A semiquantitative expression analysis was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) in eight up-regulated genes that emerged within the 1q12-q22 amplification through oligonucleotide microarray analysis. Sequences of primer pairs for each gene examined are available on request. Single-stranded complementary DNA (cDNA) was generated from total RNA of MM cell lines using the SuperScript First-Strand Synthesis system (Invitrogen) following the manufacturer's directions. The optimal annealing temperature cycle numbers were determined by performing pilot PCR. PCR products were electrophoresed in 3% agarose gel, and the band quantification was done with LAS-3000 (Fujifilm, Tokyo, Japan).

*Quantification of Expression of mRNA and DNA Copy Number of *PDZK1**

Quantifications of expression level of *PDZK1* mRNA were examined by a real-time quantitative PCR in anti-sense experiments.³⁴ DNA copy number of *PDZK1* in primary cases of MM was also determined by a real-time quantitative PCR as described.³⁵ Real-time quantitative PCR was performed using LightCycler (Roche Diagnostics) with SYBR Green according to the manufacturer's protocol. Primer-pairs for *PDZK1* are follows; forward 5'-CCCACAGTACAGCCTCACATT-3'; reverse 5'-CACATGGTGAATGGTTTCCA-3'. The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was the endogenous control (Roche Diagnostics) for mRNA expression levels. Level of mRNA expression in each sample was normalized on the basis of the corresponding *GAPDH* level, and recorded as a relative expression level. β -globin (*HBB*) served as the endogenous control for genomic DNA copy number analysis. *HBB*, which is located at 12q13 and rarely involved in MM, either in our panel or in previous studies,¹³⁻¹⁵ served as the endogenous control for genomic DNA copy number. Copy number in each tumor sample was normalized by dividing it by the corresponding *HBB* value, and recorded as copy number ratios. Duplicate PCR amplification was performed for each sample.

Assessment of Drug Sensitivity and the Effect of Anti-Sense Oligonucleotide (ASO) Treatment

Melphalan (MEL), *cis*-platin (cDDP), vincristin (VCR), dexamethasone (DEX), adriamycin (ADM), mitoxantrone (MIT), daunorubicin (DNR), and thalidomide (THAL) were purchased from Sigma (St. Louis, MO). Sensitivity of each MM cell line to those chemotherapeutic/cytotoxic reagents-induced cell death was assessed using a colorimetric assay on microtiter plates (Cell-Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan), which measures the ability of viable cells to cleave a tetrazolium salt

(WST-8) to a water-soluble formazan. In brief, MM cells of 3×10^4 in 96-well plates were exposed to various concentrations of reagents for 48 hours. WST-8 was added 2 hours before the end of culture, and absorbance was measured at 450 nm using a microplate reader (Benchmark, Bio-Rad Laboratories). Experiments were repeated three times, and each series was performed in triplicate.

Anti-sense experiments were performed as described previously,³⁴ with minor modifications. Briefly, we synthesized the following oligonucleotides containing phosphorothioate backbones (oligonucleotide phosphorothioate; Espec Oligo Service Co., Tsukuba, Japan): *PDZK1*-AS; 5'-CTGTACCTCTTTGATGAATG-3' (the anti-sense direction of *PDZK1* cDNA nucleotide 1308 to 1327; GenBank accession number NM.002614), *PDZK1*-SC; 5'-GTAAGTAGTTTCTCCATGTC-3' (the scramble control for *PDZK1*-AS1). Oligonucleotides were delivered into cells using oligofectamine (Invitrogen) according to the manufacturer's instruction. Treatment with chemotherapeutic reagents was started 24 hours after transfection of oligonucleotides, and viable cell number was assessed 48 hours after treatment (72 hours after transfection) using a colorimetric assay as described above. Experiments were repeated twice, each performed in triplicate.

Results

DNA Copy Number Aberrations Detected by CGH in MM Cell Lines

An overview of the genetic changes we detected among 37 MM cell lines is shown in Figure 1. All of lines showed detectable copy number aberrations. The average number of aberrations per cell line was 9.3 (range, 1 to 23); average numbers of gains and losses were 5.6 (range, 0 to 14) and 3.8 (range, 0 to 11), respectively. The minimal common regions of gains that were most frequently involved were at: 1q12-q24 (31 of 37, 83.8%), 7q31-q36 (13 of 37, 35.1%), 18q22-q23 (11 of 37, 29.7%), 8q23-q24 (10 of 37, 27.0%), 19p (9 of 37, 24.3%), and 16p12.1-p12.2 (8 of 37, 21.6%). The most common losses were observed at: 13q14-q32 (25 of 37, 67.6%), 14q22-q24 (17 of 37, 45.9%), 4p and 4q (10 of 37 each, 27.0%), and 6q22-q23 (8 of 37, 21.6%) (Figure 1, Table 1). The smallest regions of HLGs were seen at 1q12-q21 (11 of 37, 29.7%), 7q31-q32, and 8q23-q24 (4 of 37, 10.8% each) (Table 1).

Definition of 1q HLG Region in MM Cell Lines

We further focused on the HLG region (Table 1 and Figure 1) at 1q12-q24, because it was most frequently involved. To define the SRO for HLG at 1q12-q24, we performed FISH analysis in three MM cell lines (AMO1, KMS-11, and KM-5) that had exhibited copy number gains with the highest intensity and the narrowest regions in CGH analysis (Figure 1 and Figure 2A). We selected 26 BACs covering 1q21-1q24 and a pUC1.77 at 1q12 as

Figure 1. Summary of genetic imbalances detected by CGH in 37 MM cell lines. The 22 autosomes and X chromosome are represented by ideograms showing G-banding patterns. As judged by the computerized green-to-red profiles, **vertical lines on the left (red)** of each chromosome ideogram show losses of genomic material in cell lines, whereas those on the **right (green)** correspond to copy number gains. HLGs are represented as **yellow rectangles**. The number at the **top** of each **vertical line** corresponds to the cell line in which each change was recorded (see Table 1).

probes for the FISH experiments (Figure 2, B and C). Twelve BACs (640M16, 315I20, 373C9, 300L20, 94I2, 565E6, 47D6, 325P15, 301M17, 91G11, 763B22, and 54A4) produced high copy signals as 15 copies in AMO1 cell line (Figure 2, B and C). Notably, three BACs (337C18, 533N14, and 123P3) located between 325P15 and 301M17 were nine copies in the same cell line (Figure 2, B and C). However, in other two cell lines (KMS-11 and KM-5), the larger (~20 Mb) regions showing the high-level and constant copy number gains (KMS-11; nine copies, KM-5; six copies) between pUC1.77 and 26N3 (KMS-11) or 138F3 (KM-5) were observed (Figure 2C). Therefore, the SRO could be defined between pUC1.77 and 188M11 (SRO-I; 5 Mb, 1q12-q21.1) as the most HLG region in AMO1, except for 337C18, 533N14, and 123P3, or else between pUC1.77 and 26N3 (SRO-II; ~20 Mb, 1q12-q22) if we consider the high-level and constant copy number gains of large chromosomal unit in KMS-11 and KM-5, and might include the target genes associated with malignant phenotype of MM.

Expression Profile on 1q12-q22

To explore the up-regulated genes within the SRO, a corresponding transcriptional mapping using oligonucleotide array on 1q12-q22 was performed in two MM cell lines, AMO1 and KMS-11, both of which showed HLG indicative of gene amplification at 1q12-q22. A mRNA from Epstein-Barr virus-transformed B-lymphoblastoid cell line established from a healthy male volunteer was used as a reference.³⁶ In those experiments, information for 219 genes spanning the SRO-II region at 1q12-q22 was obtained from the NCBI database. When twofold changes were considered significant in AMO1, 31 of 219 genes were detected as up-regulated genes. Of them eight (six known genes; *KCNN3*, *SLC27A3*, *IRTA2*, *PDZK1*, *S100A6*, and *SPAP1*, and two unknown genes; FLJ22530 and FLJ23221) were up-regulated in both AMO1 and KMS-11 (Table 2). Although there were nine known cancer-related genes (*BCL9*, *MCL1*, *ARNT*, *AF1q*, *JTB*, *MUC1*, *FCGR2B*, *IRTA1*, and *PBX1*) located within the SRO-II (Figure 2C), none of them was detected as an

Table 1. Summary of 37 MM Cell Lines Used in the Present Study

No. cell lines	Tissue source	Origin	High-level gains (HLGs)
1 KM-1			1q12-q23, 7p14-p15
2 KM-4			—
3 KM-5			1q12-q22, 18q22-q23, 20p12-p13
4 KM-6			8p22-p23, 8q, 19p13.2-p13.3, 20q
5 KM-7			1q12-q23, 8q23-q24
6 KM-11			—
7 KMS-5		MM	—
8 KMS-11		MM	1q12-q21
9 KMS-18		MM	—
10 KMS-20			1q12-q22
11 KMS-24			—
12 KMS-26			12p11-p12, 16q23-q24
13 KMS-27			—
14 KMS-34			7q31-q16
15 KMS-12PE	PE	MM	3q26.3-q29
16 KMS-12BM	BM	MM	13q33-q34
17 KMS-21PE	PE	MM	—
18 KMS-21BM	BM	MM	—
19 KMS-28PE			7q31-q16
20 KMS-28BM			—
21 MOLP-2	PB	MM	1q12-q22
22 MOLP-5	PB	MM	—
23 MOLP-6	PB	MM	—
24 ILKM-2	BM	MM	—
25 ILKM-3	BM		—
26 ILKM-8			1q12-q23, 17q21
27 ILKM-10			—
28 ILKM-12			7p, 7q
29 ILKM-13			1q12-q23, 20p12-p13
30 U266	BM	MM	—
31 AMO1		PLC	1q12-q21, 2p24-p25
32 L363	PB	PCL	1q12-q23, 19p13.2-p13.3
33 OPM-2	PB	PCL	—
34 KMM1		MM	—
35 KHM-4		MM	1q12-q23
36 HS			7q31-q32, 16q22-q23
37 RPMI8226	PB	PCL	—

PE, pleural effusion; BM, bone marrow; PB, peripheral blood; MM, multiple myeloma; PLC, plasma cell leukemia; PCL, plasmacytoma.

up-regulated gene in our oligonucleotide arrays (it was not available for only *IRTA1* in oligonucleotide arrays).

Correlation between Copy Number and Expression of *PDZK1* in MM Lines

We first confirmed an increase of expression level of eight candidate targets by semiquantitative RT-PCR in two panels consisting of seven MM lines with or without HLG at 1q12-q21, respectively. Among those eight genes, *IRTA2*, *PDZK1*, and *S100A6* showed the significant difference on expression level between MM cell lines with and without HLG (Figure 3, A and B). However, the up-regulation of *IRTA2* in both AMO1 and KMS-11, compared with the lymphoblastoid cell line, did not show in conflict with oligonucleotide array analysis (Figure 3A). Of them, *PDZK1* was a notable candidate positionally and functionally, since the previous report disclosed the relevance of this gene to drug resistance³⁷ and further multiplication of 1q harboring *PDZK1* was suggested to be associated with drug resistance in ovarian cancer.¹⁹ Thus we further examined copy number status and expression levels of *PDZK1* in MM cell lines. As shown in Figure 3C, *PDZK1* copy numbers determined by FISH were posi-

tively correlated with their expression levels; seven cell lines with HLG of *PDZK1* (AMO1, 15 copies; KMS-20, 10 copies; KMS-11, 9 copies; MOLP-2, 7 copies; ILKM-8, 6 copies; KM-7, 6 copies; and KM-5, 6 copies) and KMS-21BM (7 copies), which showed *PDZK1* amplification in fact copy number by FISH, although it did not show HLG at 1q12-q21 by CGH (Figure 1, Table 1), showed higher expression levels of these genes compared with those without HLG (KMS-34, three copies; KMS-12PE, three copies; U266, three copies; KMS-12BM, two copies; KMS-28BM, two copies; HS, three copies; and KM-6, three copies).

Frequent Copy Number Gain of *PDZK1* in Primary MM Samples

DNA copy number of *PDZK1* in primary cases of MM was determined by a real-time quantitative PCR using genomic DNA obtained from 29 patients. As the normal control of diploid copy number, we first determined the copy number ratio of *PDZK1* normalized with *HBB* in DNA derived from five independent healthy volunteers. To separate cases with or without copy number gain, the mean + 2 SD of copy number ratio of *PDZK1* in normal

Figure 2. Amplicon map of the 1q12-q22 region in MM cell lines. **A:** Representative CGH image of chromosome 1 of AMO1 cell line showing distinct amplification on the 1q12-q22 region (left) and the corresponding green (gain) and red (loss) profile (right). **B:** Representative images of FISH analysis on metaphase chromosomes from AMO1 cells. The images show 15 copies with RP11-315I20 (1q21, left) and four copies with pUC1.77 (1q12-specific probe, right). **C:** Summarized results of DNA copy number analysis at 1q12-q24 by FISH in three different cell lines. The vertical axis shows the number of FISH signals achieved with the BAC probes indicated as horizontal bars. The SRO with the maximal amplification was defined between pUC1.77 and RP11-188M11, except RP11-337C18, RP11-533N14, and RP11-123P3 (SRO-I), or between pUC1.77 and RP11-26N3 (SRO-II) if the change of copy number is considered as common chromosomal gain, and these are indicated by closed arrow. The position and length of each BAC were compiled from information archived by NCBI. The position of each known oncogenes was also listed on the bottom.

controls was used as the cutoff ratio. As shown in Figure 3D, the copy number gain of *PDZK1* was detected in 9 of 29 cases (31%), even though primary samples contains only 4.7 to 87.0% (mean, 48.9%) tumor cells. Notably *PDZK1* copy number ratio of KMS-11 cell line determined by a real-time quantitative PCR was similar to that of case 18 (copy number ratio = 4.08), from which KMS-11 cell line had been established (Figure 3D). Furthermore we confirmed that *PDZK1* copy number ratios for three different MM cell lines determined by real-time quantitative PCR were clearly correlated with *PDZK1* copy numbers determined by FISH analysis (copy number ratio in real-time PCR versus copy number in FISH: AMO1, 6.9 versus 15; KMS-11, 4.1 versus 9; and KMS-34, 0.8 versus 3, respectively). These results indicate that the PCR-based method can evaluate genomic copy number change quantitatively, and the amplification of this region is not the artifact ac-

quired during or after the establishment of cell lines. Indeed, significant amplification of *PDZK1* could be really observed in one patient with MM by FISH as shown in Figure 3E.

Overexpression of *PDZK1* Contributes to the Resistance to Chemotherapeutic Reagent-Induced Cell Death

We next investigated the correlation between the overexpression of *PDZK1* affecting copy number gain and the sensitivity to various chemotherapeutic reagents in MM cell lines. Cell lines with the copy number gain and overexpression of *PDZK1*, AMO1, and KMS-11, were more resistant to the cytotoxic effect of MEL, cDDP, and VCR compared with those without their copy number gain and overexpression, KMS-34 and KMS-12PE (Figure 4A).

Table 2. Gene Expression Profile of the 1q12-21 Region for Cell Lines with High-Level Copies

No.	Symbol	Genbank (Acc. no.)	Gene name	Fold change (/LCL)	
				AMO1	KMS-11
1	<i>HAX1</i>	NM_006118	hs1 binding protein	13.50	1.52
2	<i>KCNN3*</i>	NM_002249	Potassium intermediate/small conductance calcium-activated channel, subfamily, member 3	12.67	2.22
3	<i>FLJ14225</i>	NM_024874	Hypothetical protein	11.15	1.23
4	<i>FLJ12528</i>	NM_025150	Hypothetical protein	10.29	1.36
5	<i>SLC27A3*</i>	NM_024330	Solute carrier family 27	9.43	3.70
6	<i>C1orf2</i>	NM_006589	Chromosome 1 open reading frame 2	8.61	0.96
7	<i>PP591</i>	NM_025207	Hypothetical protein	6.96	1.66
8	<i>CLK2</i>	NM_003993	cdc-like kinase 2 isoform hclk2	6.94	1.52
9	<i>SELENBP1</i>	NM_003944	Selenium-binding protein 1	6.31	1.70
10	<i>FY</i>	NM_002036	Duffy blood group	4.67	1.11
11	<i>FCGR1A</i>	NM_000566	Fc fragment of IgG, high affinity 1a, receptor for (CD64)	4.33	1.03
12	<i>FLJ22530*</i>	XM_016470	Hypothetical protein	4.05	2.04
13	<i>IRTA2*</i>	AF343663	Immunoglobulin superfamily receptor translocation associated protein 2b	4.00	3.95
14	<i>CTSK</i>	NM_000396	Cathepsin k (pseudosclerosis)	4.00	1.66
15	<i>LASS2</i>	NM_013384	LAG1 longevity assurance homolog 2	3.57	0.80
16	<i>GBA</i>	NM_000157	Glucosidase, beta; acid (includes glucosylceramidase)	3.50	1.45
17	<i>S100A12</i>	NM_005621	S100 calcium-binding protein A12	3.48	1.11
18	<i>FLJ23221*</i>	NM_024579	Hypothetical protein	3.43	2.08
19	<i>PDZK1*</i>	NM_002614	pdz domain containing 1	3.24	2.00
20	<i>NTRK1</i>	NM_002529	Neurotrophic tyrosine kinase, receptor, type 1	3.04	1.07
21	<i>PIAS3</i>	NM_006099	Protein inhibitor of activated stat3	3.01	0.63
22	<i>SLC39A1</i>	NM_014437	Solute carrier family 39 (zinc transporter), member 1	3.00	1.46
23	<i>S100A3</i>	NM_002960	S100 calcium-binding protein A3	2.88	1.18
24	<i>PDE4DIP</i>	NM_014644	Phosphodiesterase 4D interacting protein (myomegalin)	2.76	0.97
25	<i>SPRR2C</i>	NM_006518	Small proline-rich protein 2C	2.37	1.20
26	<i>S100A6*</i>	AL162258	S100 calcium-binding protein A6	2.33	2.03
27	<i>LY9</i>	AF244129	Lymphocyte antigen 9	2.33	1.50
28	<i>TXNIP</i>	NM_006472	Thioredoxin interacting protein	2.23	1.08
29	<i>NESCA</i>	NM_014328	New molecule containing SH3 at the carboxy-terminus	2.22	1.21
30	<i>VPS45A</i>	NM_007259	Vacuolar protein sorting 45A	2.13	1.03
31	<i>SPAP1*</i>	AF356276	SH2 domain containing phosphatase anchor protein 1	2.00	7.35

*Overexpressed genes in both AMO1 and KMS-11.

LCL, Epstein-Barr virus-transformed B-lymphoblastoid cell line.

Whereas, all four cell lines showed no correlation between the overexpressions and the sensitivities to DEX, ADM, MIT, DNR, or THAL (Figure 4A).

To test whether the *PDZK1* is responsible for the resistance to chemotherapeutic reagent-induced cell death in MM cells, the effect of down-regulation of *PDZK1* expression was investigated in MM cell line having HLGs of 1q12-q22 region using anti-sense technique. ASO for *PDZK1* delivered into KMS-11 cells inhibited mRNA expression of this gene efficiently (Figure 4B), whereas the control oligonucleotide (*PDZK1*-SC) did not affect the *PDZK1* expression level. We confirmed that there was no effect of ASO on cell growth of KMS-11 in the absence of drugs after its transfection (Figure 4C). *PDZK1*-AS-treated KMS-11 cells were more sensitive to MEL-, VCR-, or cDDP-induced cell death compared with oligofectamine alone (Oligo. alone) or *PDZK1*-SC-treated cells (Figure 4C), suggesting that *PDZK1*, activated through

copy number gain, may be at least one of the molecules responsible for drug resistance in MM cells.

Discussion

CGH analysis is a useful method to define regions of genomic gain/amplification and deletion in tumors with a low-proliferative fraction, including MM, which limits generation of evaluable metaphases. Karyotyping and CGH analyses have identified partial or whole 1q gain (consistently involving 1q21) in 40 to 60% of MM.¹³⁻¹⁵ In the study reported here, our CGH analysis also frequently detected the partial or whole gains of 1q in 30 of 37 MM cell lines (83.8%), consistent with previously reported CGH studies (Table 1 and Figure 1). Besides the high frequency, gain/amplification around 1q21 is of quite interest, because the high prevalence of abnormalities

Figure 3. Correlation between copy number and expression level of *PDZK1* in MM cell lines and copy number in primary MM. **A:** Semiquantitative RT-PCR of eight candidate genes was performed in each panel of seven MM cell lines with HLGs or without HLGs, respectively. Lymphoblastoid cell line was used as the control. Signal intensity was quantified with LAS-3000. **B:** Correlation between HLGs and relative expression levels was determined on eight candidate genes by a nonparametric Mann-Whitney *U*-test. *IRTA2*, *PDZK1*, and *S100A6* revealed a significant difference between HLG(+) and HLG(-). Median values are indicated with horizontal bars. Black or white circles are cell lines with HLG(+) or without HLG(-), respectively. **C:** DNA copy numbers were determined by FISH using a BAC of *PDZK1*. Although KMS-21BM never did show in HLG at 1q12-22 by CGH and listed in the group of HLG(-) in Table 1 and Figure 3A, *PDZK1* copy number unambiguously showed seven copies. The relative expression levels of each cell line were determined in **B**. **D:** Copy number ratios of *PDZK1* in 29 primary cases of MM determined by real-time quantitative PCR. The abundance of the *PDZK1* gene in genomic DNA of each sample was normalized by the corresponding endogenous control *HBB* value, and recorded as copy number ratio. DNAs from five independent healthy volunteers were used as normal controls (N). Notably, the copy number ratios for three different MM cell lines determined by a real-time quantitative PCR were clearly correlated with the copy numbers determined by FISH analysis (copy number ratio in real-time PCR versus copy number in FISH: AMO1, 6.9 versus 15; KMS-11, 4.1 versus 9; and KMS-34, 0.8 versus 3, respectively). Bar, the mean $\pm$ SD of normal controls and samples. Line, the mean + 2 SD indicating cutoff ratio for copy number gain. **E:** Amplification of *PDZK1* in one case of primary MM by FISH. Green signal of *PDZK1* (BAC:RP11-373C9) was clearly amplified, as compared to red signal for pUC1.77 (1q12-specific probe) used as the control (a). Green and red signals were co-localized in 1q12-q21 on metaphase chromosomes from normal peripheral lymphocytes (b).

involving this region has been observed in multiple tumor types and this aberration may be associated with tumor evolution and/or responsiveness to therapy in those tumors.¹⁷⁻¹⁹ Gain/amplification around 1q21 region was related to a highly aggressive phenotype with metastatic potential in osteosarcoma and squamous cell carcinoma of the lung^{17,18} and with a drug-resistant phenotype in ovarian cancer.¹⁹ In addition, patients with osteosarcoma showing 1q21 amplification have a tendency toward

shorter survival.¹⁸ These pieces of evidences suggested the existence one or more genes could contribute to acquisition of malignant phenotypes necessary for progression of MM and other tumors. Thus we further performed molecular cytogenetic and functional characterization for the chromosomal gains of 1q12-q22 region in MM cells.

We determined the critical region for HLG of 1q12-q22 in MM by FISH analysis using 26 BAC clones, and de-

Figure 4. Effect of various chemotherapeutic/cytotoxic reagents to MM cells with 1q12-q22 amplification. **A:** The dose-response curves to melphalan (MEL)-, cis-platin (cDDP)-, vincristin (VCR)-, dexamethasone (DEX)-, adriamycin (ADM)-, daunorubicin (DNR)-, mitoxantrone (MIT)-, and thalidomide (THAL)-induced cell death in the 1q12-q22-amplified cell lines, AMO1 (●) and KMS-11 (■), and the nonamplified cell lines, KMS-34 (○) and KMS-12PE (□). Cell viability was assessed by microtiter plate colorimetric assay. Each point indicates the average value for three separate experiments, each performed in triplicate. A 100% corresponds to the average value in the absence of any drug (vehicle alone). IC₅₀ value of each cell line to each reagent was shown. **B:** Specific down-regulation of mRNA expression level of *PDZK1* or by ASO. Cells were transfected with ASO (AS), oligofectamine alone (Oligo. alone), or the control oligonucleotide (SC). The quantification of mRNA levels was determined 24 hours after transfection by a real-time quantitative PCR. **Bars**, SD. a, $P < 0.05$ versus Oligo. alone; b, $P < 0.05$ versus SC. **C:** Effect of ASO on cell growth of KMS11 was investigated. In brief, 1×10^5 cells were plated in 24-well plates and after 24 or 48 hours of transfection in absence of drugs, viable cell numbers were determined by hemacytometer cell counts using trypan blue. **Bar**, the mean $\pm$ SD. **D:** The KMS-11 cells treated by *PDZK1*-ASO were incubated in the presence or absence of each drug (MEL, 10 $\mu\text{g/ml}$; VCR, 0.01 ng/ml ; and cDDP, 300 ng/ml) for 48 hours. Each value indicates the average for three separate experiments, each performed in triplicate. A 100% corresponds to the average value in the absence of any drug (vehicle alone). **Bars**, SD. a, $P < 0.05$ versus Oligo. alone; b, $P < 0.05$ versus SC.

tected more narrowed HLG region only in AMO1 cell line as SRO-I (Figure 2C). However, in KMS-11, KM-5, and the other 17 MM cell lines we investigated by FISH, it was no evidence for selectively more narrowed HLG region like in AMO1 (Figure 2, B and C, and data not shown). Therefore, we considered that SRO-II was the most frequently and commonly amplified region at 1q in our series of MM cell lines. Further, it has been reported that FISH analysis in MM patients showed the breakages in 89% of cases with 1q breaks and 1q rearrangements,

which were all unbalanced (translocations or duplications), leading to generate the aberrant heterochromatin/euchromatin junctions and gain of 1q materials in a majority of cases.¹¹ Consistent with those previous findings, the derivative chromosomes resulting in duplications and breakages with region between 1q12 and 1q21 were observed in FISH analysis for AMO1, KMS-11, KM-7, and KMS-20 (Figure 2, B and C, and data not shown).

The gene expression profiling of MM cell lines with chromosomal gain at 1q12-q22 could provide the novel

and valuable information about candidate oncogenes involving chromosomal gains of this region. We identified three up-regulated genes in MM cell lines with HLGs of 1q12-q22. Among them, *PDZK1* was identified within SRO-I, where it was the common and narrowest region in our MM cell lines bearing 1q12-q22 amplification. Further, because the chromosomal gain around 1q21 has been reported to be a potential indicator for malignant progression and/or resistance to chemotherapy,¹⁷⁻¹⁹ we focused on one gene of those candidates, *PDZK1*, which are likely to be related to drug-resistant phenotype of tumors, as a probable target for HLG. Indeed, *PDZK1* was involved in copy number gain at 1q12-q22 and consequently overexpressed in MM cell lines (Figure 3C). In primary cases of MM, this gene was frequently amplified (Figure 3D), even though we might underestimate their copy number ratios because of the contamination of normal cells in primary samples available for the present analysis. Those findings further support the hypothesis that *PDZK1* was the positional and functional target of HLG at 1q12-q22 region.

PDZK1 encodes one of the PDZ domain-containing proteins that were reported to be involved in organizing proteins at the cell membrane³⁸ and in linking transmembrane proteins to the actin cytoskeleton.³⁹ Through these interactions, PDZ domain-containing proteins regulate a diverse set of cell functions, including signal transduction, cell polarity, cell differentiation, and ion transport.^{40,41} *PDZK1* was first isolated in a yeast two-hybrid screen designed to identify proteins interacting with *MAP17*, a membrane-associated protein involved in regulation of cell proliferation.⁴² Subsequently, the interaction of *PDZK1* with *cMOAT*, which is a canalicular organic anion transporter and known as the multidrug resistance-associated protein 2 (*MRP2*) was identified.⁴³ These findings suggested that the protein cluster formed by the association of *PDZK1*, *cMOAT*, and *MAP17* may play an important role in the cellular mechanisms associated with multidrug resistance. *cMOAT* participates in the detoxification process by transporting organic anions through the cytoplasmic membrane, and prevents the accumulation of chemotherapeutic drugs, such as cDDP, resulting in resistance to such agents. Overexpression of *cMOAT* has been observed in cell lines that had developed resistance to drug, and down-regulation of *cMOAT* expression by an anti-sense cDNA sensitizes hepatocellular carcinoma cells to a number of chemotherapeutic drugs.⁴⁴ Although overexpression of *PDZK1* was reported in a variety of carcinomas,³⁷ the clinicopathological and biological significance of overexpressed *PDZK1* in tumorigenesis remains unknown.

In this study, we first reported that copy number gain/overexpression of *PDZK1* might be related to the sensitivity of cell lines to various chemotherapeutic/cytotoxic reagents, and the introduction of a specific ASO for *PDZK1* sensitizes MM cell lines to those reagents. Those findings suggest that *PDZK1* is one of potential targets for chromosomal gain of 1q12-q22 region and associated with drug resistance of MM. In addition, it is possible that this gene might be an appropriate candidate for the target of novel therapeutic protocols. To understand the

biological and clinicopathological significance of overexpression of this gene, further experiments using larger sets of primary samples and/or cell lines for the expression and functional characterization will be needed.

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