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Deletions of *CDKN2C* in Multiple Myeloma: Biological and Clinical Implications

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Abstract

Purpose: Deletions of chromosome 1 have been described in 7-40% of cases of myeloma with inconsistent clinical consequences. *CDKN2C* at 1p32.3 has been identified in myeloma cell lines as the potential target of the deletion. We tested the clinical impact of 1p deletion and used high resolution techniques to define to role of *CDKN2C* in primary patient material.

Experimental Design: We analyzed 515 cases of monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM) and newly diagnosed multiple myeloma (MM) using fluorescence in situ hybridization (FISH) for deletions of *CDNK2C*. In 78 myeloma cases, we carried out Affymetrix SNP mapping and U133 plus 2.0 expression arrays. In addition, we performed mutation, methylation and western blotting analysis.

Results: By FISH we identified deletion of 1p32.3 (*CDKN2C*) in 3/66 MGUS (4.5%), 4/39 SMM (10.3%) and 55/369 MM cases (15%). We examined the impact of copy number change at *CDKN2C* on overall survival (OS), and found that the cases with either hemizygous or homozygous deletion of *CDKN2C* had a worse OS compared to cases which were intact at this region (22 months vs. 38 months; $P = 0.003$). Using gene mapping we identified three homozygous deletions (HD) at 1p32.3, containing *CDKN2C*, all of which lacked expression of *CDKN2C*. Cases with HD of *CDKN2C* were the most proliferative myelomas, defined by an expression-based proliferation index, consistent with its biological function as a cyclin-dependent kinase inhibitor.

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Statement of Clinical Relevance

We use high-resolution SNP-based mapping arrays to identify hemi- and homozygous deletions of *CDKN2C*, at 1p32. In the cases with deletion, expression of the gene was low suggesting it is affected by the genetic loss. Deletion was associated with an increase in proliferation index, defined by expression criteria, suggesting these cases may have a distinct clinical outcome and that deregulation of the G1/S transition point of the cell cycle is important. As such, we carried out a comprehensive assessment of amplification and deletion of other positive and negative regulators of this transition point in the cases from our series, the results of which suggest that it is predominantly *CDKN2C* which is important. Subsequently, FISH was used to determine the clinical importance in a set of 515 presenting MGUS, SMM and myeloma cases. Significantly more deleted cases were seen in myeloma than in MGUS cases, suggesting *CDKN2C* may be important in disease progression. Up to 15% of myeloma cases have any deletion of *CDKN2C,* which results in a worse overall survival, making the detection of this deletion clinically important.

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Conclusions: Our results suggest that deletions of *CDKN2C* are important in the progression and clinical outcome of myeloma.

Keywords

CDKN2C gene; Homozygous deletion; Multiple Myeloma; mapping array; expression array

Introduction

Multiple myeloma (MM) is a mature B-cell malignancy characterized by the accumulation of clonal plasma cells in the bone marrow. During a normal immune response, following an encounter with their cognate antigen, B cells undergo a marked proliferative response passing through a germinal center reaction before undergoing terminal plasma cell differentiation. This process of terminal differentiation is closely linked to cessation of cell cycle and *CDKN2C* ($p18^{INK4c}$) is important in linking these two processes (1). These normal plasma cell functions are “hijacked” in myeloma by a range of molecular mechanisms, one example of which is homozygous deletion which inactivates both copies of a gene contained within it (2).

The *CDKN2C* gene has been mapped to chromosome 1p32.3. Deletions of 1p have been identified in approximately 7-40% of cases of myeloma using cytogenetics, FISH and CGH (3-11). These techniques have mapped the site of the recurrent deletion to 1p12-p21, though some studies have focused on 1p35-p36, the location of the *TP73* gene, and 1p34, the location of the *LAPTM5* gene (12, 13). More recently, it has been possible to map recurrent deletions of 1p in more detail using high resolution techniques, combining aCGH or 50K SNP based mapping with expression data (14, 15).

Using high resolution mapping, deletions of *CDKN2C* have been identified in approximately 40% of myeloma cell lines studied (16, 17). However, only a few studies have examined primary patient material, with homozygous deletion of the *CDKN2C* gene having been reported in one case of 20 samples by PCR-SSCP and in about 2% in 261 cases using RT-PCR assay, western blot and expression array (17, 18). These initial data suggest that inactivation of *CDKN2C* may be important in the initiation and progression of myeloma and, as such, could be an important prognostic factor (17). Combined expression and high resolution 500K gene mapping array analysis provide a new tool with which to determine the biological and clinical implications of deletions of *CDKN2C* in newly diagnosed myeloma. In this study we have used this approach in combination with FISH to examine a large series of presenting myelomas as well as cases with monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM).

Materials and Methods

Human Myeloma cell lines and cell culture

We analyzed Human Myeloma derived tumor cell lines (HMCLs) (H929, JIM-1, JIM-3, KMS-11, KMS-12-BM, KMS-26, LP-1, RPMI8266 and U266). All cell lines were acquired from either ATCC or DSZM, with the exception of KMS-11, which was kindly provided by Dr. Otsuki (Kawasaki Medical School, Japan), and JIM-1 and JIM-3 by Birmingham University (Birmingham, United Kingdom). All HMCLs were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (Invitrogen Life Technologies). Cultures were maintained in exponential growth phase at 37°C in a humidified atmosphere of 95% air / 5% carbon dioxide.

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Sample preparation

Bone marrow (BM) aspirates were received by the Leukaemia Research Fund UK Myeloma Forum Cytogenetics Database after informed consent. A total of 515 BM samples were included in this study. Patients were diagnosed with either MGUS (n=66), SMM (n=39) or MM (n=410; 369 analyzed by FISH, 78 analyzed by array, with an overlap of 37 cases). Patients were newly diagnosed and had not undergone any previous treatment. Clinical data were only available on 148 patients. In 78 samples adequate tumor RNA and adequate tumor and germline DNA were available for gene expression and gene mapping analysis, respectively. Plasma cells were selected as previously described, to a purity of >90% using CD138 microbeads and magnet-assisted cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany) (19).

FISH analysis

Fluorescence in situ hybridization (FISH) was performed using standard approaches aimed at identifying translocation partners: t(4;14), t(6:14), t(11;14), t(14;16), t(14;20), cases with split 14 but unidentified partners, and hyperdiploid status by examining chromosomes 3, 4, 5, 7, 9, 11, 13, 14, 15 and 17 using previously described probes (20). Hyperdiploidy was defined primarily on the results from chromosomes 5, 9 and 15 (21) but modified by the results from other probes used (22). Deletion of 1p32.3 was deduced from loss of the probe RP11-278J17 grown and labeled in the laboratory. In cases with discrepancies between SNP mapping data and FISH results, a smaller probe (RP11-116M11) was tested. The probe RP11-418J17 located at 1p12 was used as a control. RP11-278J17 is a 130 kb probe which is situated from the 5′ end of *CDKN2C* and covers the inter-genic region to C1orf185. RP11-116M11 is 77 kb and covers the 5′ end of *FAF1* and the first and second exons of *CDKN2C*. The two probes overlap by 4 kb, covering the first 2 exons of *CDKN2C* (Supplementary Fig. 1).

RNA/DNA extraction

Patient plasma cells for RNA and DNA extraction were frozen in RLT buffer (Qiagen, Valencia, CA) immediately after selection. Nucleic acids from tumors and cell lines were extracted and quantified as previously described (15). Matched germline DNA from 78 patients was also extracted as previously described.

Genome Mapping and Expression analysis

250 ng of DNA were used for hybridization to the GeneChip Mapping 500K Array set (250K Nsp and 250K Sty) according to manufacturers' instructions (Affymetrix, Santa Clara, CA). For expression arrays, 100 ng of total RNA were amplified using a 2-cycle target labelling kit (Affymetrix) as per manufacturers' instructions. 15 μg of amplified cRNA were hybridized to Human Genome U133 plus 2.0 arrays. Arrays were washed on an Affymetrix Fluidics Station 450 and scanned using a GeneChip Scanner 3000. Data from gene expression arrays were normalized using dChip via the default invariant set normalization method and the model-based expression summarization.

Copy number analysis

SNP genotypes were obtained using Affymetrix GCOS software (version 1.4) to obtain the raw feature intensity which was processed using the Affymetrix GTYPE software (version 4.0) to derive SNP genotypes. The data output from GCOS and GTYPE were analyzed using dChip (23). The control samples were assigned a copy number of two and were used as a reference set to calculate copy number in tumor samples. Median smoothing with a window size of 11 was used to infer copy number along each chromosome. All results were verified using outputs from CNAG (24).

Loss of Heterozygosity analysis

Loss of heterozygosity (LOH) analysis was performed with dChip using a Hidden Markov Model to infer the probability of LOH based on the paired control/tumor samples, using an average heterozygosity rate of 0.26.

Integration of SNP mapping and expression array data

The samples were divided into two groups based on presence or absence of loss or gain at the region of interest compared with cases without an alteration. We focused on the expression of the genes located at the region of interest.

Expression-based proliferation index

The expression-based proliferation index (PI) (25) was determined using the median level of expression of genes that are associated with proliferation (*TYMS*, *TKI*, *CCNB1*, *MKI67*, *KIAA101*, *KIAA0186*, *CKS1B*, *TOP2A*, *UBE2C*, *ZWINT*, *TRIP13* and *KIF11*), scaled to the maximum value among all samples (26). The PI was used to divide the cases into quartiles comprising of Low (L), Low Intermediate (LI), High Intermediate (HI) and High (H) groups.

q-PCR analysis

The cases with homozygous deletion of *CDKN2C* were validated as being deleted using q-PCR on a real-time ABI 7500 PCR machine (Applied Biosystems, Foster City, CA), Power SYBR Green (Applied Biosystems, Foster City, CA) and specific primers: *CDKN2C* 3F, 5′ - CAATGGCTCAGTTTTGCTGAATAA - 3′; *CDKN2C* 3R, 5′ - GTAAGATCTGCCTGCCAAAAGC - 3′, corresponding to the nucleotides of exon 3. The copy number in each of the three samples was normalized relative to the copy number of the *PRKCQ* gene: 3F, 5′ - CCTGGGACAGCACTTTTGATGC - 3′; *PRKCQ* 3R, 5′ - CACGGTGGTTTCAGAGATGAGGTC - 3′. Data evaluation was carried out using the ABI 7500 SDS software (Applied Biosystems).

Mutational analysis

As tumor DNA is limited whole genome amplification (WGA) was performed using the Repli-g kit (Qiagen) with 25 ng of input DNA. This approach was used in subsequent mutational analysis studies. For mutational analysis, the coding region was analyzed with primers designed to amplify exons of *CDKN2C*: *CDKN2C* 1F, 5′ - CCGGAGTCATTAACCAG - 3′; *CDKN2C* 1R, 5′ - AAATATGGCAACCAACTAGG -3′; *CDKN2C* 2F, 5′ - TTTTGGGCCCATTTAAGACGTTC - 3′; *CDKN2C* 2R, 5′ - TAGGCACCAAGGTGGACGGGACA - 3′. PCR was performed with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s. A final extension of 72°C for 5 min concluded the PCR. PCR products were purified using Ampure PCR clean-up beads (Agencourt Bioscience Corp., Beverly, MA). Products were sequenced directly using BigDye v3.1 (Applied Biosystems, Foster City, CA) on an ABI 3130xl Genetic Analyser (Applied Biosystems). 53 samples were sequenced for mutations in *CDKN2C*, of which 9 were hemizygous, and 44 were diploid for this locus.

Methylation analysis

Genomic DNA (10 ng) was treated with sodium bisulfite using EpiTect® Bisulfite Kit (Qiagen). The fragment of interest was amplified from bisulfite-modified DNA and sequenced as described (27). As a positive control for methylation we used a Hodgkin Lymphoma cell line, L-540, which was kindly provided by Dr. Sanchez-Beato (CNIO, Spain).

Western blot analysis

Whole cell lysates were made by washing 10×10^6 MM HMCLs growing exponentially in cold PBS followed by lysis in cell lysis buffer (50mM Tris –HCl, pH 7.5; 150mM NaCl; 1% (v/v) Triton X-100 + 0.5% (w/v) Na-Deoxycholate; 1mM EDTA; 1mM PMSF) containing a cocktail of protease inhibitors (Roche Applied Science, UK). Protein concentration was estimated by using the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., Rockford). Twenty micrograms of total proteins were fractionated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with polyclonal anti-p18 (Santa Cruz Biotechnology) antibody followed by chemiluminescence detection using ECL Plus™ (GE Healthcare, UK). Blots were stripped and immunoblotted with anti-α-tubulin antibody to control for protein loading.

Statistical analysis

Overall survival (OS) was calculated from the date of diagnosis using the Kaplan-Meier method, with the log rank test used to calculate significance. The results were considered statistically significant when $P \leq 0.05$. Statistical analysis was performed using the SPSS 14.0 software (SPSS, Chicago, IL). The identification of statistical differences between the disease states was determined using hypergeometric function analysis.

Results

Homozygous deletion of 1p identified by gene mapping analysis

Following a detailed analysis of the gene mapping profile of chromosome 1p, we found 3 homozygous deletions (median size 0.43 Mb; range 0.39-1.74 Mb), comprising 4% of all cases (Fig. 1*A* and Supplementary Figure 1). The homozygous deletions affect two genes at 1p32.3, *CDKN2C* and *FAF1*. With the exception of MM1.r, MM1.s and KMS-26, *FAF1* was expressed in all myeloma cell lines studied (data not shown), which is consistent with previous observations (17). However, the expression of *CDKN2C* varies greatly across cell lines by as much as 7500-fold, compared to only 15-fold for *FAF1,* suggesting that its expression level may be affected by molecular mechanisms important myeloma progression.

As *CDKN2C* is known to affect cell-cycle regulation, which is of great importance in the pathogenesis of myeloma, we decided to concentrate on characterizing *CDKN2C* in this study. While we do not discount the potential relevance of *FAF1*, and plan to study this gene further, the focus of this study was *CDKN2C* which is a good candidate gene in myeloma.

Homozygous deletions of this region including the *CDKN2C* gene were confirmed by q-PCR of DNA copy number (data not shown). Reviewing the mapping data further identified another nine cases with LOH of chromosome 1p of which four were interstitial (14.32-90.10 Mb) and one additional case had uniparental disomy (UPD) of 1p (Fig. 1*B*). These nine cases with hemizygous deletion/UPD, together with the three samples with homozygous deletion, represent 15% of the total cases analyzed and define a minimally altered region at 1p32.3, containing *CDKN2C*.

FISH analysis of 1p32.3

The results obtained by mapping data were consistent with the results of FISH analysis using a probe to 1p32.3 (Fig. 1*C*). As a result of this analysis we only found one discrepancy where SNP data showed a homozygous deletion whereas FISH data suggested a hemizygous deletion. However, when a smaller FISH probe was used homozygous deletion in this sample was confirmed.

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We extended the analysis using FISH to analyze a further 484 plasma cell neoplasms. The results of this analysis showed that hemizygous deletion of 1p32.3 was present in 3/66 MGUS cases (4.5%), in 4/39 SMM cases (10.3%) and in 55/369 MM cases (15%) consistent with a statistically significant difference in distribution between MGUS vs. MM ($P = 0.018$) (Fig. 1*D*).

We looked at the impact of the deletion of the *CDKN2C* locus on OS, and found that loss of either one or two copies of 1p32.3 was associated with a worse median OS of 22 months compared to 38 months in cases with intact 1p32.3 ($P = 0.003$) (Fig. 2). In a comparison of the baseline levels of hemoglobin, platelet count, lactate dehydrogenase, C-reactive protein, calcium, serum creatinine, age and International Staging System (ISS) we found that while deletion of 1p32.3 was associated with high creatinine level ($P = 0.03$) no other statistically significant associations with baseline variables were identified (Table 1).

To further show that this region is important, we also carried out FISH analysis on the same dataset using a probe at 1p12, which did not show a statistically significant association for OS between cases with deletion of 1p12 and those with intact 1p12 ($P = 0.125$)

Correlation of gene mapping changes with expression patterns

The expression pattern of *CDKN2C* was examined in the total data set, specifically comparing cases with and without homozygous deletion (Table 2; Fig. 3). The distribution of the expression pattern highlights the strong correlation of low expression of *CDKN2C* with homozygous deletion. In addition, three of nine (33%) cases with hemizygous deletion and 19 of 66 (29%) cases without alterations of 1p32.3 also expressed *CDKN2C* at a similar low level. *CDKN2C* was strongly overexpressed in six cases: one case had a hemizygous deletion and the other five cases were diploid at the *CDKN2C* locus.

Analysis of gene mapping and expression data of CDKN2C and its effect on proliferation

CDKN2C is a negative regulator of cell cycle progression and cases lacking *CDKN2C* would be expected to have a high rate of cell cycling. To test this hypothesis, the expression level of *CDKN2C* was correlated with an expression-based proliferation index (PI). The PI was used to divide the cases into quartiles and the three cases with homozygous deletion of *CDKN2C* were shown to be the most proliferative myelomas (Table 2). The level of expression of *CDKN2C* was also correlated with proliferation defined by this index. Contrary to what would be expected, cases with a high expression level were also seen in the group with an increased PI (data not shown), suggesting that mechanisms of resistance to the inhibitory effects of CKIs are present and could result in G1/S deregulation.

Correlation of expression array data and protein levels of CDKN2C

In order to determine whether the expression of *CDKN2C* at the protein level correlates with the expression array data, we examined a panel of HMCLs using SNP mapping, expression array analysis and western blotting. CDKN2C protein was expressed in eight HMCLs (JIM3, JIM1, KMS-12-BM, KMS-11, U266, H929, RPMI8266 and LP-1). Expression array data showed that six HMCLs expressed *CDKN2C* at levels equivalent to high expression in patient samples. *CDKN2C* was absent in the cell line with homozygous deletion (KMS-26) of 1p32.3 locus. These results confirm that the gene expression of *CDKN2C* is mirrored by protein expression (Supplementary Fig. 2).

Analysis of the residual allele in cases with hemizygous deletion of 1p32.3

The status of the residual allele in cases with a hemizygous deletion was examined using a comprehensive mutation and methylation analysis of the *CDKN2C* locus. This analysis did not detect either mutation or methylation changes at this locus, suggesting that it is silenced

by other mechanisms or its biological effect is reduced through hemizygosity. Despite this negative result, mutation screening identified nine polymorphisms of *CDKN2C* (data not shown).

Variations at other regions affecting the G1/S transition point

The prevalence of *CDKN2C* loss suggests that other changes affecting the G1/S transition point may be important in the pathogenesis of myeloma. To analyze this hypothesis further we looked for copy number changes affecting other genes acting at the G1/S transition point, in particular looking for deletion/LOH of other CKIs and amplification of cyclin-dependent kinases (CDK). This analysis identified an additional nine cases with hemizygous deletion at 6p, 9p, 12p and/or 19p, within which *CDKN1A* (p21), *CDKN2A* (p16)/*CDKN2B* (p15), *CDKN1B* (p27) and *CDKN2D* (p19) are located (Table 3). The most frequently deleted gene was *CDKN1B* with hemizygous deletion identified in 6 cases. However, there was no clear association between copy number change and expression levels (data not shown).

An alternative way to deregulate the G1/S transition point is amplification and overexpression of a positive regulator such as cyclin D-*CDK4/6*. Analyzing this possibility we identified 39 cases (50%) with gains of 6p, 7q, 11q and/or 12p within which *CCND3*, *CDK6*, *CCND1* and *CCND2* are located (Table 4). However, these chromosomes are frequently present in increased copy number in myeloma, and we were unable to correlate this increased copy number with significantly increased expression levels of the genes contained within these regions, effectively ruling this out as the mechanism of G1/S transition deregulation in these cases.

The translocation/cyclin D (TC) classification of myeloma is based on the presence of defined chromosomal translocations and/or overexpression of a D group cyclin (26). D cyclins act at the G1/S transition point and, therefore, we investigated whether loss or inactivation of *CDKN2C* acts together with this mechanism to deregulate G1/S, or whether it is present within a distinct group of myeloma. This analysis showed that there was no particular D cyclin expression group in which *CDKN2C* was affected (data not shown).

It has been postulated that it is the ratio of CKIs and CDKs that is important in controlling cell cycle progression and we wished to explore this relationship further within this data set. We found that while the cases with homo/hemizygous deletion of *CDKN2C* showed high level expression of *CDK4* or *CDK6*, we could not demonstrate a consistent relationship between the levels of expression of these factors through the remainder of the data set. In order to explore the relationship of *CDK4/6* with *CDKN2C* further we looked at the level of CDK expression in cases with low *CDKN2C* and vice-versa but cases with this pattern were not numerous enough to allow us to reliably determine the clinical outcome. We did, however, identify 4 cases with low *CDKN2C* expression and intermediate/high level *CDK4/6*, the clinical outcome of which did not differ from the remainder of the cases. In an exploratory analysis examination of the impact of *CDK6* expression alone on outcome showed that cases with high *CDK6* expression compared with low expression were associated with an adverse prognosis, with an overall survival of 40 months vs. not reached, $P = 0.047$ (Supplementary Fig. 3). We confirmed our results on a large independent dataset (GSE2658), consisting of 559 cases treated with Total Therapy 2 (TT2) from the University of Arkansas (28). In this dataset, cases in the fourth quartile of *CDK6* expression had an inferior OS in comparison with the remainder ($P = 0.003$). In contrast, no such association was seen for *CDK4* expression (data not shown).

Discussion

While, deletions of *CDNK2C* have been identified previously in myeloma cell lines (16, 17) and a limited number of patients (17, 18) we have been able to extend the analysis further. In this study, chromosome 1 was scanned using SNP mapping and expression analysis in 78 presenting myeloma samples. A cluster of three homozygous deletions located at 1p32.3 was identified. The size of these deletions was in the range of 0.39 to 1.74 Mb (median size 0.43 Mb) and defined a minimally deleted region containing *CDKN2C*. This gene is a good candidate for involvement in myeloma pathogenesis because of its physiological role in inhibiting cell cycle progression.

Homozygous deletions are important markers of genes that may be important in the progression of the disease, as they require two rounds of deletion and selection, demonstrating the importance of obliterating expression of that gene in the cell. Although only 3 cases with homozygous deletion are detected, in our data series, it demonstrates the importance of *CDKN2C* in these cases, and it may be that inactivation in the other cases occurs through disruption of cell cycle regulatory elements, which are upstream of *CDKN2C* activation (as we could not demonstrate inactivation through mutation or methylation of the remaining allele). The homozygous deletions detected in our study could not distinguish between *CDKN2C* and *FAF1* and as these are situated proximal to one another they may highlight the importance of *FAF1*. However, it has been shown that homozygous deletions only affect *CDKN2C* in three myeloma cell lines, indicating that *CDKN2C* is in fact the targeted gene in this region (17).

It has been reported that when a gene is occasionally homozygously deleted and frequently hemizygously deleted, that they are often haploinsufficient. The frequency of homozygous deletions depends on the timing of the first deletion; early versus late onset (29). In our study we found that hemizygous deletion is frequent and homozygous deletion rare, indicating that this gene is a candidate for haploinsufficiency. In addition, a study in mice examining the function of *CDKN2C* as a tumor suppressor shows that this gene can be haploinsufficient as a tumor suppressor (30).

The importance of the G1/S transition point in the pathogenesis of myeloma has been previously recognized and genes regulating this checkpoint have been used as the basis for the TC classification (26). In this study, deletion of *CDKN2C* segregates independently between the TC groups, apparently acting in concert with overexpression of any of the D group cyclins to deregulate G1/S.

The current analysis in newly presenting patients suggests that deletion of *CDKN2C* is frequent (15% of cases) and is associated with decreased *CDKN2C* expression levels and increased proliferation, as would be expected from its physiological role. In addition, we demonstrated that cases with deletion of *CDKN2C* have a worse overall survival than those lacking this alteration, highlighting the clinical importance of this deletion. We extended the analysis of the clinical relevance of deletion of 1p32.3 by examining its importance in the multi-step model of progression from normal through MGUS to myeloma. Deletions of *CDKN2C* were observed in 4.5% of MGUS cases, 10.3% of SMM cases and 15% of MM cases, suggesting that deletion of *CDKN2C*, associated with increased proliferation, provides a mechanism of progression from MGUS to myeloma.

A surprising finding was the presence of six cases (7.7%) over-expressing *CDKN2C*. Counter-intuitively, four of these cases (5.1%) also had an increased proliferation index. This suggests that these cases have developed resistance to the inhibitory effects of *CDKN2C*, although the mechanism leading to this is uncertain (17). The possible explanation might be that G1 progression is governed by the balance between positive

(CDKs and cyclins) and negative regulators (CKIs) and not by the level of any single cell cycle regulator alone. Alternatively, as we know that *CDKN2C* is required for negative cell cycle control during the differentiation of B-cells to plasma cells, it could be expressed in cycling normal plasma cell precursors, and does not imply a resistance to *CDKN2C*. To gain insight into these potential mechanisms further, we looked at the expression patterns of these genes and found that the proliferating cases with overexpression of *CDKN2C* had a high level of expression of *CDK4* consistent with the possibility that the inhibitory effects of *CDKN2C* could have been over-ridden by either deregulated cyclin D or *CDK4/6* expression in some cases of myeloma (31). However, even in such cases it would be predicted that the proliferation of myeloma cells would be more rapid in the absence of *CDKN2C* and this paradox requires further investigation.

While this study has focused on copy number changes, we also examined the potential for point mutations or methylation to affect the residual *CDKN2C* allele. Loss of function by deletions, mutations and methylation of the CKIs has been previously described. However, despite having carried out an extensive analysis we could not define inactivating events on the residual allele and our results would be consistent with a primary molecular mechanism of *CDKN2C* inactivation in patient samples being via deletion and possibly haploinsufficiency. Others have found mutations in myeloma cell lines (KMS-12BM) but we did not find this mutation in our laboratories cell lines.

In addition to *CDKN2C*, a number of other molecules are important in controlling the G1/S transition point. Due to the global nature of the mapping and expression analysis carried out in this study, in addition to analyzing *CDKN2C*, we were able to analyze changes impacting on the other CKI loci affecting this transition point. This analysis showed relatively frequent loss of *CDKN1B*, but the number of cases was insufficient to demonstrate an association between deletion and expression. Looking at the role of this gene further and whether its loss may interact with *CDKN2C* loss, we observed that *CDKN1B* copy number was normal in all cases with hemizygous deletion of *CDKN2C*, while it was reduced to one copy only in cases where *CDKN2C* copy number was normal, suggesting that deletion of either one of these genes may be of importance. Functionally, we know that *CDKN2C* plays an important role in end-stage PC differentiation and consequently the loss of one copy of *CDKN1B* would not be predicted to affect G1 progression in plasma cells in presence of both copies of *CDKN2C*. In contrast, loss of one copy of *CDKN2C*, even in the presence of both copies of *CDKN1B*, would be predicted to significantly alter G1 progression consistent with our results and a report that *CDKN2C* is haploinsufficient in mediating tumor suppression (30). However, the frequency of both *CDKN2C* and *CDKN1B* deletions in this series warrants further investigation determine the relative importance of *CDKN1B* compared to *CDKN2C* in a large data set.

In addition to studying the negative regulation of G1/S, we were able to analyze the impact of increased copy number at the *CDK4/6* and the D group cyclin loci. We looked at the genomic structure at the *CDK6* locus and showed that there was frequent copy number gain of *CDK6* in hyperdiploid MM, based on its location at 7q21. This is perhaps not surprising as it is one of the odd numbered chromosomes frequently gained in myeloma. However, we also found gain of this region in nonhyperdiploid myeloma. The finding of an association of high *CDK6* expression with adverse overall survival is interesting but requires testing further in additional data sets. We could not find a firm correlation between *CDKN2C* and *CDK6* expression or with *CDK6* copy number, suggesting that additional mechanisms influence CDK expression. However, these data confirm the clinical importance of cell cycle regulation and are consistent with in vitro studies (31), suggesting that *CDKN2C* acts in cooperation with over expression of *CDK4/6* to deregulate the G1/S transition point and

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the association between *CDKN2C-CDK4/6* being potentially more important than the over expression of the D group cyclins alone.

In summary, high resolution mapping and expression analysis were performed in primary patient material to understand the role of *CDKN2C* in MM. SNP mapping identified homozygous deletion at 1p32.3 which was validated by FISH analysis. Cases with homozygous deletion had a low expression of *CDKN2C* and were the most proliferative myelomas, consistent with its biological function. The analysis of patients with MGUS, SMM and MM showed an increased incidence of deletion of 1p32.3 in disease progression and patients with loss of *CDKN2C* have a worse overall survival. Lack of mutations and methylation in cases without deletion suggest that the main mechanism of loss of function of *CDKN2C* is by deletions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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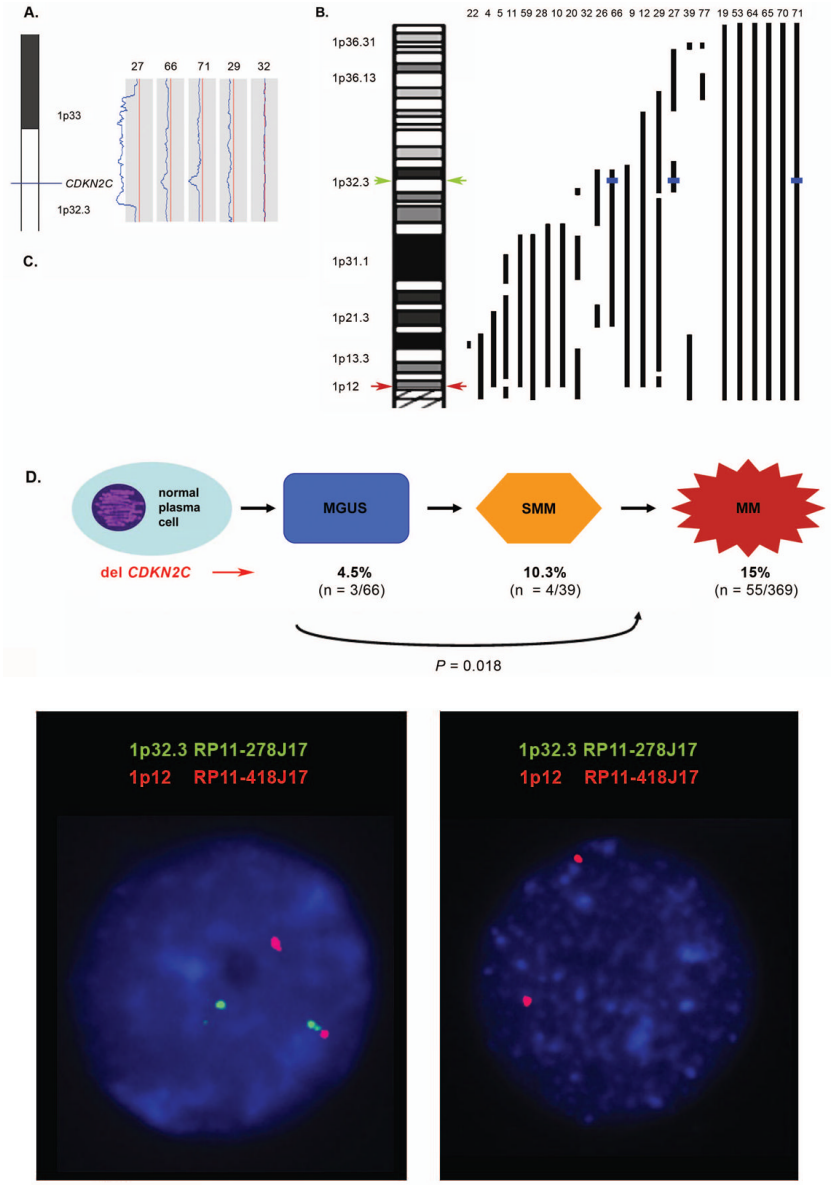

Figure 1.

Deletions of 1p as determined by SNP array. (**A**) Copy number (CN) is shown in the grey panel to the right of the ideogram, where the line indicates the CN at the point on the chromosome. Homozygous deletion is observed in 1p32.3 in three cases (27, 66 and 71), hemizygous deletion is shown in case 29, and a sample without deletion of 1p32.3 in case 32. (**B**) The positions of deletions in individual samples are shown as black vertical lines to the right of the ideogram. The green arrow at 1p32.3 indicates the location of *CDKN2C* and of BAC RP11-278J17. The red arrow at 1p12 indicates the location of BAC RP11-418J17. Cases 27, 66 and 71 had homozygous deletion (blue line) and case 19 had UPD of 1p. (**C**) FISH of chromosome 1p. An example of normal FISH signal for BACs at 1p32.3 and 1p12 (case 22) (left) and an example of homozygous deletion at 1p32.3 with normal signal at 1p12 (case 27) (right). (**D**) Deletion of 1p32.3 (*CDKN2C*) in the model of myeloma pathogenesis. MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma.

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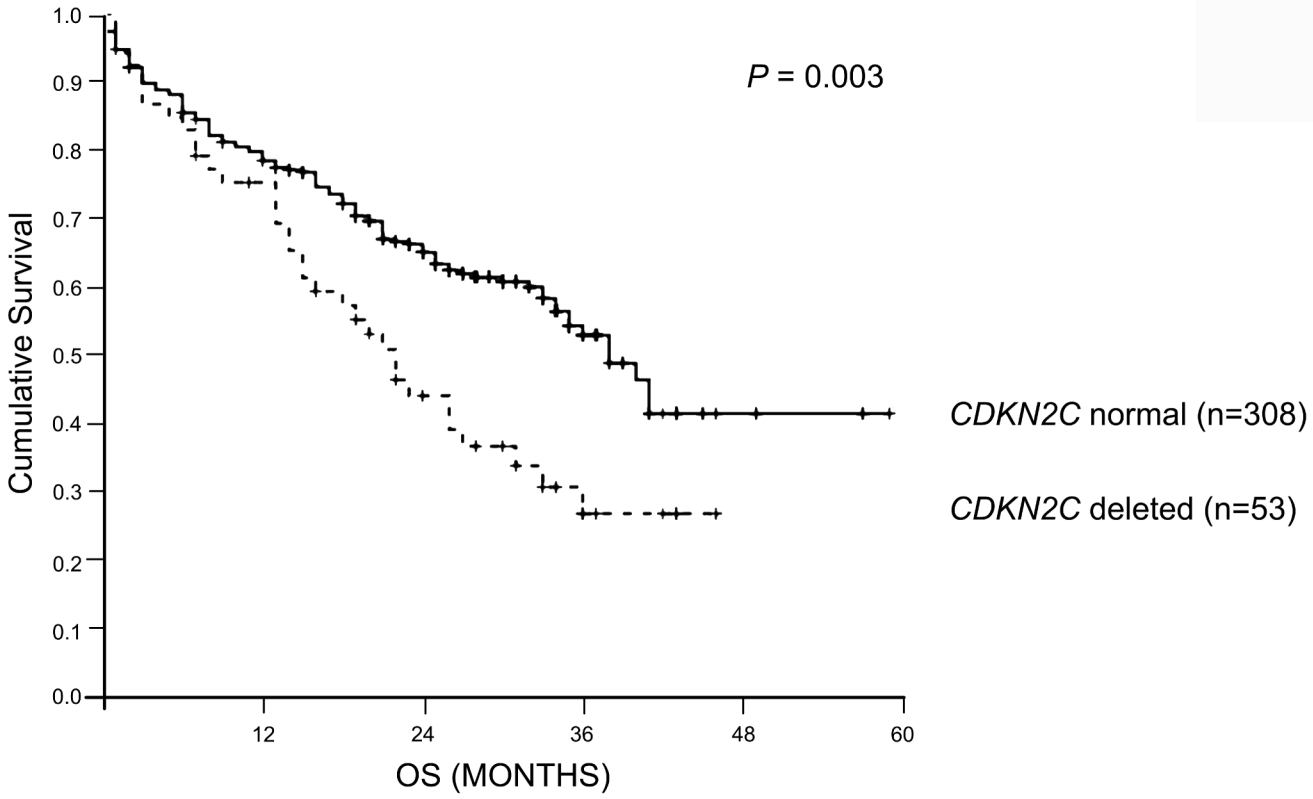

Figure 2.
Effect of status of *CDKN2C* on overall survival. Kaplan-Meier estimates of overall survival in cases with alterations of *CDKN2C* versus cases without alterations of this gene (*P* = 0.003).

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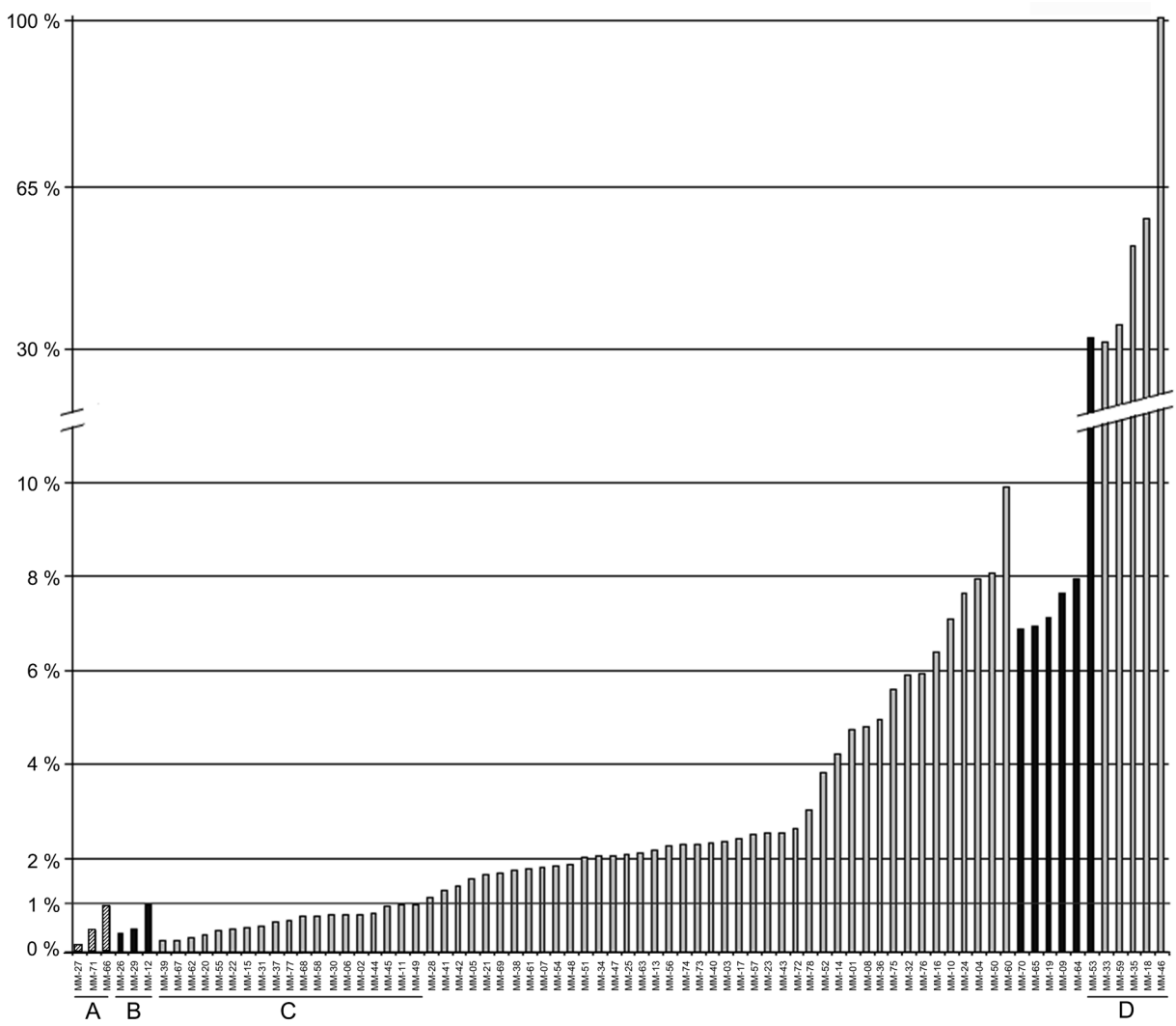

Figure 3.

Distribution of the level of expression *CDKN2C*. Sample expression data are expressed as a percentage of the highest value. The level of expression of *CDKN2C* was divided into quartiles, 0<2%, 2<4%, 4<10% and 30<100% (y-axis). Cases are grouped as homozygous (hatching), hemizygous (black) and intact *CDKN2C* (white). Cases with homozygous deletion (**A**) and those cases with similar expression levels but with hemizygous deletion (**B**) or intact *CDKN2C* (**C**). One case with hemizygous deletion and five cases without alterations of 1p32.3 showed the highest level (30-100%) of expression *CDKN2C* (**D**).

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Table 1

Association between baseline clinical variables and 1p32.3 (*CDKN2C*) status

Clinical data MM	All cases		No del(1p32.3)		del(1p32.3)		
	n	(%)	n	(%)	n	(%)	p-value
International Staging System (ISS)							
ISS 1	45	30.4	39	31.5	6	25.0	0.163
ISS 2	56	37.8	49	39.5	7	29.2	0.120
ISS 3	47	31.8	36	29.0	11	45.8	0.052
Hb <10 g/dl	71	34.0	59	34.3	12	32.4	0.149
Plt <150 × 10^9/l	26	13.4	22	13.7	4	12.1	0.220
LDH ≥200 U/l	106	85.5	91	85.0	15	88.2	0.287
CRP ≥10 mg/l	63	44.7	51	43.6	12	50.0	0.150
Calcium ≥2.6 mmol/l	46	23.4	35	21.9	11	29.7	0.098
Creatinine >140 μmol/l	47	24.0	34	21.3	13	36.1	**0.030**
Age≥70 years	134	35.5	105	34.1	29	42.0	0.051

Abbreviations: del(1p32.3), deletion of 1p32.3; Hb, hemoglobin; plt, platelet count; LDH, lactate dehydrogenase; CRP, C-reactive protein.

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Table 2

Gene mapping and expression data of *CDKN2C* and expression-based proliferation index

Gene mapping and expression data of *CDKN2C*

CDKN2C mapping status	expression data †			
	0 < 2%	2% < 4%	4% < 10%	30% < 100%
Normal	30	18	13	5
Hemyzigous deletion	3	0	5	1
Homozygous deletion	3	0	0	0

Gene mapping data of *CDKN2C* and proliferation index

CDKN2C mapping status	expression-based proliferation index ‡			
	L	LI	HI	H
Normal	19	18	17	12
Hemyzigous deletion	1	1	3	4
Homozygous deletion	0	0	0	3

† Expression data are grouped according to the values of expression in four groups: 0<2%, 2<4%, 4<10% and 30<100%.

‡ The expression-based proliferation index was divided into quartiles: L, Low; LI, Low-intermediate; HI, High-intermediate; H, High.

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Table 3

Gene mapping data of the genomic regions around the cyclin-dependent kinase inhibitor loci

Status of other CKI loci in cases with Homozygous deletion of *CDKN2C*

Case	***CDKN2C* (p18) 1p32**	***CDKN1A* (p21) 6p21.2**	***CDKN1B* (p27) 12p13.1-p12**	***CDKN2A* (p16) / *CDKN2B* (p15) 9p21.3**	***CDKN2D* (p19) 19p13.2**
MM-27	0	+	N	−	N
MM-66	0	N	N	N	++
MM-71	0	N	N	N	+

Status of other CKI loci in cases with Hemizygous deletion of *CDKN2C*

Case	***CDKN2C* (p18)** 1p32	***CDKN1A* (p21)** 6p21.2	***CDKN1B* (p27)** 12p13.1-p12	***CDKN2A* (p16) / *CDKN2B* (p15)** 9p21.3	***CDKN2D* (p19)** 19p13.2
MM-9	−	N	N	++	+
MM-12	−	N	N	+	+
MM-19	−	N	N	+	+
MM-26	−	N	N	+	+
MM-29	−	N	N	N	N
MM-53	−	N	N	+	+
MM-64	−	N	N	+	+
MM-65	−	N	+	N	+
MM-70	−	N	N	−	+

Cases identified with hemizygosity at others CKI loci

Case	***CDKN2C* (p18)** 1p32	***CDKN1A* (p21)** 6p21.2	***CDKN1B* (p27)** 12p13.1-p12	***CDKN2A* (p16) / *CDKN2B* (p15)** 9p21.3	***CDKN2D* (p19)** 19p13.2
MM-7	N	N	−	N	N
MM-10	N	N	−	+	+
MM-15	N	−	N	N	N
MM-17	N	N	−	N	N
MM-20	N	N	N	−	N
MM-21	N	N	−	+	+
MM-32	N	N	N	−	N
MM-34	N	N	−	N	+

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Status of other CKI loci in cases with Homozygous deletion of *CDKN2C*

Case	*CDKN2C* (p18) 1p32	*CDKN1A* (p21) 6p21.2	*CDKN1B* (p27) 12p13.1-p12	*CDKN2A* (p16) / *CDKN2B* (p15) 9p21.3	*CDKN2D* (p19) 19p13.2
MM-46	N	N	−	N	+

Abbreviations: CKI, cyclin-dependent kinase inhibitor; “0”, without copy of chromosome; “−”, one copy of chromosome; “N”, two copies of chromosome; “+”, three copies of chromosome; “++”, four copies of chromosome.

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Table 4

Gene mapping data of the genomic regions around the cyclin dependent kinases and cyclin D genes

		Total number of cases with copy number gain at the *CDK* loci					
FISH	**Case**	***CDKN2C*** **1p32.3**	***CDK4*** **12q14.1**	***CDK6*** **7q21-q22**	***CCND1*** **11q13**	***CCND2*** **12p13.32**	***CCND3*** **6p21.1**
HRD	MM-3	N	N	N	+	N	N
HRD	MM-5	N	N	N	+	N	N
HRD	MM-9	−	N	+	N	N	N
HRD	MM-10	N	N	N	+	N	N
HRD	MM-12	−	N	N	+	N	N
HRD	MM-18	N	N	+	+	N	N
HRD	MM-19	−	N	+	+	N	N
HRD	MM-21	N	N	+	+	N	N
HRD	MM-22	N	N	+	+	−	N
HRD	MM-26	−	N	N	+	N	N
HRD	MM-30	N	N	N	++	N	N
HRD	MM-31	N	−	+	+	N	N
HRD	MM-34	N	N	N	+	−	N
HRD	MM-37	N	N	+	N	N	N
HRD	MM-40	N	N	N	+	N	N
HRD	MM-42	N	N	+	+	N	N
HRD	MM-47	N	N	N	+	N	N
HRD	MM-48	N	N	N	+	N	N
HRD	MM-53	−	N	N	N	N	N
HRD	MM-55	N	N	+	+	N	N
HRD	MM-56	N	N	N	+	N	N
HRD	MM-64	−	N	+	+	N	N
HRD	MM-70	−	N	+	+	N	N
HRD	MM-72	N	N	+	N	N	N
HRD	MM-73	N	N	+	+	N	N
HRD	MM-77	N	N	+	+	N	N
HRD UNK	MM-46	N	N	+	+	−	N

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Total number of cases with copy number gain at the *CDK* loci

FISH	Case	*CDKN2C* 1p32.3	*CDK4* 12q14.1	*CDK6* 7q21-q22	*CCND1* 11q13	*CCND2* 12p13.32	*CCND3* 6p21.1
UNK	MM-32	N	N	N	+	N	N
UNK	MM-52	N	N	+	+	N	N
UNK	MM-68	N	N	N	+	N	N
t(4;14)	MM-65	−	N	N	+	+	N
t(4;14)	MM-66	0	N	++	+	N	N
t(4;14)	MM-74	N	N	N	N	N	+
t(6;14)	MM-29	−	N	N	N	N	N
t(6;14)	MM-69	N	N	N	N	N	+
t(11;14)	MM-7	N	−	N	+	−	N
t(11;14)	MM-57	N	N	N	+	N	N
t(11;14)	MM-61	N	N	N	+	N	N
t(11;14)	MM-78	N	N	N	+	N	N
t(14;16)	MM-24	N	N	+	N	N	N
t(14;16)	MM-27	0	N	N	−	N	+
−13	MM-71	0	N	+	N	N	N

Abbreviations: CDK, cyclin-dependent kinase; HRD, hyperdiploid; UNK, IgH rearrangement with unidentified partner chromosome; “0”, without copy of chromosome; “−”, one copy of chromosome; “N”, two copies of chromosome; “+”, three copies of chromosome; “++”, four copies of chromosome.