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Original Article

Down-regulation of Cyclin D3 by Small-interfering RNA Induces Cell Cycle Arrest and Apoptosis through the Dissociation of $p27^{Kip1}$ in a t(6 ; 14) (p21 ; q32) Positive Myeloma Cell Line

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Translocation of chromosome t (6 ; 14) (p21 ; q32) results in overexpression of the cyclin D3 gene (*CCND3*), and is a recurrent genetic alteration in multiple myeloma. To elucidate the biological role of the overexpression of the cyclin D3 protein (CCND3) in t (6 ; 14) (p21 ; q32), we transfected a *CCND3*-specific, small-interfering RNA (siRNA) into KMM-1 cells carrying t (6 ; 14) (p21 ; q32). Following transfection, CCND3 expression levels decreased with maximal effect after 24 hours. While CCND3 expression was down regulated the rate of proliferation in transfected KMM-1 cells was half that in control cells. Cell-cycle analysis revealed that transfection resulted in transition fron G1 to S being blocked, and the transfected cells underwent apoptosis. Immunoprecipitation experiments demonstrated that CCND3 formed a complex with the majority of $p27^{kip1}$ in KMM-1 cells under steady-state conditions. When CCND3 expression was down regulated the $P27^{kip1}$ shifted to cyclin E protein (CCNE) and formed a complex. Our results show that CCND3 is essential for the cellular growth of t (6 ; 14) (p21 ; q32)- positive myelomas and that CCND3 sequesters $p27^{kip1}$ from CCNE, resulting in functional inactivation of its anti-proliferative role. Modification of CCND3 and $p27^{kip1}$ interaction may be a novel therapeutic approach for t (6 ; 14) (p21 ; q32)-positive myeloma.

Key words cyclin D3, myeloma, apoptosis, $p27^{kip1}$, t (6 ; 14) (p21 ; q32)

INTRODUCTION

Multiple myeloma is a neoplasm arising from terminally differentiated B-cells producing immunoglobulin protein. Accumulated evidence in recent years has shown that various oncogenes involved in translocation of the immunoglobulin heavy chain gene (*IGH*) play a pivotal role in the pathogenesis of this disease. Recurrent chromosome translocations involving of *IGH* gene seen in myeloma are t (11 ; 14) (q13 ; q32), t (4 ; 14) (p16 ; q32), t (6 ; 14) (p25 ; q32), and t (14 ; 16) (q32 ; q23). The t (11 ; 14) (q13 ; q32) is also found in the vast majority of mantle cell lymphomas[1-4], whereas other translocations are restricted to myelomas[4,5]. More recently, the *cyclin D3 (CCND3)* gene, at 6p21, has been shown to be a target of t (6 ; 14) (p21 ; q32) in myeloma, as well as in mature B-cell malignacies[6,7]. These *IGH* translocations bring the oncogenes near the regulatory elements of *IGH* and result in the deregulated expression of oncogenes. As a consequence of deregulation, such tumor cells appear to obtain a growth advantage through several pathways.

Cyclins D1 (CCND1), D2 and D3 are all synthesized in response to mitogenic stimulation and have a short half-life[8]. D-type cyclins form complexes with cyclin-dependent kinases (CDK, mainly CDK4 and CDK6). The CCND/CDK complex is then able to phosphorylate and inactivate Retinoblastoma protein (Rb), which initially binds to E2F transcriptional factors[9,10]. Once phosphorylated, Rb loses the capacity to stay bound to E2F and release transcription factors into the nucleus[9,10]. E2F induce the expression of positive cell cycle regulators, such as cyclin E, and promote cell cycle progression[9,10]. Withdrawal of this mitogenic stimulation attenuates the synthesis of D-type cyclins and, in turn, the catalytic activity of CDK[9,10]. In this context, the overexpression of D-type cyclin resulting from *IGH* translocation is speculated to disrupt cell-cycle regulation, and activate the CDK/Rb/E2F pathway, leading to autonomic cell proliferation.

The cell cycle is also controlled by negative regulator families. The INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$), which binds CDK4 and CDK6 but not other CDK,

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inhibits the kinase activity of CDK4 and CDK6 upon binding[9,10]. Regulators in the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) inhibit not only CDK4 and CDK6 but also other cyclin-dependent kinases, such as cyclin E- and cyclin A-dependent kinases[9,10]. In general, loss of INK4 and Cip/Kip function is associated with a poor prognosis in many human cancers[11-14]. However, overexpression of $p27^{Kip1}$ has been found in a subset of aggressive, diffuse large cell lymphomas and in Burkitt lymphomas[15,16]. It has been suggested that in tumor cells expressing both $p27^{kip1}$ and CCND3 at high levels sequestration of $p27^{Kip1}$ by CCND3 inhibits the function of $p27^{kip1}$ as a negative cell cycle regulator.

To test the hypothesis that the down-regulation of CCND3 causes an increase in free $p27^{kip1}$ and the recovery of its role as a negative regulator we transfected *CCND3*-specific small interfering RNA (siRNA) into a t(6; 14)(p21; q32)-positive myeloma cell line, in which CCND3 is overexpressed, to directly inhibit the expression of CCND3. We analyzed the correlation between inhibition and cell growth, induction of apoptosis and dissociation of $p27^{kip1}$ from CCND3.

MATERIALS AND METHODS

Cell line

The KMM-1 myeloma cell line with t(6; 14)(p21; q32) was kindly provided by Prof. Takemi Otuski, Department of Hygiene, Kawasaki Medical School (Okayama, Japan) and Prof. Huh Nam Ho, Department of Cell Biology, Okayama University Graduate School of Medicine and Dentistry (Okayama, Japan) through the Riken Bio-resource Center (Ibaragi, Japan). This cell line has been shown to express six times the normal level of CCND3[6].

Preparation of small interference RNA

A 21-nucleotide RNA that silences *CCND3* was chemically synthesized at Nihon Bio Service (Saitama, Japan). The siRNA sequences targeting *CCND3* (Acc. No. BC011616) corresponded to the coding region from 174 to 192 relative to the first nucleotide of the start codon. The nucleotide sequences were designated D31A (GAUGCUGGCUUACUGGAUGTT), and D31B (CAUCCAGUAAGCCAGCAUCTT). To anneal siRNA 50 μM of each single strand was incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, and 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C. KMM-1 cells were cultured in PRMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C. One hour before transfection, KMM-1 cells were diluted in Opti-MEM (Invitrogen, Carlsbad, CA) without serum so that the cell concentration would be approximately 5.0×10^5/ml at the time of transfection with siRNA/Lipofectamine 2000 (Invitrogen) complexes. The siRNA was diluted in Opti-MEM medium to a concentration of 33 nM. Lipofectamine 2000 was diluted in Opti-MEM just before use, as recommended by the manufacturer. Lipofectamine 2000 and siRNA were mixed gently and incubated for 5 min at room temperature. The siRNA/Lipofectamine 2000 complexes were added to KMM-1 cells and subsequently incubated at 37°C in 5% CO_2 for 4 h without serum. Opti-MEM medium with FBS was then added for further incubation. The transfection experiments were performed independently three times.

MTT assay

Fifty μl of diluted KMM-1,10 μl of siRNA/Lipofectamine 2000 complex and 200 μl of opti-MEM with FBS were incubated in 96-well microtiter plates. 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and the cells were incubated at 37°C for 3 h. After incubation, 100 μl of acidified isopropanol containing 4% (v/v) Triton X-100 was added to each well and the optical density was measured at 560 nm with a micro plate reader (Vmax : Molecular Devices, Sunnyvale, CA). The MTT assay was performed in triplicate and Student's t-test was used for statistical analysis. The transfected cells were harvested and used for Western blot and flow cytometer analyses.

Western blot analysis

The KMM-1 cells were harvested and washed in cold PBS, and then dissolved in SDS sample buffer. Fifty μg of total protein were separated on 15% polyacrylamide gel and transferred to a Hybond-C Extra nitrocellulose (Amersham Bioscience, Piscataway, NJ). Immunoblots were probed with the following anti/bodies : anti-cyclin D1 (BD Pharmingen, San Diego, CA) anti-cyclin D2 (BD Pharmingen), anti-cyclin D3 (BD Pharmingen), anti-cyclin E (BD Pharmingen), anti-$p27^{Kip1}$ (BD Parmingen) and anti-actin (Sigma). HRP-conjugated IgG (Amersham Bioscience) was used as a secondary antibody. Blots were visualized using ECL Plus Western Blotting Detection Reagents (Amersham Bioscience). Band intensity was quantified by densitometry analysis using NIH image software. The quantity of CCND3 was calculated by the ratio of CCND3 to Actin.

Immunoprecipitation assay

KMM-l cells (6×10^6) were harvested, washed with cold PBS, and then dissolved in lysis buffer (50 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l dithiothreitol (DTT), 10 mmol/L beta-glicerophosphate, 1 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l EDTA, 5 mmol/l

EGTA, 1 mmol/l phenyl-methylsulfonyl fluoride (PMSF), 5 μg/ml leupeptine, 2 μg/ml aprotinine). The cells were sonicated and supernatants were cleared by centrifugation at 12,000 g for 15 min at 4℃. Approximately 500 μg of total protein was incubated with 2 μg of the anti-$p27^{kip1}$ rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of protein A sepharose fast-flow sepharose beads (Amersham Biosciences). After gentle rotation for 24 h at 4℃ the beads were pelleted, washed three times with lysis buffer and resuspended in 10 μl of SDS sample buffer. Samples were denatured for 5 min at 90℃ and centrifuged at 12,000 g for 5 min. Ten μl of supernatant was separated by electrophoresis in 15% polyacrylamide gel and transferred to a Hybond-C Extra nitrocellulose membrane. Immunoblots were probed with anti-cyclin D3, anti-cyclin E, and anti-$p27^{kip1}$ antibodies as appropriate for Western blotting.

BrdU staining and flow cytometry analyses

To measure the S phase fraction in myeloma cell lines KMM-1 cells (1×10^6) were exposed to 10 μg/ml of bromodeoxyuridine (BrdU) for 30 min. After cells were harvested and washed in cold PBS, cell-cycle analysis was performed using a BrdU Flow Kit (BD Bioscience Pharmingen) according to the manufacturer's directions. Cells were analyzed by flow cytometric analysis with an EPICS flow cytometer (Beckman Coulter Inc, Fullerton, CA).

Detection of apoptosis

Apoptosis was quantified from the expression of Annexin V/PI stained with a Mebcyto Apoptosis kit (Medical & Biological Laboratories, Nagoya, Japan) in cells detected on an EPICS V flow cytomer.

RESULTS

Down-regulation of cyclin D3 expression by siRNA

Before the siRNA transfection experiments we determined the cell growth curve of the KMM1 cell line. Cells growing at a logarithmic rate were subjected to transfection experiments with several concentrations of siRNA of the laminin and *CCND3* genes. Expression of CCND3 was successfully down regulated at the protein level by transfecting with siRNA that was specific for *CCND3*. As shown in Fig. 1, the level of CCND3 clearly started to decline 16 h after transfection, reached a minimum after 24 h and subsequently recovered to the basal level at 72 h. These results indicated that siRNA significantly inhibited CCND3 expression from at least 24 to 48 h after transfection. Because several reports have documented that the loss of one D-type cyclin can be compensated by the expression of another, we further examined whether the down-regulation of cyclin D3 induces D1 or D2 expression in the KMM1 cell line. However, neither D1 nor D2 was expressed when CCND3 down-regulation was induced by siRNA (data not shown).

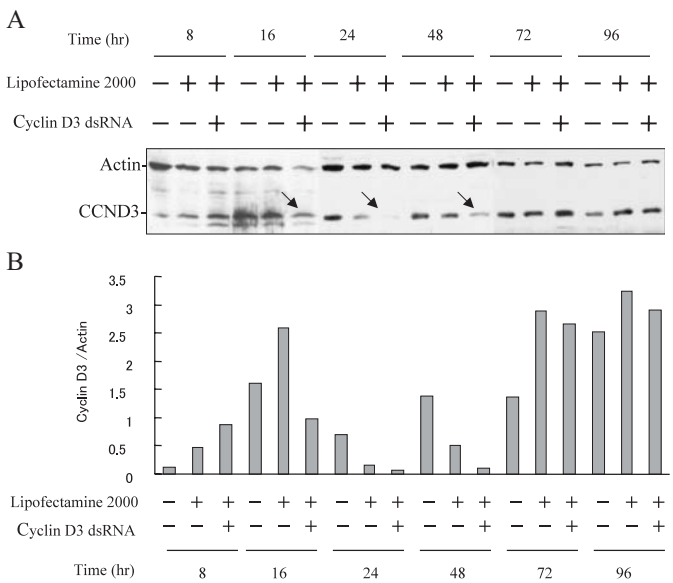

Fig. 1. Transfection of *cyclin* D3-specific siRNA reduced cyclin D3 protein in KMM1 cells.
(A) Cell lysates at 8, 16, 24, 48, 72 and 96 h after transfection were subjected to Western blot analysis. The blot was probed simultaneously with anti-cyclin D3 and anti-actin antibodies. Cyclin D3 expression was significantly decreased at 16, 24, and 48 h after transfection (*arrows*). The expression of actin protein showed internal control. (B) Relative intensity of CCND3 is shown as the CCND3/Actin ratio.

Down-regulation of CCND3 induced cell cycle arrest and apoptosis

To investigate the possible biological effects of *CCND3* siRNA, we assessed cell proliferation after transfection using the MTT assay. As the doubling time of the KMM-1 cell line in the logarithmic proliferation phase was estimated at 24 h, the effect of down-regulation of CCND3 expression on cell growth presumably was seen at 72 h following transfection. The proliferation of siRNA-transfected cells was half that of control cells (Fig. 2). A previous report suggested that the overexpression of *CCND1* resulted in the acceleration of cell cycle progression in a t(11; 14) (q13; q32)-positive myeloma cell line[17]; therefore, the effect of *CCND3* siRNA on the cell growth of KMM1 might be explained simply by the suppression of cell cycle progression. In addition to determining inhibition of cell proliferation, the cell assay with viable cells revealed that cells in the S phase were significantly decreased as cells accumulated in the G1 phase, suggesting that arrest occurred at G1 (Fig. 3). Taken together, the results suggest that inhibition of cell growth arose from interference

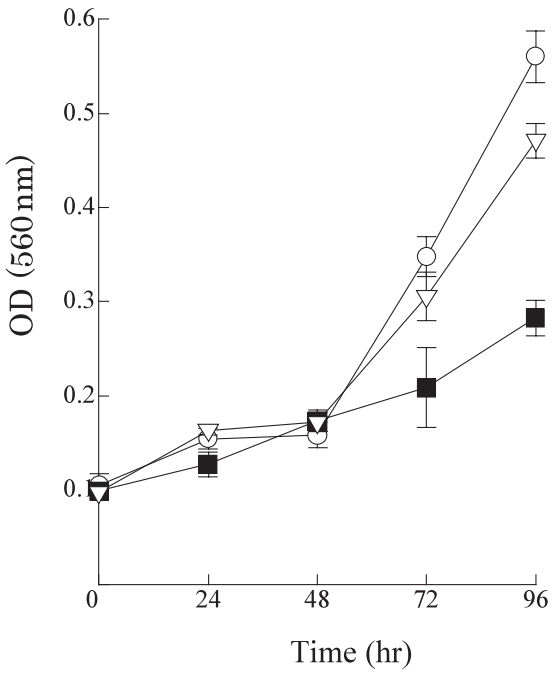

Fig. 2. The siRNA-transfected cells show retarded growth.
Cell growth was analyzed by MTT assay at 24, 48, 72, 96 h following siRNA transfection. Because KMM-1 cells were cultured initially at a low cellular density, the cells grew very slowly until reaching the logarithmic proliferation phase (48 h). The cell growth curve shown in the figure demonstrated that growth retardation occurred at 72 h after transfection. Solid boxes, open circles and open triangles represent *CCND3*-specific siRNA transfected cells, untreated control cells, and lipofectamin controls, respectively. Bars represent standard deviation.

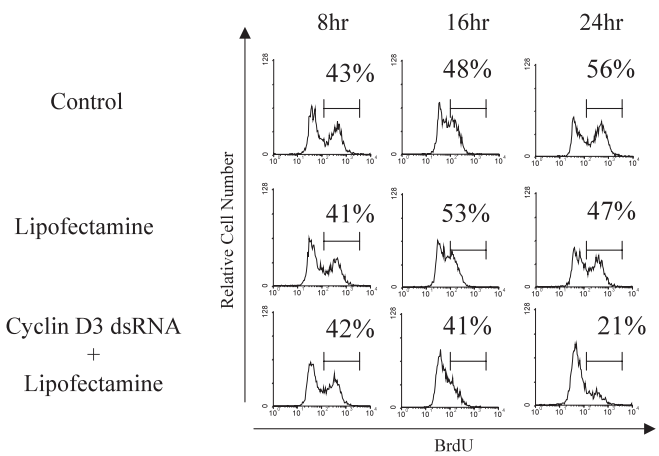

Fig. 3. siRNA transfection caused G1 arrest in KMM-1 cells.
Each cell (control, lipofectamin only and lipofectamin with siRNA) was incubated with BrdU and subjected to flow cytometry for cell-cycle analysis. In siRNA-transfected cells, the BrdU-positive fraction was decreased at 24 h (21%) after transfection indicating cell cycle arrest at the G1 phase.

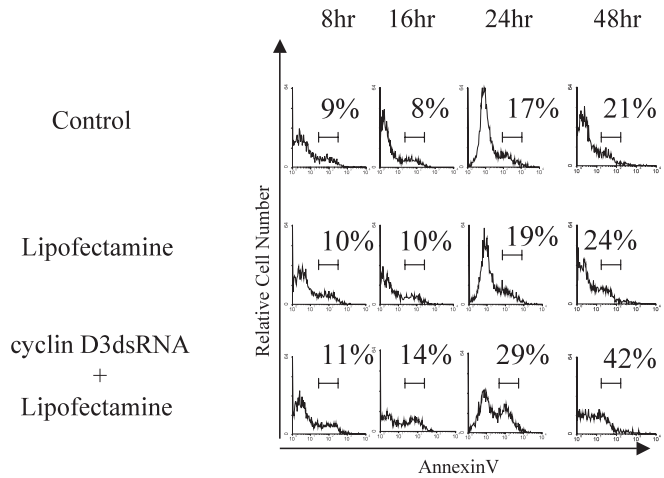

Fig. 4. siRNA-treated cell showed apoptotic cell death.
Cells were harvested at 8, 16, 24, and 48 h after siRNA transfection and subsequently stained with Anenexin. In siRNA-transfected cells the Annexin-positive fraction was increased at 24 h (29%) and 48 h (42%) indicating that apoptosis was induced by siRNA treatment.

in the progression from the G1 to S phases. Transition from the G1 to S phase is one of the major checkpoints of the cell cycle : determining cell proliferation, differentiation and apoptosis. To examine the fate of cells arrested in G1, we performed an experiment using Annexin staining to detect apoptotic cells. As shown in Fig. 4, the proportion of apoptotic cells increased among *CCND3* siRNA-transfected cells (Fig. 4). Thus, the down-regulation of CCND3 appeared to inhibit growth, arrest at G1 and increase apoptosis.

$P27^{kip1}$ sequestered by CCND3 combines with cyclin E following transfection of siRNA

To analyze the mechanism of apoptosis seen when CCND3 reduction was induced, we examined the interactions of $p27^{kip1}$, CCND3, and CCNE. Using immunoprecipitation, the majority of $p27^{kip1}$ was seen to form a complex with CCND3 (Fig. 5). When CCND3 expression was reduced by the addition of siRNA, the amount of CCND3 combining with $p27^{kip1}$ decreased. Moreover, the amount of CCNE bound to $p27^{kip1}$ increased.

DISCUSSION

Multiple myeloma is a uniformly fatal disease, with the mean survival just three or four years. The pathogenesis of this disease includes cell to cell interaction, activation of NF-κB, loss of tumor suppressor genes and chromosome translocations[18-21]. Chromosome translocation involving the immunoglobulin heavy chain gene at 14q32 is the most common genetic aberration in this disese[20,22,23]. The *IGH* translocations seen in B-cell malignancies are associated closely

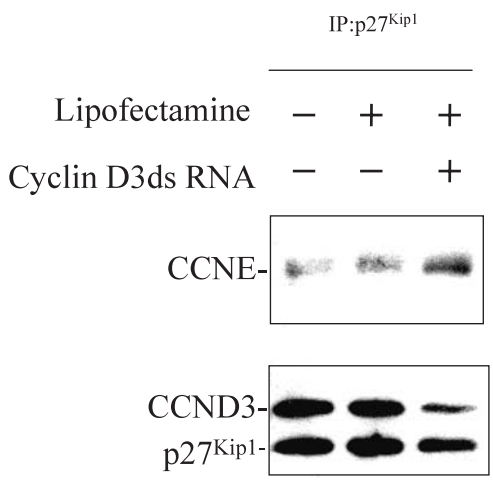

Fig. 5. $p27^{kip1}$ shifted to cyclin E following cyclin D3 down-regulation.
Whole cell lysates extracted from 24 h after treatment were subjected to immunoprecipitation analyses. The cell lysates were immunoprecipitated with rabbit anti-$p27^{kip1}$ and Western analysis was performed subsequently. As shown in the figure, $p27^{kip1}$ combined with cyclin E increased while that with cyclin D3 decreased in siRNA-transfected KMM1 cells. IP ; immunoprecipitation.

with the disease entity, however, those seen in myeloma are various and include t (11 ; 14) (q13 ; q32), t (4 ; 14) (p16 : q32), and t (6 ; 14) (p21 ; q32)[22]. Each *IGH* translocation may define the clinical characteristics and predict the prognosis of myeloma patients[24-26]. Moreover, a novel therapeutic strategy is now under development that targets the deregulated expression that results from *IGH* translocations[27, 28].

Deregulation of D-type cyclins occurs in almost all myeloma cells through *IGH* translocations or other events[29]. The overexpression of CCND3 seen in myeloma is thought to be a consequence of translocation between the *CCND3* locus and one of the immunoglobulin gene loci[6]. Overexpression of CCND1 has been shown to hasten progression through the cell cycle in t (11 ; 14) (q13 ; q32)-positive cells[17,30] ; however, the role of CCND3 overexpression has not been documented well. In this study, we transfected *CCND3*-specific siRNA into a t (6 ; 14) (p21 ; 32) - positive cell line, KMM-1, and silenced CCND3 expression at the protein level. Transfection of siRNA successfully and transiently reduced the level of CCND3, resulting in growth inhibition, cell cycle arrest, and apoptosis. These observations indicated that CCND3 is crucial for cell cycle progression and the survival of t (6 ; 14) (p21 ; q32)-positive myeloma cells.

Several reports have documented that CCND3 overexpression is associated with a high level of $p27^{kip1}$ expression in mature B cell malignancies[15,16,31]. In addition, CCND3 has been reported to bind $p27^{kip1}$, resulting in the loss of the anti-proliferative function of $p27^{kip1}$. The t (6 ; 14) (p21 ; q32)-positive myeloma cell line, KMM-1, expressed both CCND3 and $p27^{Kipl}$; thus, we focused on analyzing the interaction of CCND3 and $p27^{Kipl}$ during the silencing of CCND3 expression. The immunoprecipitation experiment (Fig. 5) showed that the vast majority of $p27^{Kipl}$ formed a complex with CCND3 protein, but not with CCNE, under steady-state conditions in KMM-1 cells. This result indicated that $p27^{Kipl}$ was sequestered from CCNE, and was thus functionally inactivated as an anti-proliferative molecule. When CCND3 expression was down-regulated $p27^{Kipl}$ became free from CCND3 and shifted to CCNE, inhibiting cell-cycle progression. Alternatively, CCND3 overexpression interfered with the formation of a $p27^{Kipl}$/CCNE complex and abrogated the anti-proliferative effect of $p27^{Kipl}$. Supporting our result, an earlier report[15] demonstrated that CCND3 overexpression could overcome G1 arrest mediated by $p27^{kip1}$ *in vivo*. Although the detailed mechanism was not fully examined in the presented study, cell-cycle arrest in G1 appeared to trigger the apoptosis of KMM-1 cells. The vast majority of $p27^{kip1}$ in KMM-1 cells bound to CCND3, as shown in Western blot analysis using the anti-$p27^{kip1}$ antibody for immunoprecipitation (Fig. 5), there should be some fraction of CCND3 not bound to $p27^{kip1}$. The fraction of overexpressed CCND3 that did not bind to $p27^{kip1}$ presumably promotes the cell-cycle progression of KMM-1 cells.

The deregulation of a particular oncogene involved in *IGH* translocation is thought to be the initial event in the development of myelomas. A transgenic model mouse that mimicks the deregulation of *C-MYC* caused by chromosome translocation showed tumor regression after *C-MYC* gene expression was modified in vivo[32, 33]. This result indicates that the modification of gene expression may be of therapeutic value, although genetic alteration is an early event in tumorigenesis. Various gene-silencing techniques are under development[34-36] and *BCL-2* anti-sense RNA therapy has been reported to improve clinical symptoms and outcomes in non-Hodgkin lymphoma patients[37]. Our results suggest that interaction of CCND3 with $p27^{kip1}$ may be a novel therapeutic approach to treat neoplasms that over-express CCND3 and $p27^{kip1}$.

In conclusion, this study showed that the down-regulation of CCND3 expression resulted in cell-cycle arrest and apoptosis via the dissociation of $p27^{kip1}$ from CCND3 in t (6 ; 14) (p21 ; q32)-positive cells.

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