Targeting Cyclin D1 in Non-small Cell Lung Cancer and Mesothelioma Cells by Antisense Oligonucleotides

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Abstract. *Background: Cyclin D1 overexpression exists in multiple types of cancer and is a potential chemopreventive or therapeutic target. Materials and Methods: Non-small cell lung cancer and mesothelioma cells were incubated with antisense oligonucleotides (ASO) to cyclin D1 (CD1) and evaluated for effects on cellular proliferation, apoptosis, expression of cell cycle-specific proteins, and protein phosphorylation states. Results: ASO to CD1 inhibited proliferation of non-small lung cancer cells and mesothelioma cells. ASO induced apoptosis as determined by TUNEL assay. Western blot analysis of cell lysate showed that ASO inhibited the de novo synthesis of CD1, CD3, and CDK2 in multiple cell lines. Immunoprecipitation and immunoblotting with phosphoantibodies demonstrated that CD1, CD3, and CDK2 exist in a phosphorylated state. Conclusion: The work demonstrates that non-small cell lung cancer and mesothelioma cells respond to ASO-mediated cellular growth inhibition. These findings make ASO to CD1 attractive as a potential therapeutic for mesothelioma and non-small cell lung cancer.*

Complexes of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors are implicated in the various stages of cell cycle progression in mammalian cells (1). CD1, CD2, and CD3, along with CDK4 and CDK6 have been established to play a key role for the cell to enter into, and progress through, the G1 phase. Once bound to CD1, CDK4 or CDK6 phosphorylates retinoblastoma protein (pRb) (2-6). The activity of pRb is controlled by both inactivating phosphorylation and physical association with growth-promoting factors, and it must be phosphorylated for the repression and release of E2F family of transcription factors for the G_1 to S transition to occur (7). CD1 has also been described as a protooncogene since cancerous cells exhibiting uncontrolled cell growth exhibit overexpression of CD1(8-9). Increased expression of CD1 has been documented in prostate cancer, squamous cell carcinoma, and colorectal cancer (10). CD1 antisense oligonucleotides (ASO), alone or in conjunction with other therapeutics/agents, have been reported to inhibit growth of cells from various cancer cells, *e.g.* colon (11), lung (12), pancreas (13), squamous cell carcinoma (14, 15), and hepatocellular carcinoma (16), to name a few. There is no report of any growth inhibition of mesothelioma cells by ASO to cyclin D1; hence, this work was undertaken targeting pleural mesothelioma cells along with non-small cell lung cancer cells by ASO to determine growth inhibition and to elucidate possible mechanisms of action of depleted CD1 on other cyclin proteins.

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Key Words: Antisense oligonucleotide, cyclin D, cyclin E, cyclin-dependent kinase, retinoblastoma protein, non-small lung cancer cells, pleural mesothelioma cells, apoptosis, kinases, phosphatases.

Materials and Methods

Production of ASO. CD1 ASO 5'-GGA GCT GGT GTT CCA TGG-3' was complementary to the translation start site of the CD1 cDNA, and sense oligomer 5'-CCA TGG AAC ACC AGC TCC-3' (SO) representing nucleotide 208-225, was used as control. Both sequences were synthesized at the University of Minnesota Oligonucleotide and Peptide Synthesis Facility.

Reagents. Various antibodies used included mouse anti- CD1, CD2, CD3, cyclin E, CDK2, (Invitrogen, Carlsabad, CA, USA); anti-actin (Ambion Anti, Foster City, CA, USA; Cell Signaling Technology, Danvers, MA, USA); anti-CDK4 (Invitrogen); rabbit anti-pRb, -CD1, -CD2; (Epitomics, Burlingame, CA, USA); antiphosphoserine, -phosphotyrosine, -phosphothreonine, -phosphatases (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and horse radish peroxidase-conjugated goat antimouse/rabbit antibodies (Thermo Scientific, Rockford, IL, USA). All reagents used were analytical grade.

Cell culture. Non-small lung cancer cells (H661 and H2030), mesothelioma cells (H2373, H2596, and H2461), and additional non-small cell lung cancer cells (2009, Rb negative control line)

(American Type Culture Collection) were maintained at 37˚C in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and 1× concentration of an antibiotic/antimycotic reagent (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2.

Effect of ASO on cell proliferation. Live cells as determined by Trypan blue dye exclusion assay (3000 cells/well) were plated in 96-well plates (3000 cells/100 μl medium/well). On the second day, the medium was aspirated and the cells were transfected with oligofectamine 2000 with or without ASO or SO at different concentrations for 4 h in serum-free medium followed by the addition of fetal calf serum (50 μl of RMPI medium containing 30% fetal calf serum added to 100 μl of transfected cell medium). After 72 h of incubation, 10 μl of solution from Cell Counting Kit-8 (Dojindo Laboratories, Kunamoto, Japan) was added to each well and plates were re-incubated for another 2 h and read at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The reduction in optical density represented the reduction in mitochondrial succinate dehydrogenase activity, hence the reduction in surviving cell numbers.

In situ apoptosis detection. The type of cell death (necrosis/apoptosis) was evaluated *via* TUNEL assay using the commercial ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit (Chemicon International, Temecula, CA, USA). The method utilizes conjugation of digoxigenin- nucleotides with free 3'-OH termini of fragmented DNA strands, and (*via* terminal deoxynucleotidyl transferase) the conjugant reacts with anti-digoxigenin antibodies. The bound peroxidase reacts with its substrate 3,3'-diamino benzidine (DAB) that generates chromogenic DNA.

Cells were grown in 16-well chamber slides (3000 cells/well) as described in the proliferation assay section. The cells were treated with 1 μM of ASO as well as nonspecific oligonucleotides as a control. After 72 h of incubation, media were aspirated and replaced with 200 μl of Histochoice tissue fixative (Amresco, Solon, OH, USA). The slides were transferred to 4˚C for at least overnight until the next step. After removing the media, each well was treated with reagents from the kit as described in the protocol by the manufacturer, with a modification that all the reagents were scaled down to 1/5th. After removing the gaskets around the wells, the processed cell lines were dehydrated in ascending grades of ethanol, cleared in xylene, and finally mounted using DPX mountant (Sigma, St. Louis, MO, USA), observed under light microscopy, and photographed (×400).

Effect of ASO on cellular proteins. Three cell lines (H661, H2371, H6421) showing proliferation inhibition (≥45%) by ASO at 1 μM concentration were selected to study the molecular mechanisms by which ASO acts on CD1 and other cell cycle proteins. Live cells as tested above were plated on 100 mm plates (0.5×10^6 cells/plate). On day 2, the medium was aspirated, cells were washed with serum-free medium and transfected with oligofectamine 2000, with or without ASO or sense oligomers at 0.1 μM, for 4 h in serum-free media followed by the addition of fetal calf serum (8 ml of RMPI medium containing 30% fetal calf serum added to 16 ml of transfected cell medium). After 72 h of incubation, cells were harvested and processed for Western blot analysis.

Immunoblotting. Cells were washed with cold phosphate-buffered saline and lysed in lysis buffer (50 mM Tris, pH 7.40, 1% Triton X-100 containing 1× protease inhibitor mixture (Sigma, St. Louis, MO, USA) and 1× phosphatase inhibitor mixture A & B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 10 min. Cellular debris was pelleted by centrifugation at 14000 rpm at 4˚C for 10 min, and the supernatant was transferred to a fresh prechilled tube. Protein content was determined by Bio-Rad protein DC assay. Twenty-five micrograms of protein were mixed in equal proportions (v/v) with 2× Laemmli sample buffer and heated at 95˚C for 5min. Samples were subjected to electrophoresis on 10% or 4-20% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. Membranes were rinsed in Tris-buffered saline, 0.05% Tween 20 (TBST) and incubated in blocking buffer (5% nonfat milk/bovine serum albumin made in TBST) for 2 h at room temperature followed by incubation overnight (4˚C) with primary antibodies (10 μg/10 ml) made in TBST containing 5% BSA. Following three 5-min washes in TBST, blots were incubated with appropriate secondary antibodies (1:5000 dilution in blocking buffer) for 1 h and then subjected to 3 washes (10 min each) in TBST. Signals were developed with a chemiluminescence reagent (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL, USA) and exposure to x-ray films. X-ray film was processed on an Agfa-CP1000.

Immunoprecipitation. Cells were grown as above to 100% confluency. After 24 h media were discarded and cells were washed with cold PBS. Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, containing 1× protease inhibitor mixture and 1X phosphatase inhibitor mixture; Santa Cruz Biotechnology) and left on ice for 10 min. Protein content was determined by Bio-Rad protein DC assay. Cell lysates were diluted to 1.0 mg/ml. Five micrograms of anti- CD1, CD2, CD3 and CDK2 antibodies were added to each of 1 ml of lysate from different cell lines. The tubes were kept on a rotatory shaker for 1 h in the cold room. Fifty microliters of protein A/G sepharose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added, and incubation with shaking continued overnight. The tubes were spun at 10000 × g for 5 min and the supernatant was discarded. The agarose gel beads were washed 3 times in Tris HCl buffer (10 mM, pH 7.4) containing 0.05% Triton X-100, then incubated with shaking (at 4˚C for 10 min) and centrifuged at 10000 × g. Sepharose beads were resuspended in 2× Laemmli sample buffer, heated at 95˚C for 5 min and processed for immunoblotting using phosphoantibodies as described above.

Results

The effect of ASO on cellular growth is shown in Figure 1. All the cell lines, except for H2030 and H2596, exhibited 48-95% inhibition of cellular growth upon treatment with 10 μM of ASO, while no change in growth pattern was observed after treatment with the SO (data not shown). At a lower concentration (1.0 μM), three of the cell lines showed 43-94% inhibition. The fastest growing cell line (H661) exhibited maximum inhibition (94%), while slowest growing cell line (H2373), had the minimum inhibition (43%). Cell line H2461 exhibited 52% inhibition.

ASO-treated cell lines exhibited condensed fragmented nuclei covered with membrane, while the control cells grew normally with dividing nuclei apparent at various stages

(Figure 2). On the basis of cellular growth inhibition we selected three cell lines (H661, H2373, H2461) to study possible mechanisms. The effect of ASO on cell cycle-specific cellular proteins is shown in Figure 3. Western blot analysis of cellular proteins showed remarkable effects on the cell cycle proteins tested.

Western blot analysis showed that all the proteins reacting with their respective antibodies showed up as discrete bands approximately in range as outlined by the vendor of the antibodies i.e. CDK2 (33 kDa), CDK4 (34 kDa), CD1 (36 kDa), CD2 (34 kDa), CD3 (34 kDa), β-actin (42 kDa), pRb (110 kDa), and phosphatase (46 kDa). Western blot analysis of lysate of cells treated with ASO show that ASO inhibited the *de novo* synthesis of CD1 more than 75% in H661, 50%, in H2461, and to a lesser extent in H2373 cells. A similar pattern was observed for CD3, while there was no apparent change in cyclin E. Cyclin D2 was decreased in H661 cells. CDK2 expression in cell line H661 remained unaffected but there was about a 50% decrease in H2461 cells as well as in H2373 cells. CDK4 expression at 34 kDa, though it appeared to decrease in ASO-treated H661 cells, was difficult to evaluate in other cell lines. It also appeared to exist as a dimer (68 kDa) as well as degraded. The major degraded products moved along with the dye front (10% gel). Along with the right-sized bands, we observed slow moving trailing bands of CDK2, CD1, and CD3 that tended to disappear upon ASO treatment selectively in different cell lines. In the CD1 immunoblot there is complete disappearance of this slow moving band from almost all the cell lines; even the normal band is hardly visible. The same blot treated with anti-CDK2 shows complete disappearance of the slow moving band in H2461 and H2373 cells; however, in H661 cells it remained unaffected. The β-actin control on the same blot did not show any slow moving part. The CD3 immunoblot appeared like that of CD1 except that ASO-treated 661 cells had a lesser amount of both slow moving bands as well as the band front. ASO-treated 2373 cells had faster band fronts for CD1 and CD2 as compared to all other bands in all of the blots. Stripping the blot and reprobing with phosphatase antibodies (PP1) increased the intensity of bands in ASO-treated cell lysate as compared to control.

There was no change in the concentration pRb in cell line H661 and H2373 while in H2461 cells a high molecular weight, trailing, phosphorylated band was observed in control, which disappeared upon ASO treatment. The Western blot results of lysate from normally growing cells immunoprecipitated with antibodies to CD1, CD2, CD3, CDK2 and immunoblotted with anti-phospho antibodies are shown in Figure 4. All the immunoprecipitated proteins showed the existence of serine and threonine phosphorylation of CD1, CD2, CD3, and CDK2 and tyrosine phosphorylation of CD3 and CDK2. The results in the case of CD1 and CD2 tyrosine phosphorylation appear to be inconclusive because of the presence of very close double bands.

Discussion

Various inhibitors of CDKs and their complexes have been reported to inhibit cellular growth and have been implicated as cancer therapeutics or chemopreventive agents, (17-35) and several are in clinical trials (36). ASO to CD1 alone or in conjunction with other therapeutics/agents inhibits growth of various cells lines (10-16). We observed that CD1 ASO have varied effects on cellular proliferation inhibition of non-small lung cancer cells as well as pleural mesothelioma cells. The apparent reduction in cell number following treatment with ASO has been attributed, at least partially, to programmed cell death as determined by *in situ* peroxidase apoptosis detection. Targeting CD1 not only depleted CD1 but also affected the expression of CD2, CD3, CDK2. Slow moving bands that appeared in blots of CD1, CD3, and CDK2 in normal growing cells almost disappeared or decreased in intensity in ASO-treated cells. Ruling out the low likelihood of any other posttranslational modifications because of the intranuclear site of these proteins and absence of other consensus sequences such as NXG/S, the stripped blot was reprobed with antiphosphatase antibodies. The result showed elevation of phosphatase level in the ASO-treated cells, thereby implicating the role of phosphatases in dephosphorylation of these proteins leading to inhibiting the cell division, and hence, the cell number. CD1 splice variant CD1b does not have the T286 phosphorylation site required for nuclear transportation (37). Phosphorylation of CD1 [T286] has been implicated in its proteosomal degradation (38), while degradation of CD3 has been reported to be independent of phosphorylation at T283 (39). However in our experiment it is obvious that overexpression of protein phosphatases during the CD1 down-regulation may lead to dephosphorylation of phosphorylated CD1 and CD3 and CD2. This effect may be responsible for blocking the cell cycle from entering into the S phase from the G1 phase. Since in our experiment CD1 synthesis was blocked at the transcriptional level, we cannot label it as proteosomal degradation that has been targeted, as reported earlier (33-34). Blocking CD1 synthesis or enhancing its proteosomal degradation both prevent cell entry into S phase, where dephosphorylation may play an important role. Cyclin D1 has been reported to initiate the phosphorylation of Rb that disrupts the Rb-mediated transcriptional repression leading the cell to enter into the S stage (37). Phosphorylation of cyclins, as those of CDKs, may be an essential requirement for this phosphorylation in a co-operative chain reaction.

We utilized the NetphosK server (http://www.cbs.dtu.dk/services/NetPhos/) to identify various predicted phosphorylation sites that have been noted in cyclins and CDKs (40), and the data has been tabulated as below (Table 1). Cell lysate immunoprecipitated with anti-CD1, -CD2, -CD3, and -CDK2 and immunoblotted with phosphoantibodies showed serine and

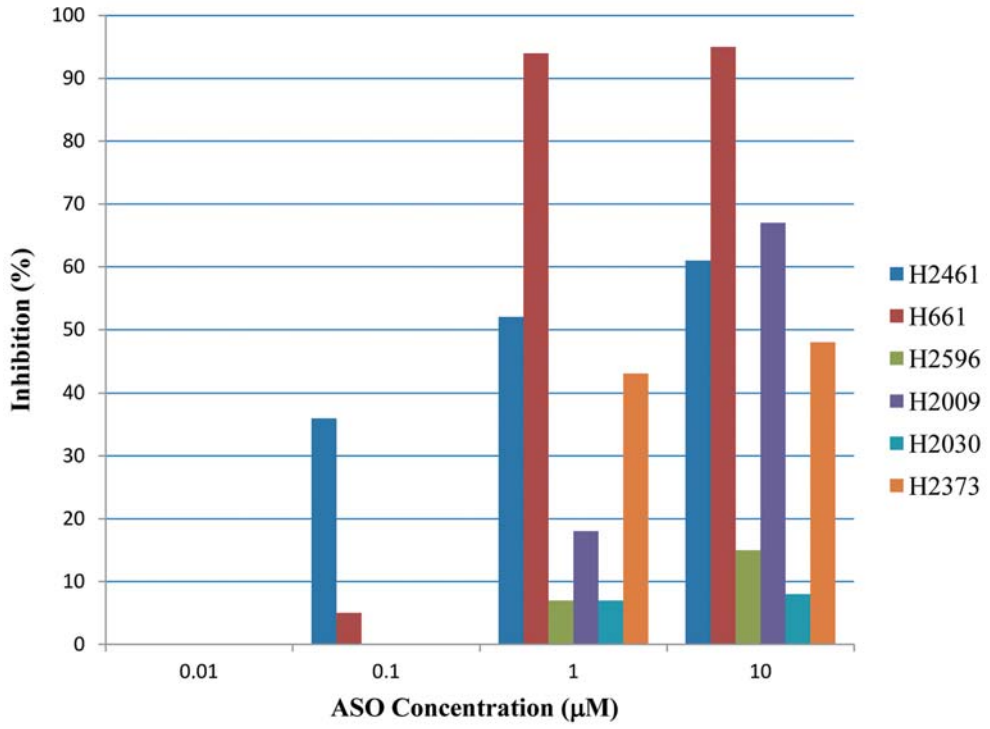

Figure 1. *Effect of cyclin D1 antisense oligonucleotide (ASO) on cancer cells.*

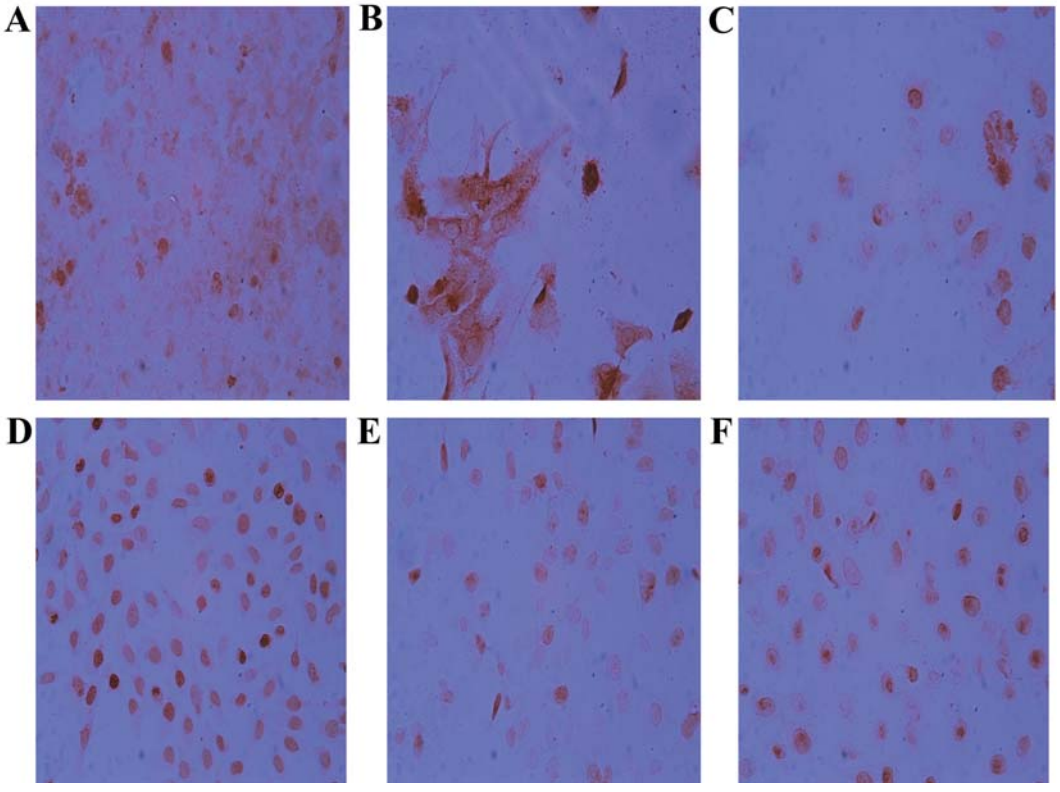

Figure 2. *Microphotograph of mesothelioma and non-small lung cancer cells (661, 2373, 2461) treated with cyclin D1 ASO. Cells were grown in 16 well chamber slides (3000 cells/well) and treated with 1 μM of ASO (A, B, C) as well as non-specific oligonucleotides as a control (D, E, F), respectively, for 72 hrs [Light microscopy].*

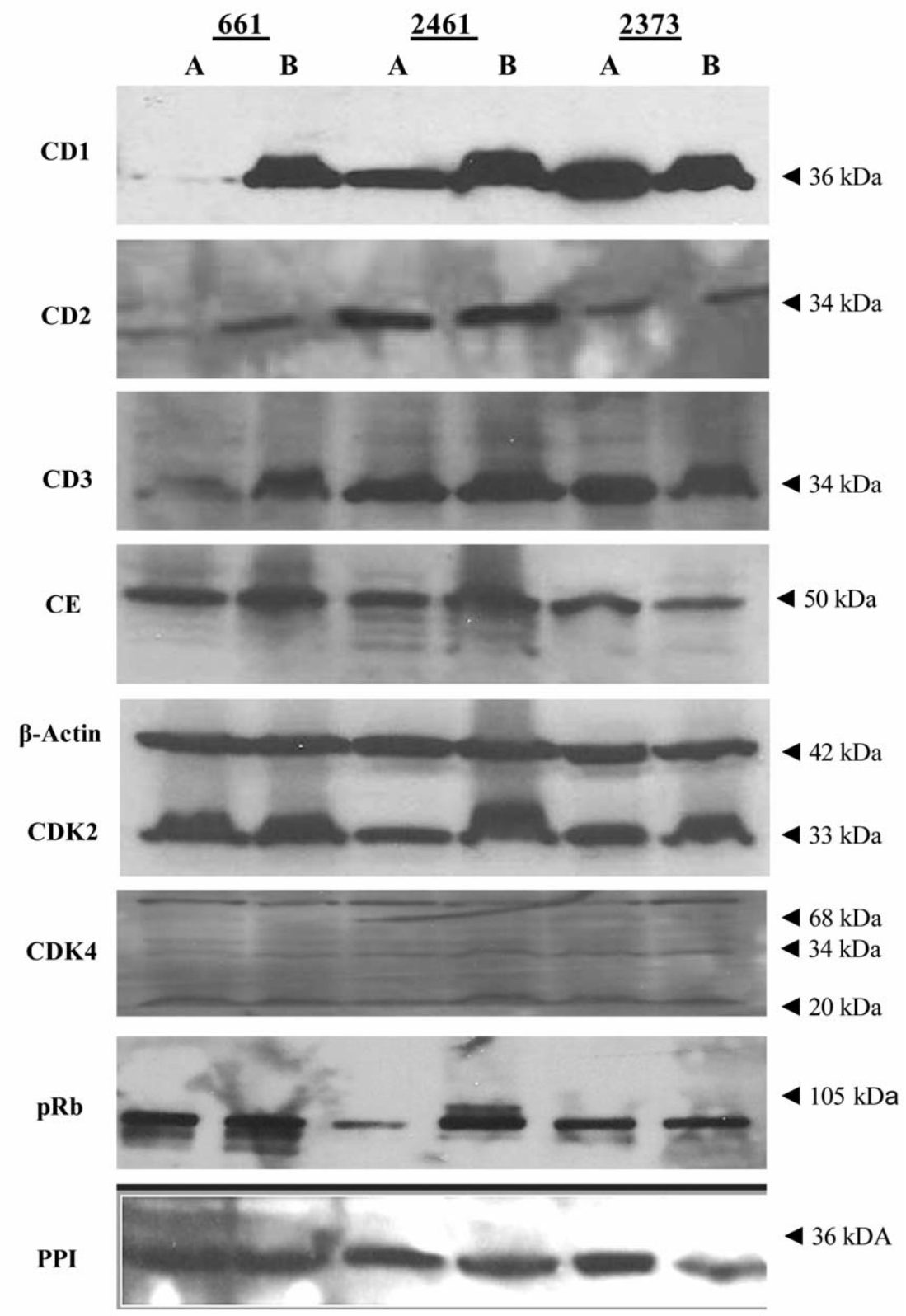

Figure 3. *Western blot analysis of lysate of cells (661, 2461, 2373) treated with cyclin D1 antisense oligodeoxynucleotides (A) and control (B). Cellular protein (25 μg) resolved by SDS-PAGE (10% gel), blotted onto PVDF membrane and immunoblotted with various antibodies. CD1, cyclin D1, CD2, cyclin D2, CD3, cyclin D3, CE, cyclin E, CDK4, cyclin-dependent kinase 4, pRb, retinoblastoma protein, PP1, phosphatase antibodies, kDA, kilodalton.*

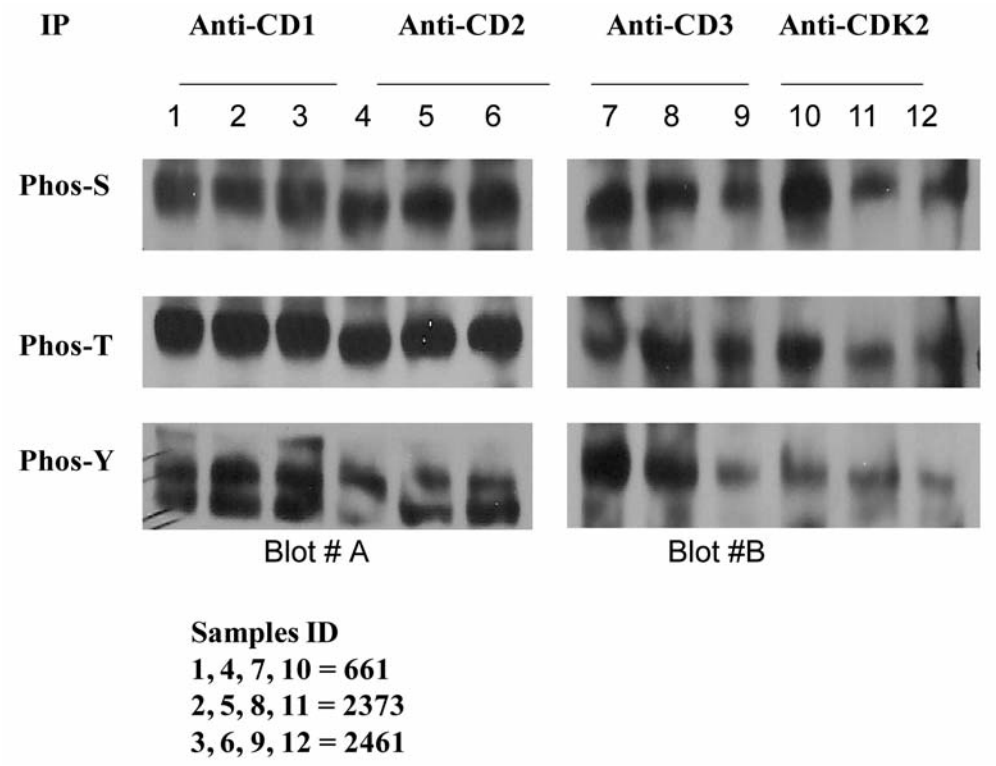

Figure 4. *Detection of phosphorylated cyclin proteins (CD1, CD2, CD3 and CDK2) of normally growing mesothelioma and non-small lung cancer cells (H661, H2373, H2461) cells by immunoprecipitating the cell lysate by anti-CD1, -CD2, -CD3 and -CDK2 and immunoblotting with antibodies to phosphoserine (Phos-S), phosphothreonine (Phos-T), and phosphotyrosine (Phos-Y). Immunoblots obtained by immunoprecipitating the cell lysates by anti-CD1 and -CD2 (BlotA) and anti-CD3 and anti-CDK2 (BlotB) respectively, were used and reused for immunoblotting with all the three anti-phosphoantibodies after stripping and reprobing.*

threonine as well as tyrosine phosphorylation on CD1, CD2, CD3, instead CDK2. Although there is no predicted phosphorylation site for tyrosine in CD1 as per its sequence analysis (netPhos), we did observe a band at the appropriate site. This band may represent a CD1 co-immunoprecipitated protein other than CD1 that may have tyrosine phosphorylated sites. While CD2 has an equal number of predicted phosphorylation sites to those of CD1, there appeared to be a single band of CD2 in all the cell lines treated (H661, H2373, H2461). It could be possible that a) there is only weak phosphorylation and the bands remained non-highlighted in the blot; b) CD2 may be phosphorylated as soon as it is synthesized; or c) glycosylation may have prevented phosphorylation at these sites, a mechanism named yin-yang regulation. However, its role as phosphorylated or unphosphorylated remains to be elucidated. Although CDK4, as well as cyclin E, has a fairly large number of predicted phosphorylation sites, they were not included in the immunoprecipitation/Western blot study, since there was no apparent change in cyclin E in any of the cell lines treated with ASO. Anti-CDK4 showed a number of bands in the blots where the data from immunoprecipitation would have been inconclusive.

Table I. *Predicted phosphorylation sites on the cyclin and cyclin-dependent kinase (CDK). The predicted phosphorylation sites of cyclins and CDK were determine by loading their deduced amino acid sequence onto the Netphos server: (http://www.cbs.dtu.dk/services/NetPhos/).*

Protein	Number of predicted phosphorylation sites			
	Serine	Threonine	Tyrosine	Total
Cyclin D1	5	4	0	9
Cyclin D2	5	3	1	9
Cyclin D3	14	5	2	21
Cyclin E	12	4	6	23
CDK2	3	5	2	10
CDK4	8	8	1	17

There are several families of kinases involved in phosphorylation of these various sites (http://www.cbs.dtu.dk/services/NetPhos/K). Among these, protein kinase C (PKC) has the highest score for phosphorylation prediction for CD1 (0.83, S55), CD3 (0.87, S263), cyclin E (0.87, S405), CDK2 (0.87; S206), and CDK4 (0.89, S36); while protein kinase A

(PKA) involvement (0.71) at S276 is predicted in CDK2. Although the phosphorylation of threonine and its role in CD1 (T286) and CD3 (T283) is documented (37-39), the role of phosphorylation at the serine that accounts for the highest scores for predicted phosphorylation *via* PKC lacks evidence. While there are plenty of predicted sites for threonine, there are no reports on the predicted serine- or tyrosine-phosphorylation sites. Phosphorylation inhibition or dephosphorylation of these cyclin proteins would appear to have potential as anticancer therapy.

Acknowledgements

M.A.K. received an IASLC Young Investigator Award, 2007-2009.

Conflict of Interest

The Authors have no conflict of interest.

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Received July 14, 2011
Revised September 18, 2011
Accepted September 19, 2011