Volume : 2 | Issue : 8 | August 2013 • ISSN No 2277 - 8179

Blocking of CDK4 Gene Expression by Antisense Oligos Induce Apoptosis and Decreases the Proliferation of A549 Cells

Biotechnology

KEYWORDS : CDK4-antisense, A549, apoptosis, RT-PCR

Babu R L	Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India
Vijayalakshmi E	Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India
Naveen Kumar M	Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India
Rajeshwari H. Patil	Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India
S Chidananda Sharma	Department of Microbiology and Biotechnology, Bangalore University, Bangalore-560 056, Karnataka, India

ABSTRACT

Cell cycle is a highly regulated process and can be broadly divided into G1, S, G2 and M phases. Cyclins Ds interact and activate cyclin dependent kinases CDK4/6. CDKs are also controlled by family of CDK inhibitory proteins. CDK4 is expressed in a variety of normal cells, but over expressed in human tumours. Blocking of CDK4 expression could be an attractive strategy for the treatment of cancers. Hence, in the present study CDK4-antisense oligos were used for the blocking of CDK4 expression in A549 cells. A549 cells transfected with CDK4-antisense oligos inhibit the proliferation of cells in a dose dependent manner and show increased expression of a pro-apoptotic Bax gene by 35%, and caspase-8 by 3 folds. Further, reduction in the expression of cyclin D1 and anti-apoptotic Bcl-2, suggested that the blocking of CDK4 expression in A549 cells inhibit the cell cycle with induced apoptosis.

INTRODUCTION

Cell cycle plays an important role in the growth and development of all the organisms. In cancer many genes are mutated and exhibit altered levels of expression/ proteins (Lapenna & Giordano, 2009). Such changes contribute to deregulation of cell cycle kinases, which is often associated with aberrant cell division and uncontrolled proliferation. Cell division is a highly regulated process and can be broadly divided into G1, S, G2 and M phases. Cyclins Ds interact with CDK4/6 and activate kinases. Cyclin E1 activates CDK2 and CDK3 and is expressed in late G1 and early S phase. Cyclin A also activate CDK2 and appear during G1 and S phase. Activities of cyclin dependent kinases (CDKs) are controlled by cyclins and family of CDK inhibitory proteins (INK4), kinase inhibitory proteins (KIP) and thereby keep the check on the cell cycle (Hanahan & Weinberg, 2000; Hartwell & Weinert, 1989). Deregulation of cell cycle control is one of the initial events in the development of most of the cancers (Hanahan & Weinberg, 2000). CDK4 is expressed in a variety of normal cells and is often over expressed in human tumours (e.g. malignant melanoma, glioma, sarcoma and carcinomas of the breast, colon, lung, ovary and oral cavity). CDK4 constitutes the catalytic subunit of a heterodimeric Ser/Thr protein kinase, important in regulating retinoblastoma protein function. The retinoblastoma tumor suppressor a nuclear phosphoprotein (pRB) regulates progression through the G1-to-S phase transition during cell cycle (Weinberg, 1995). Loss of RB is well documented and p16-cyclin D1-CDK4/6- RB pathway is disrupted in many human tumors (Sherr, 1996). Extracellular signals induce the expression of cyclin D1 in cells entering the cell cycle. Cyclin D1 is one of the target genes of E_2, that exerts proliferative effect (Roy & Thompson, 2006), in estrogen responsive cells. Cyclin D1-CDK4/6 an activated complex phosphorylates RB. pRB dissociates from the inactive transcription factor E2F. Activated E2F in turn activates many genes that lead the cell cycle from G1 to S phase (Massague, 2004; Sherr, 1996; Weinberg, 1995). The p16 acts as a tumor suppressor molecule (Lukas et al., 1995), inhibits CDK4/6 by maintaining RB in its hypo-phosphorylated E2F in associated state and prevents G1-to-S phase progression (Li, Poi, & Tsai, 2011; Witkiewicz, Knudsen, Dicker, & Knudsen, 2011). Inactivation of p16 would result in the continued activation of CDK4/6 and RB phosphorylation, that facilitates a loss of control over cell cycle arrest (Sherr, 1996) with oncogenic transformation and tumor development (Paternot et al., 2010; Rocco & Sidransky, 2001).

Although, CDK4/6 is not essential for the cell cycle (Santamaria et al., 2007; Thoms, Dunlop, & Stark, 2007), CDK4 has been shown to be critical for proliferation and malignancy. However, overexpression of CDK4 was reported in oral squamous cell carcinoma (Poomsawat, Buajeeb, Khovidhunkit, & Punyasingh, 2010), pancreatic endocrine tumors (Lindberg, Hessman, Akerstrom, & Westin, 2007), nasopharyngeal carcinoma(Fang et al., 2008) and lung cancer (Dobashi, Goto, Fukayama, Abe, & Ooi, 2004; Wikman et al., 2005), suggested that CDK4 is a key factor in promoting the initiation, progression and development of tumours in humans. Many cancer drugs (Staurosporines, Flavones, Paullones, Indirubin, and Hymenialdisine etc.,) have been characterized and extensively used for the cancer treatment but, they lack specificity and most of the time patients develop multidrug resistance. Hence, the selective blocking of CDK4 expression by specific CDK4 antisense technology could be an attractive strategy for targeting different types of tumours. The objective of this study was to examine the effect of CDK4 antisense oligonucleotides on the proliferation, expression of genes involved in cell cycle regulation and apoptosis in lung epithelial A549 cells.

Materials and Methods:

Materials

Human lung epithelial cells (A549) were purchased from NCCS (Pune, India), primer (forward and reverse) sequences of cell cycle regulators and Apoptotic genes (Babu et al., 2013) were purchased from *Sigma*-Aldrich (St Louis, USA). CDK4-sense oigos (20mer) sequence (5'ATGGCTACCTCTCGATATGA-3') and antisense oligo sequence (5'-TCATATCGAGAGGTAGCCAT-3') were designed and purchased from Eurofins MWG/Operon India Pvt. Ltd. (Bangalore, India). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, RPMI-1640, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Trypan Blue chemicals were purchased from Himedia (Mumbai, India). Oligo dT's and Super script reverse transcriptase were obtained from Invitrogen BioServices India Pvt. Ltd (Bangalore, India), Taq DNA Polymerase (1U/µl) was purchased from Merck (Mumbai, India). Turbofect transfecting reagent was purchased from Fermentas (MD, USA).

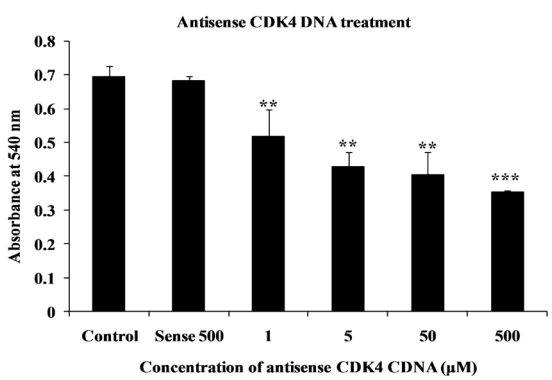
Cell culture and treatments:
A549 cells were cultured in 6 wells plate (10^5 cells/well) or 96 wells plate (2 x 10^3cells/well) using RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and with or without 10% fetal bovine serum at 37° C with the supply of 5% CO_2 for 24 h. The cells grown in 96 wells plate were used for MTT assay and cells in 6 wells plate were used for reverse transcription-polymerase chain reaction (RT-PCR).

Cell viability / MTT assay:
The effect of antisense CDK4 DNA (20-mer) using Turbofect (fermentas) transfecting agent on proliferation of A549 cells was analyzed by MTT assay as per the protocol described earlier (Sharma et al., 2007).

RNA isolation and semi quantitative RT-PCR:
The effect of CDK4-antisense or sense oligos on the expression of cell cycle regulators and apoptosis activator genes were analyzed by semi-quantitative RT-PCR using specific primers as per the protocol described earlier(Babu et al., 2013).

Results:
Effect of CDK4-antisense and sense oligos on the proliferation of A549 cells:
To investigate the effect of CDK4-antisense oligos on the proliferation and the viability, the A549 cells were treated with different concentrations oligos and the viability of the cells were assayed by MTT. The results show that the proliferation of A549 cells were significantly decreased with the addition of CDK4 antisense oligos in a dose dependent manner and maximum of 50% decrease in cell proliferation was seen at 500µM concentration ($p < 0.001$) of antisense DNA, while there was no significant difference CDK4-sense oligos treated cells compared to control (Fig 1), suggested that antisense CDK4 sequence used has the anti-proliferative effect.

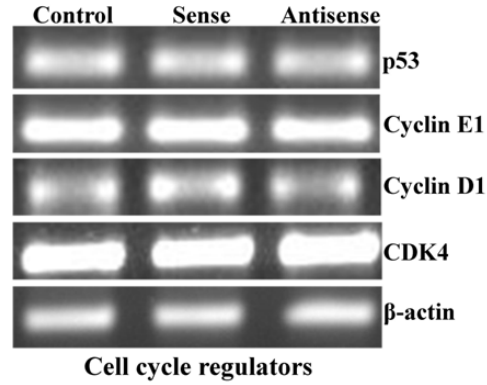
Fig 1. Effect of CDK4-antisense oligos on the proliferation of A549 cells:



*Lung epithelial A549 cells were treated with or without CDK4-antisense oligo nucleotides (1µM-500µM) in a 96 wells plate for 48 h and the cell growth were analyzed by MTT assay. Results were expressed as relative proliferative index compared to control (mean ± SD, n=8). Values are significantly different from control, **P <0.01, *** P <0.001 by student t-test. The results were shown as representative of three independent experiments.*

Effect of CDK4-antisense and sense oligos on cell cycle regulators and apoptosis:
To study the effect of CDK4-antisense and sense oligos on the expression of genes of cell cycle regulators and cell apoptotic activators, the cells were treated with antisense and sense oligos, reverse transcribed and amplified by using specific primers. The CDK4-atisense treatment show decreased transcriptional activity of cell cycle regulator, Cyclin D1 by more than 20 % and marginal decrease of P53 gene (Fig 2). However, CDK4-antisense and sense found to have no effect on the expression of Cyclin E1 and CDK4 gene (Fig 2).

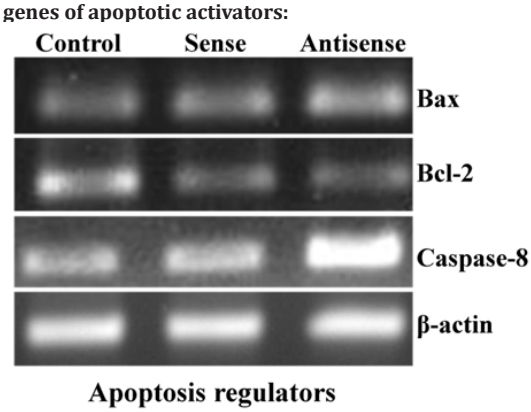
Fig 2. Effect of CDK4-antisense oligos on the expression of genes of cell cycle regulators:



A549 cells were treated with or without CDK4- antisense (500µM) or CDK4-sense (500µM) for 24 h in a 6 wells plate. The cDNA was prepared from total RNA and subjected to 30 cycles of PCR using specific primers of cell cycle regulators and β-actin was used as positive control and for normalization. The mRNA levels were analysed on 1% agarose gel and the band intensity was compared to control. The results were shown as representative of three independent experiments.

The CDK4-antisense treatment significantly inhibits the expression of anti-apoptotic gene Bcl-2 by more than 30 % while, induced the pro-apoptotic genes Bax by 35 % and Caspase-8 by 3 folds. However, CDK4-sense oligos treatment shows little or no effect on the expression of the cell cycle regulators or apoptotic genes studied (Fig 3).

Fig 3. Effect of CDK4-antisense oligos on the expression of genes of apoptotic activators:



A549 cells were treated with or without CDK4- antisense (500µM) or CDK4-sense (500µM) for 24 h in a 6 wells plate. The cDNA was prepared from total RNA and subjected to 30 cycles of PCR using specific primers of apoptosis regulators and β-actin was used as positive control and for normalization. The mRNA levels were analysed on 1% agarose gel and the band intensity was compared to control. The results were shown as representative of three independent experiments.

Discussion:
Cancer is the multi process controlled phenomena and lung cancer is the most prevalent malignancies found in the world. Many protein kinases orchestrate the complex events of cell cycle, and their activity is often deregulated in cancer cells.

Although CDK4 and CDK6 are dispensable for driving the cell cycle (Santamaria et al., 2007; Thoms et al., 2007), some reports show the absolute requirement for oncogenic transformation of many tissues including lung. In most of the cases the cancer cells are addicted to higher levels of CDK4 activity (Paternot et al., 2010). Thus CDK4 is an important target gene for the therapeutic development and prognosis of the cancer. In the

present study we attempted to block the expression of CDK4 by antisense oligos to mitigate the proliferation of A549 cells. Treatment of cells with CDK4-antisense oligos found to inhibit the proliferation of cells in a dose dependent manner and also induced the transcripts of pro-apoptotic genes Bax and caspase-8. Further, the blocking of CDK4 gene expression show the decreased expression of cyclin D1 a cell cycle regulator.

However, in our studies the treatment of cells with antisense oligos, the expression of CDK4 mRNA was not decreased. In contrary, a study from Wu et al., 2011, using lentiviral vector containing shRNA which target and stably knock down the expression of CDK4 in A549 cells the CDK4 expression was significantly reduced with decreased proliferation. In our studies the CDK4 expression was not reduced, probably because the cells were treated only for 24 h with antisense oligos. In this short time the antisense oligos might be subjected to degradation by DNAses. To obtain complete knock down of CDK4 expression in A549 cells, the antisense Phosphothioate oligonucleotides, a second generation antisense oligos characterised to be safer with increased half life need to be designed (Zhou, He, Chen, Wang, & Wang, 2003). Some more experiments need to be designed and carried out to fine tune about the requirement of different transfecting agents, targeting and time required for maximum effect on the decreased expression of the CDK4 mRNA transcripts. However, our findings undoubtedly establish that CDK4-antisense oligos blocked the expression of CDK4 as the proliferation of A549 cells was greatly reduced with increased expression of proapoptotic Bax and caspase-8 genes.

Both the activation of CDK4/6 and CDK2 by cyclin D1 and cyclin E1 respectively, are required for cell cycle progression through G1/S transition. It is a well known fact that the classical mitogen-activated protein kinase (MAPK) pathway is a key component in the transduction of signals that lead to growth and transformation of many cells into cancer. Activator protein-1 (AP-1) is a family of transcription factors, found to be regulated by MAPK signaling cascades. AP1 factors control rapid responses to stimuli and controls the regulation of various intracellular events.

Studies have shown that AP-1 transcription factor modulates cyclin D1 transcription (Phuchareon, J. & Tokuhisa, T, 1995) and promote cell cycle through MAPK (Cargnello, M. & Roux, P. P, 2012). MAPK in turn activate other set of AP-1(Jun-Jun or Jun-Fos) that induce genes involved in proliferation/transformation activity. Studies using immune cells demonstrated that, CDK4 phosphorylates c-Jun, that helps in the formation of AP-1 transcription complex with broad spectrum of cellular functions (Vanden Bush & Bishop, 2011). Thus, CDK4 plays an important role in the regulation of AP1 activities mediating cell proliferation and tumor development. Studies carried out on the expression of AP-1 transcription factors in A549 cells show that both Jun (c-Jun, Jun-D, Jun-B) and Fos (c-Fos, Fos-B, Fra-1and Fra-2) mRNAs were prominently expressed, while Fos-B was not expressed (unpublished results). Poor expression of Fos-B mRNA in A549 cells was in agreement with MCF-7 cells (Babu et al., 2013), where Fos-B expression was down regulated compared to normal mammary cells in humans(Milde-Langosch, Kappes, Riethdorf, Loning, & Bamberger, 2003). Probably, in healthy tissues the Fos-B is expressed in larger amounts, while in malignant tissues the expression is down regulated and that needs to be investigated. Thus the present study could be used to block the CDK4 expression by antisense oligos that may further help in the identification of AP1 factors used as the downstream substrate by CDK4 enzyme. The study may also help to understand the role of ERK1/2 in JNK/AP1/Cyc D1-CDK4 signal pathway.

Conclusion:

The blocking of CDK4 expression by 2nd or 3rd generation of antisense oligo sequences could be further used to target and treatment of different types of cancer, a novel unique and potentially useful therapeutic modality.

Acknowledgements:

The authors wish to express their gratitude to University Grant Commission (UGC), New Delhi for providing fellowship to BRL, EV and Major Research Project Grant F. No. 34-250/2008 (SR) to SCS.

REFERENCES

Babu, R. L., Naveen Kumar, M., Patil, R. H., Devaraju, K. S., Ramesh, G. T., & Sharma, S. C. (2013). Effect of estrogen and tamoxifen on the expression pattern of AP-1 factors in MCF-7 cells: role of c-Jun, c-Fos, and Fra-1 in cell cycle regulation. Mol Cell Biochem, 380(1-2), 143-151. | Cargnello, M., & Roux, P. P. (2012) Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. Microbiol Mol Biol Rev, 76(2), 496. | Dobashi, Y., Goto, A., Fukayama, M., Abe, A., & Ooi, A. (2004). Overexpression of cdk4/cyclin D1, a possible mediator of apoptosis and an indicator of prognosis in human primary lung carcinoma. Int J Cancer, 110(4), 532-541. | Fang, W., Li, X., Jiang, Q., Liu, Z., Yang, H., Wang, S., . . . Yao, K. (2008). Transcriptional patterns, biomarkers and pathways characterizing nasopharyngeal carcinoma of Southern China. J Transl Med, 6, 32. | Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. Cell, 100(1), 57-70. | Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science, 246(4930), 629-634. | Lapenna, S., & Giordano, A. (2009). Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov, 8(7), 547-566. | Li, J., Poi, M. J., & Tsai, M. D. (2011). Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. Biochemistry, 50(25), 5566-5582. | Lindberg, D., Hessman, O., Akerstrom, G., & Westin, G. (2007). Cyclin-dependent kinase 4 (CDK4) expression in pancreatic endocrine tumors. Neuroendocrinology, 86(2), 112-118. | Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., . . . Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature, 375(6531), 503-506. | Massague, J. (2004). G1 cell-cycle control and cancer. Nature, 432(7015), 298-306. | Milde-Langosch, K., Kappes, H., Riethdorf, S., Loning, T., & Bamberger, A. M. (2003). FosB is highly expressed in normal mammary epithelia, but down-regulated in poorly differentiated breast carcinomas. Breast Cancer Res Treat, 77(3), 265-275. | Paternot, S., Bockstaele, L., Bisteau, X., Kooken, H., Coulonval, K., & Roger, P. P. (2010). Rb inactivation in cell cycle and cancer: the puzzle of highly regulated activating phosphorylation of CDK4 versus constitutively active CDK-activating kinase. Cell Cycle, 9(4), 689-699. | Phuchareon, J., & Tokuhisa, T. (1995) Deregulated c-Fos/AP-1 modulates expression of the cyclin and the cdk gene in splenic B cells stimulated with lipopolysaccharide. Cancer Lett. 92(2):203-208. | Poomsawat, S., Buajeeb, W., Khovidhunkit, S. O., & Punyasingh, J. (2010). Alteration in the expression of cdk4 and cdk6 proteins in oral cancer and premalignant lesions. J Oral Pathol Med, 39(10), 793-799. | Rocco, J. W., & Sidransky, D. (2001). p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res, 264(1), 42-55. doi: 10.1006/excr.2000.5149 | Roy, P. G., & Thompson, A. M. (2006). Cyclin D1 and breast cancer. Breast, 15(6), 718-727. | Santamaria, D., Barriere, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., . . . Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. Nature, 448(7155), 811-815. | Sharma, C. S., Sarkar, S., Periyakaruppan, A., Barr, J., Wise, K., Thomas, R., . . . Ramesh, G. T. (2007). Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. J Nanosci Nanotechnol, 7(7), 2466-2472. | Sherr, C. J. (1996). Cancer cell cycles. Science, 274(5293), 1672-1677. | Thoms, H. C., Dunlop, M. G., & Stark, L. A. (2007). CDK4 inhibitors and apoptosis: a novel mechanism requiring nucleolar targeting of RelA. Cell Cycle, 6(11), 1293-1297. | Vanden Bush, T. J., & Bishop, G. A. (2011). CDK-mediated regulation of cell functions via c-Jun phosphorylation and AP-1 activation. PLoS One, 6(4), e19468. Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell, 81(3), 323-330. | Wikman, H., Nymark, P., Vayrynen, A., Jarmalaite, S., Kallioniemi, A., Salmenkivi, K., Anttila, S. (2005). CDK4 is a probable target gene in a novel amplicon at 12q13.3-q14.1 in lung cancer. Genes Chromosomes Cancer, 42(2), 193-199. | Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P., & Knudsen, E. S. (2011). The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments. Cell Cycle, 10(15), 2497-2503. | Zhou, Y. Q., He, C., Chen, Y. Q., Wang, D., & Wang, M. H. (2003). Altered expression of the RON receptor tyrosine kinase in primary human colorectal adenocarcinomas: generation of different splicing RON variants and their oncogenic potential. Oncogene, 22(2), 186-197.