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# Designing siRNA for Silencing Polo-Like Kinase 1 (Plk1) Gene of Prostate Cancer

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**Abstract:-**Prostate cancer is the most commonly occurring cancer in American men, next to skin cancer. Existing treatment options and surgical intervention are unable to manage this cancer effectively. Therefore, continuing efforts are ongoing to establish a novel mechanism based targets and strategies for its management. PLK1 plays a key role in the mitotic entry of proliferating cells and regulates many aspects of mitosis which are necessary for successful cytokinesis. PLK1 is overexpressed in many tumour types with aberrant elevation frequently constituting a prognostic indicator of poor disease outcome and our study indicate that PLK1 could be an excellent target for the treatment as well as chemoprevention of prostate cancer.

**Keywords:** Prostate cancer, PLK1 gene and siRNA

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## Introduction

Prostate cancer (PCa) is the second leading cause of cancer deaths in men (Landis et al., 1998; Jemal et al., 2004; Reagan-Shaw and Ahmad, 2005). Prostate cells may spread (metastasize) from the prostate gland to other parts of the body, especially the bones and lymph nodes. PCa may cause pain, difficulty in urinating, erectile dysfunction, blood in the urine or semen as pointed out by Talcott et al. (1998). Out of every three men who are diagnosed with cancer each year, one is diagnosed with PCa. No one knows the exact causes of PCa. It has become one of the most common cancer in males in several developing countries including India (Quinn and Babb, 2002; Hebert et al., 2006). Currently available therapies such as surgery, radiation, chemotherapy, hormonal and other therapies have a number of side effects (Daskivich and Oh, 2006; Gallagher and Gapstur, 2006). The existing treatment approaches and surgical intervention have not been able to effectively manage this dreaded cancer and, therefore, continuing efforts are ongoing to explore novel targets and strategies for the management of PCa as pointed out by Reagan-Shaw and Ahmad (2005). Thus, there is an urgent need to intensify our efforts to better understand this disease and develop novel approaches and strategies for the management of PCa. A clear understanding of genetic controls of cellular proliferation and cell division may provide the basis for the rational design of specific targets and therapeutic strategies for the management of PCa. In this regard, new molecular targets for

 

diagnosis and therapy of cancer disease are desperately needed.

RNAi is a new technique that is considered as a major technology breakthrough. Since short sequences of nucleic acids are known as oligos, these are often referred to as RNAi oligos. These RNA's are then transfected into cells and the effects of "gene silencing" are analyzed. The key to the success of the technique is designing the best RNAi oligos from what we know of the gene sequence. The gene may be several thousand bases in length and the oligos are only 21-23 long so there are many options.

siRNA technology is useful in DNA-directed RNAi enabling multiple applications of RNAi, effective delivery of siRNA targeted against HPV oncogenes, real-time PCR, advancing RNAi and microRNA studies, new technologies to accelerate small-molecule screening of cell signalling pathways, chemical modifications of synthetic siRNA and effective delivery of functional siRNAs into cells.

PLKs belong to a family of serine/threonine kinases and are the human counterpart of polo in Drosophila melanogaster and of CDC5 in Saccharomyces cerevisiae (Golsteyn et al., 1994; Glover et al., 1998; Donaldson et al., 2001b). In mammals, there are four different Plks that all participate in the cell cycle regulation (Weerdt et al., 2006). Plk1 is the best characterized member in this group. Plk1, a mitotic cyclin-independent serine-threonine kinase, is a member of a family of Plks involved in a wide variety of cell cycle processes. In mammalian cells, Plk1 is primarily localized at the centrosome where it is responsible for centrosome separation and maturation (Lane and Nigg, 1996). Plk1 has been shown to be intimately involved in spindle formation and chromosome segregation during mitosis and, therefore, in the regulation of cell cycle (Liu and Erikson, 2002).

More recent studies demonstrated that polo kinase activity plays a pivotal role in the separation of sister chromatids during mitosis. Cdc5 is required to phosphorylate the cohesin subunit Scc1, to facilitate its cleavage by separase at the onset of anaphase (Alexandru et al., 2001). Plx1 is also the key regulator for the cleavage-independent dissociation of cohesin from chromosome in Xenopus (Sumara et al., 2002). Plk1 expression and activity are strongly linked to proliferating cells. Using a variety of methods, including small-molecule inhibition of Plk1function and/or activity, apoptosis in cancer cell lines, cell cycle arrest in normal cell lines, and antitumor activity in vivo have been observed by Lansing et al. (2007)

In view of this, this study was designed to investigate the hypothesis that polo-like kinase 1 (Plk1) has a critical role in the development of PCa, and the silencing of Plk1 will result in elimination of human PCa cells. Thus, if the hypothesis is tested to be true, it is conceivable that gene therapeutic approaches aimed at Plk1 or the pharmacological inhibitors of Plk1 may be developed for the management of prostate cancer.

## Materials And Methods

### Sequence Retrieval

#### a. *Nucleotide – NCBI (National Center for Biotechnology Information)*

The mRNA sequence of human Plk1 was retrieved from NCBI using Entrez tool in both Genbank and FASTA format.

#### b. *Protein-Swissprot*

The protein sequence of Plk1 was retrieved from UniProt database of SwissProt. The accession number is P53350.

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## Protein Domain and Family Analysis

### a. *InterPro*

InterPro is an integrated documentation resource for protein families, domains and sites. The protein sequence of polo-like kinase 1 (Plk1) of human was analyzed for presence of functional domains using Sequence-Motif and Sequence-Cluster Methods (ProDom). ProDom uses PSI-BLAST to find homologous domains that are clustered in the same ProDom entry.

### b. *Pfam*

Pfam is a database of multiple alignments of protein domains or conserved protein regions. The protein sequence of polo-like kinase 1 (Plk1) of human was analyzed for protein family.

## siRNA Design

### *Ambion - siRNA Design*

There are several methods for preparing siRNA, such as chemical synthesis, in vitro transcription, siRNA expression vectors, and PCR expression cassettes. Irrespective of which method one uses, the first step in designing a siRNA is to choose the siRNA target site. The siRNA target sites were chosen based on the guidelines of Ambion. Using these guidelines, approximately half of all siRNAs yield >50% reduction in target mRNA levels.

## Finding siRNA Targets

The selection of the siRNA target sequence is purely empirically determined, as long as the target sequence starts with GG and does not share significant sequence homology with other genes as analyzed by BLAST search.

## Ambion siRNA Target Finder

Using AMBION siRNA Target Finder, target sequences were retrieved for the genes associated with human polo-like kinase 1 (Plk1).

## Blast Search Against Target Organism Database

Blast was performed for the mRNA sequence of polo-like kinase 1 (Plk1) of human using BLASTN 2.2.17

## Designing Template

## siRNA Template Design Tool

Using AMBION siRNA Template design tool, SENSE and ANTI – SENSE RNA Oligonucleotides Template were created.

## Designing Controls

A complete siRNA experiment should include a number of controls to ensure the validity of the data. Two important controls are:

- **A negative control siRNA with the same nucleotide composition as your siRNA but which lacks significant sequence homology to the genome**. To design a negative control siRNA, scramble the nucleotide sequence of the gene-specific siRNA and conduct a search to make sure it lacks homology to any other gene.
- **Additional siRNA sequences targeting the same mRNA**. Perhaps the best way to ensure confidence in RNAi data is to perform experiments, using a single siRNA at a time, with two or more different siRNAs targeting the same gene. Prior to these experiments, each siRNA should be tested to ensure that it reduces target gene expression by comparable levels.

Once the target sequence of the siRNA has been chosen, the siRNA Template design tool can be used to determine the sequences of the sense and antisense siRNA oligonucleotide templates for use with the Silencer siRNA Construction Kit. The program will

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add "CCTGTCTC" (complementary to the T7 Promoter Primer supplied in the kit) to the 3' end of each oligonucleotide.

### Designing Hairpins

❋ Researchers who initially reported the use of siRNA expression vectors to induce RNAi had different design criteria for their inserts encoding the expressed siRNA. Most of the designs have two inverted repeats separated by a short spacer sequence and ended with a string of T's that served as a transcription termination site. These designs produce an RNA transcript that is predicted to fold into a short hairpin siRNA. The selection of siRNA target sequence, the length of the inverted repeats that encode the stem of a putative hairpin, the order of the inverted repeats, the length and composition of the spacer sequence that encodes the loop of the hairpin, and the presence or absence of 5'-overhangs, vary among different reports.

### Loop for Short Hairpin siRNAs (shRNA)

Researchers have tested a variety of sequences for the loop between the two complementary regions of a shRNA, ranging from 3 to 9 nt in length. Similar effectiveness has been obtained for loops of 5, 7 or 9 nt. We use a 7 nt loop sequence (TCAAGAG) for the psiRNA vectors.

The insert design tool will generate hairpin siRNA-encoding DNA oligonucleotide insert sequences from an input siRNA target sequence, for cloning into a pSilencer siRNA Expression Vector. The program will add the loop sequence and overhangs for cloning.

### Sequence Retrieval

#### a. *NCBI-mRNA*

The mRNA sequence of human Plk1 was retrieved from NCBI's (National center for Biotechnology information) using Entrez tool in both Genbank and FASTA format.

The sequence in fasta format is given below.

**>gi|34147632:1-2204 Homo sapiens polo-like kinase 1 (Drosophila) (PLK1), mRNA**

The protein sequence in fasta format:

**>gi|21359873|NP005021.2| polo-like kinase [Homo sapiens]**

#### b. *Uniprot - Protein*

The protein sequence of Plk1 was retrieved from uniprot database. The accession number is P53350. The sequence length is 603AA and its molecular weight is 68255Da.

### 2. Sequence Analysis

The sequence was analyzed to find the functional domains and protein family using Interpro and Pfam databases.

#### a. *InterPro*

The results showed that it contains two important domains namely, IPR000959 which is a POLO box duplicated region and IPR000719 which is a Protein kinase domain as shown in figure 1.

## RESULTS AND DISCUSSION

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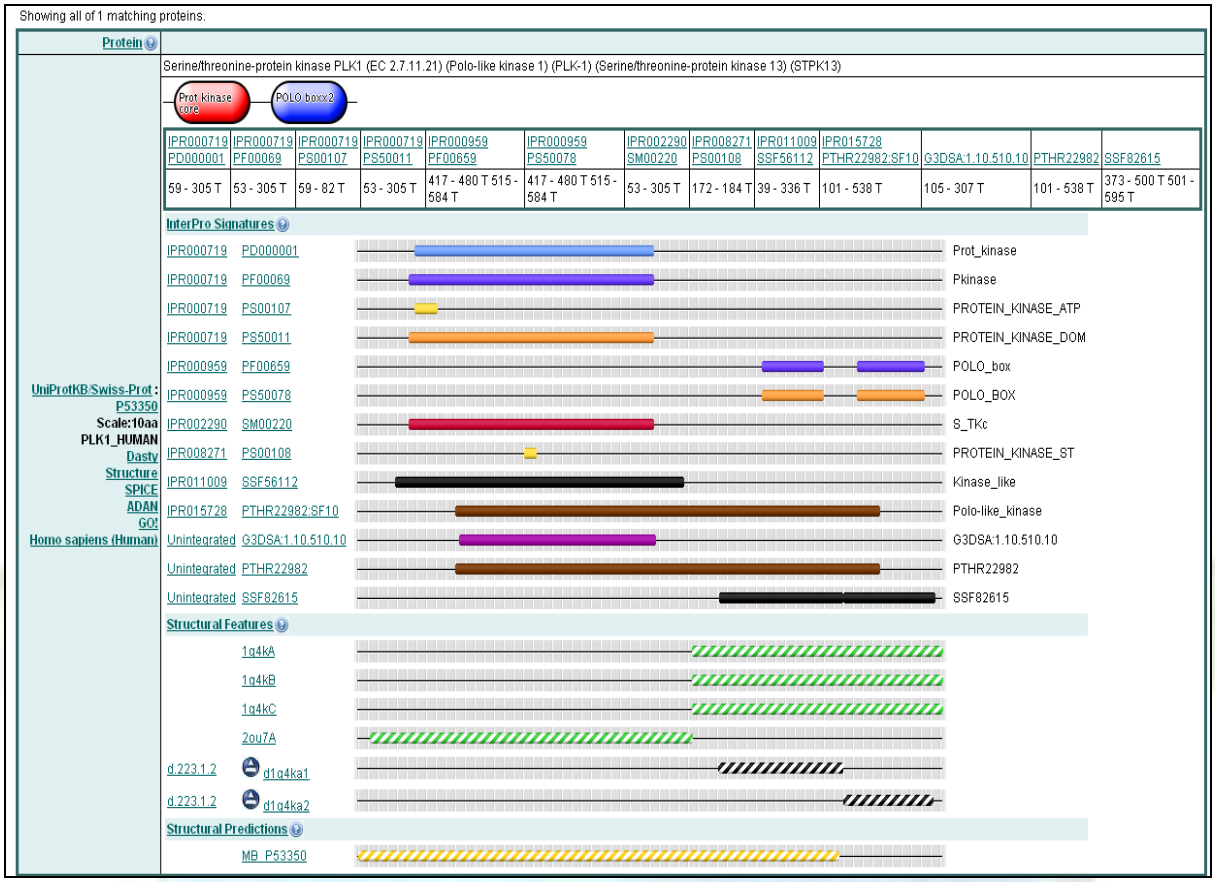


**Fig.1: Diagrammatic representations of the Protein kinase domains**

## b. *Pfam*

The protein sequence of polo-like kinase 1 (Plk1) of human was analyzed for protein family with the protein and domains. The results show that it belongs to two protein families' protein kinase and POLO box and details about them are shown below. Protein kinase domain is from 53rd residue to 305th residue. POLO box is in two regions. One is at 417 to 480 and another at 515 to 584.

## SiRNA Designing

The first step in designing a siRNA is to choose the siRNA target site. The guidelines below for choosing siRNA target sites are based on both the current literature and on empirical observations by scientists at Ambion. Using these guidelines, approximately half of all siRNAs yield >50% reduction in target mRNA levels. The blast results indicate that out of the targets selected many targets had alignments that showed less than 17 contiguous residues to human genome as shown in the table 1.

**Table 1: siRNAs target site with gene sequence**

| Target Sequence Number | Target Sequence | Position in gene sequence |
|---|---|---|
| 16 | AAACCGAGTTATTCATCGAGA | 629 |
| 50 | AAAAAGAAGAACCAGTGGTTC | 1192 |
| 64 | AAGAAGATCACCCTCCTTAAA | 1542 |

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Recent reports by van Vugt et al. (2004a,b) implicated an involvement of Plk1 in the resumption of cell cycle re-entry after checkpoint activation through DNA-damaging agents. It is therefore not surprising that targeted interference with Plk1, primarily by antibodies, antisense, or small interfering RNA technology but also with small-molecule inhibitors (McInnes et al., 2005,2006; Schmidt and Medema, 2006), has been reported to result in a blockade in mitosis with subsequent induction of cell death (Lane and Nigg, 1996).

Several studies have shown that this motif recognition model is also crucial for the targeting of Plk1 to specific subcellular locations (Seong et al., 2002). Interestingly, Sak, the fourth member of the pololike kinase family, contains only one polo box. Crystal structure analyses of the polo box motif have shown that the Sak polo box forms a homo dimer in vitro and in vivo and localizes to centrosomes and the cleavage furrow during cytokinesis (Leung et al., 2002).

Although Plk1 is often overexpressed in human cancers, the Plk1 gene is rarely amplified, indicating that transcriptional transcriptional or post-transcriptional regulation of Plk1 are affected in cancer cells. The association of Plk1 overexpression with cancers could be explained as a result of high mitotic index of tumor cells since Plk1 levels are cell cycle-regulated with a peak during mitosis (Golsteyn et al., 1995; Hamanaka et al., 1995). Indeed, early studies of Plk1 by Hamanaka et al. (1994), Holtrich et al. (1994) and Golsteyn et al. (1994) in different fetal and adult tissues have shown that Plk1 levels are much higher in thymus, spleen and testis, which have more proliferating cells. However, Plk1 overexpression has been indicated as the cause of tumor formation instead of being the consequence of high mitotic index during tumor cell proliferation. Overexpression of Plk1 in NIH3T3 fibroblasts transformed the cells into oncogenic foci in soft agar and more importantly lead to tumor formation when injected into nude mice as observed by Smith et al. (1997).

However, due to the intrinsic problems including dose-limiting side effects, inadequate penetration into the tumor tissues, and degradation by endogenous nucleases, it is difficult to achieve a consistently high efficacy (Zhang et al., 2007). Optimization of the delivery system is ongoing, for example using ASO-loaded HAS nano particles (Spänkuch et al., 2008). Using small molecules to inhibit Plk1 is another approach, as these molecules are easier to be delivered into cells and are less likely to be degraded. For most chemical inhibitors, they are designed to suppress important functional domains. For Plk1, one important domain is the serine/threonine kinase domain at the N-terminus.

RNAi is a powerful tool for post-transcriptional down regulation of endogenous genes. Small double-stranded RNAs named siRNAs or miRNAs depending on their origin abrogate gene expression by sequence-specific binding of cognitive mRNA. Alternative attempts for prolonged RNAi-based gene silencing have been made through application of Pol III (RNA polymerase III)-promoter driven expression of shRNAs that are subsequently processed to siRNAs (Sui et al., 2002).

## Conclusion

PLK1 function appears to be essential for centrosomes-mediated microtubule events and consequently for spindle assembly. siRNAs targeted against human PLK1 may be

 

valuable tools as antiproliferative agents that disaplay activity against a broad spectrum of prostate cancer.

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