ESTIMATION OF HOMOZYGOTE RECESSIVE AND HETEROZYGOUS CDK3 DISTRIBUTION IN RANDOMLY SELECTED CANCER SUBJECTS

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ABSTRACT: Cdk3 is well known cell regulating protein and has prominence role in cancer development. Studies relating to different phenotypes in various cancers would estimate the frequency of distribution among Indian patients suffering from cancer. Such distribution of Cdk3 was found to be 55.5% in coastal Andhra which is homozygote recessive and 44.4% being heterozygous.

KEY WORDS: Cdk (Cyclin-dependent kinase), Cell regulating protein, Polymorphism and SNP (Single Nucleotide Polymorphism).

INTRODUCTION: Cyclin-dependent kinases (cdk) are serine/threonine protein kinases that play essential roles in the control of cell cycle progression by interacting with a variety of regulators and substrates. In the mammalian cell cycle, the transition from the G₀/G₁ phase to S phase, in which DNA replication occurs, has been shown to be regulated by cyclin- dependent kinases (cdks). Activities of cdks are controlled by association with cyclins and reversible phosphorylation reactions. An additional level of regulation is provided by inhibitors of cdks. Two families of these inhibitors have been described, those that interact with a wide range of cyclin/cdk complexes, including p21, p27 and p57, and those that only inhibit cdk4 and cdk6, including p15, p16, p18 and p19 [1]. In eukaryotic cells, cell cycle progression is driven by the sequential and periodic activation of cyclin/cdks, and dysregulation of the cell cycle is associated with cancer development [2-4]. Over expression of cdk6 has been observed in lymphomas, leukemias, and melanomas due to chromosomal translocation [5, 6].

Cdks are closely associated with human cancer pathogenesis. Cdk3 is an important regulator of cell cycle. The activity of cdk3 is first observed in early G₁ phase [16] and reaches a peak in mid-G₁ [7]. A dominant-negative cdk3 was shown to induce G₁ arrest, which could not be rescued by cdk2, indicating that cdk3 plays an important role for G₁ exit to S entry [8-9]. Recently, cdk3 was found to form a complex with cyclin C and phosphorylate the retinoblastoma protein (pRb) at serine 807/811, which is required for G₀-G₁ transition [10]. Furthermore, cdk3 seems to be expressed in various normal human tissues and cancer cell lines including glioblastoma and neuroblastoma cells [10-12].

Therefore, since CDK3 polymorphism is in a gene coding for a cell cycle protein and is in an intron region with no known diagnostic value and also the SNP had known allele frequencies in multiple populations, and the two alleles were both common, we have undertaken this project to find out the frequency of polymorphisms in India as preliminary study. The allele frequencies in our population are not available.

MATERIALS AND (METHODS)[11]

Sample Collection: 5ml EDTA Blood sample from 10 healthy volunteers of age group 20 to 35 were taken for the study. DNA was isolated using salting out method. The extracted DNA was subjected to PCR and subsequent restriction digestion to have RFLP patterns using Hpa II.

DNA Extraction by Salting out Method: In brief, 125ul of whole blood was taken and 125ul of TKM-1 buffer (Tris HCl 10mM, KCl 10mM, MgCl₂ 10mM and EDTA 2mM) was added along with 2-3 drops of Triton - X 100 to remove RBC. Centrifuged at 2700rpm and the supernatant was removed. This step is repeated. Once white pellet is obtained, added 80ul of TKM-2 buffer (Tris HCl 10mM, KCl 10mM, MgCl₂ 10mM, EDTA 2mM and NaCl 0.4M) and 10% 12.5ul of SDS(sodium dodecyl sulphate). This mixture was incubated at 55°C to dissolve the pellet for 15 min. After dissolution of the pellet freshly prepared 35ul of 5 M NaCl was added and incubated for 10 min. Centrifuged at 12,000rpm to collect the supernatant DNA in fresh tube containing 100% ethanol. After final washing with 70% alcohol, centrifuged to remove the supernatant and air dry the pellet and further dissolved in 100ul TE buffer.

Agarose gel for Electrophoresis: Weighed 0.8g of agarose in 40ml of 1X TBE Buffer in a micro-oven and added 4μL of Ethidium Bromide (EtBr) as soon as it is removed from oven and mixed to make it uniform. Pour it in the prepared gel casting plate and allow it to solidify. After solidification remove the comb and place it in gel tank containing TBE buffer. Place loading place towards negative cord plug and the samples are loaded into the wells along with ladder. Voltage was set to 100V and the gel was inspected until the loading buffer band reaches the near to the opposite end. After running the gel completely, bands were observed on UV Transilluminator.

PCR Setup: The PCR is set up with 20pM of forward and reverse primers. The reaction is setup in 20ul of reaction volume with final concentration of 50mM KCl, 10mM Tris, and 200mM dNTP's and 1.5mM MgCl₂. Reaction were setup under the cycling parameters 94° C for 4min, 94° C for 1min, 55° C for 1min, 72° C for 1min, 72° C for 10 min. The number of PCR cycles was 35.

Primers: The resultant PCR product was loaded in 2% Agarose Gel Electrophoresis the expected band size is 308bp which is run along with the DNA ladder. From the resultant PCR product 5ul of the sample is used for the Restriction Digestion.

RESULTS AND DISCUSSION: Table 1: Restriction Digestion of the sample was done using Hind III Restriction Enzyme. Reaction is set up for 20μl of reaction volume with the final concentration of 10mM Tris-HCl, 10mM and enzyme 0.4μg/100μl. Distributed 10μl of reaction mix in 10 tubes as shown below in table. The expected band size after digestion is 209bp and 99bp.

Sl/No	Sample ID	Restriction digestion Mixture	PCR Product
1	SD3 166r	10μl	10μl
2	SD2 157r	10μl	10μl
3	SD3 294r	10μl	10μl
4	SD4 304r	10μl	10μl

5	SD2 166r	10µl	10µl
6	SD2 274r	10µl	10µl
7	SD2 56r	10µl	10µl
8	SD1 185r	10µl	10µl
9	SD1 119r	10µl	10µl
10	SD2 226r	10µl	10µl

Based on the banding patterns observed in gel image 1 and gel image 2 the samples are interpreted as Homozygote dominant, Homozygote recessive and Heterozygous. The RFLP results are shown in Image1 & 2. All samples were successfully amplified except one. In sample number SD2 274 has undigested parental band is observed. Since the 306bp band is faint when compared to rest of the bands, it should be undigested band. From the population we have selected the frequency of Homozygote recessive is 55.5%, heterozygote is 44.4% as mentioned in the Table 2.

Gel image 1

In figure 1, lane 2 showed undigested product and lane 3 showed digested product of sample SD3 166. Digestion with HPA 2 restriction enzyme produced 2 bands 209 bp and 99 bp indicating both alleles have G polymorphs which are true with sample number 157 of which undigested and digested products are shown in lane 4 and 5 respectively. Lanes 9 and 10 indicates undigested and digested products of SD4 304 no Amplicon. Corresponding undigested and digested products are nor seen. Probably it's due to isolation of DNA was faulty. Lanes 11 and 12 show the undigested and digested products of SD2 166 sample indicate 308 bp, 209 bp and 99 bp respectively indicating 2 alleles have G in place of A.

Gel image 2

In figure 2, lane 2 showed undigested product and lane 3 showed digested product of sample SD3 274r. Digestion with HPA 2 restriction enzyme produced 2 bands 209 bp and 99 bp indicating both alleles have G polymorphs which are true with sample number SD2 56 of which undigested and digested products are shown in lane 4 and 5 respectively. In sample SD2 274 a faint 308 bp is seen, it is probably undigested product. Lanes 8 and 9 indicates undigested and digested products of SD1 119. Lanes 10 and 11 show the undigested and digested products of SD2 226 sample indicate 308 bp, 209 bp and 99 bp respectively indicating 2 alleles have G in place of A.

Sl No	Sample ID	Bands detected (bp)	Results
1	SD3 166r	209 bp and 99 bp	Homozygous Recessive
2	SD2 157r	209 bp and 99 bp	Homozygous Recessive
3	SD3 294r	308 bp, 209 bp and 99 bp	Heterozygous
4	SD4 304r		N/A
5	SD2 166r	209 bp and 99 bp	Homozygous Recessive
6	SD2 274r	209 bp and 99 bp	Homozygous Recessive
7	SD2 56r	209 bp and 99 bp	Homozygous Recessive
8	SD1 185r	308 bp, 209 bp and 99 bp	Heterozygous
9	SD1 119r	308 bp, 209 bp and 99 bp	Heterozygous
10	SD2 226r	308 bp, 209 bp and 99 bp	Heterozygous

Table 2: The CDK3 RFLP –Interpretation

The nucleotide sequence selected for PCR:

5' AAGGGCGTGT AGCACAGCAT AAAGACAGAG CTAATCAAT GAGCGCCACT TTCACAGGGA
 AGATAAATAC TGCACCTTATC CTGGGGGAGG CTTCCAGGTT GAACAATCAG TATACCCAAG
 CCAGTTGTGT ACAAAGGTCA GGAAAGAGAC CCTGGCCTTG GACTCAGAAA GTGCCAGGGT
 TATGTAAGAG GCTGGCTGAT GAGGGGAAAC TGTAGTCGGA GCAGCAGCTG GAGCCCACAT
 GCACCTACCA TGAGCAGGTC CCTGCCCTCT GGCTCCAGAT TGGGCACAAT CTCTTCCAGT CCCTTCCTGG
 T 3' the nucleotide position 96 could be A or G. The site which the restriction enzyme Hpa II

recognizes is 5' CCGG 3'. Hpa II cuts the sugar phosphate backbone between the two C's at the 5' end of the sequence: 5' C|CGG 3'.

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