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Original Research

Hypo/unmethylated promoter status of Cdk2 gene correlates with its over-expression in ovarian cancer in north Indian population

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Abstract: Cdk2 has been seen to play crucial roles in cell cycle regulation particularly in G1 to S phase transition and it is known to be involved in cancer progression and cancer cell proliferation. The aim of this study was to evaluate (i) the expression of cdk2 in malignancy of ovarian tumors and (ii) correlation between expression and DNA methylation. Clonal bisulfite sequencing and quantitative PCR analysis was performed for promoter methylation and expression analysis respectively in normal and ovarian tumor tissues. Increased methylation level was observed in normal ovarian tissues than ovarian carcinoma. An inverse relationship was found with expression levels of Cdk2 and DNA methylation.

Key words: Cdk2, Ovarian Cancer, Promoter methylation.

Introduction

Ovarian cancer, an abnormal growth of ovarian cells can arise either from epithelial, germ or stromal cells of which the organ is made (1). Out of these, malignant epithelial carcinomas are the most common ones and 85-90% of the ovarian cancer patients suffer from this form of ovarian malignancy (2). As of now, no definite reason for the development of ovarian cancer is known. A few set of risk factors such as age, obesity, reproductive history, birth control, diet, breast cancer history and some genetic factors (both inherited and acquired) are known to be involved (3-4). As there is no definite known reason for the cancer formation and the survival rates of cancer patients being very less, prognosis and diagnosis becomes a major concern. A large number of studies have previously been conducted on genes implicated in ovarian cancer (5-8). In most instances, the promoter region of such genes is found to be substantially hypermethylated and an inverse relation between expression levels and methylation status of such genes is generally found (9-10). DNA methylation is a biochemical process involving the covalent addition of a methyl group to C-5 position of cytosine. It is the most frequently occurring epigenetic event in the mammalian genome and has been observed to occur in both somatic and embryonic stem cells (11). In embryonic cells, de novo methylation takes place, which is involved in the rearrangement of methylation pattern during embryogenesis or differentiation processes in adult cells (12). A large number of methylation studies have been conducted on various genes implicated in all kinds of cancers. Abnormal DNA methylation can lead to cancer mainly by spontaneous deamination of 5-methyl cytosine to thymine, by global hypomethylation and by promoter hyper or hypomethylation. The abnormal methylations of promoter regions have been extensively studied in all kinds of cancers. Tumor suppressor genes such as APC(13), BRCA1(14), CDH1(15), GSTP1(16), PTEN(17), RASSF1A(18), STK(19), TP53(20) have been widely found to be hyper-methylated in all kinds of cancers, whereas genes such as AKT1(21), ERBB2(22), HRAS (23) and many more have been found be hypomethylated.

Cyclin dependent kinase 2 (Cdk2) is a catalytic subunit of the cyclin-dependent protein kinase complex, essential for cell cycle G1/S phase transition (24). This protein associated with the regulatory subunits of the complex including cyclin A or E, CDK inhibitor p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B) (25). CDK2 is known to be involved in cancer progression and cancer cell proliferation (26). Cdk2 gene product indirectly acts as pleiotropic effector of cell physiology and plays an important role in the control of gene expression involved in the cell division cycle, apoptosis, cell differentiation and cell migration (27-28). Therefore cdk2 warrants epigenetic analysis for validating their potential role on ovarian cancer onset and progression.

The aim of this study was to evaluate whether the expression of cdk2 is related to the malignancy of ovarian tumors and whether its expression is regulated by DNA methylation. Epithelial ovarian tumors are classified as benign, borderline, and malignant on the basis of their potential for locally invasive and metastatic nature (29). Various reports indicate that the progress of ovarian carcinomas is associated with the alterations of some cell cycle proteins. Expression of p27kip1, cyclin D1, cyclin E, and cdk2 as well as cdk4 and p16has been investigated in epithelial ovarian carcinomas (30-31). The aims of the current study are (1) to investigate whether the expressions of cdk2 is associated with the malignant progression of ovarian tumors, (2) to analyse promoter DNA methylation of cdk2 and its correlation with the

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expression. Cdk2 expression has been investigated by immunohistochemistry in a series of 79 epithelial ovarian tumors and it was reported that cdk2 was expressed in a large part of ovarian tumors (32). The expression of cdk2 gradually increased from benign to borderline to malignant tumors. CDK2 inhibition sensitizes cyclin E1-driven ovarian cancer cells to platinum-based chemotherapy (33).

Materials and Methods

The 31 ovarian tumor samples used in this study were obtained from patients that underwent surgery at two institutions, Regional Cancer Center, Kamala Nehru Memorial Hospital, Allahabad and King George's Medical University, Lucknow. Tissue samples were collected with the informed consent of the patient. Work plan was approved by institute ethics committee. The age of the patients ranges from 34-68 years. Out of thirty samples, 5 were benign and 25 were malignant with grade 1 and 3. All the samples were CA-125 Positive (range: 33-217). Among the tumor samples collected, there was one clear cell, one endometrioma, three mucinous adenocarcinoma (ADEN) and 26 serous papillary adenocarcinoma samples. Tumor tissues were flash-frozen in liquid nitrogen and stored at -80°C until processed. Twenty six serous epithelial ovarian carcinoma tissues and six normal ovarian tissues were further processed for methylation and expression analysis. Cdk2 promoter region was retrieved from EPD (Eukaryotic Promoter database). MethPrimer was used for designing the primers. For Cdk2, -175 to +18bp relative to transcription start site covering 14 CpG sites Most of the methylation sensitive transcription factor binding sites were present in these regions of promoters.

DNA Extraction

DNA extraction from both tissues and peripheral blood was performed. For tissues, cells were lysed first and further digested with proteinase K (50µg/ml) in presence of SDS for 12-14 hr at 37°C. Phenol/ phenol: chloroform:isoamylalcohol/ chloroform:isoamyl alcohol extraction was done at 25 °C. Finally precipitation of DNA was done with 1/30 volume of 3M sodium acetate (pH-5) along with two volume of chilled ethanol. DNA from blood was isolated by lysis of RBCs followed by lysis of lymphocytes by SDS. DNA was extracted with chloroform and precipitated with sodium per chlorate and two volumes of ethanol.

Sodium bisulphite conversion, PCR and sequencing

The isolated DNA was treated with sodium bisulfite using BisulFlash DNA modification kit, supplied by Epigentek. Approximately 1µg of the isolated DNA was processed for bisulphite conversion using the protocol described in the user manual. Regions of interest were then amplified using gene specific primers for the bisulphite converted DNA (Primer sequences for Cdk2 are FP: 5'-GTTTTTAA GAATTAAATTTGTT-TGGG-3'and RP: 5'TTTAACCAACTTAAAACAA-TATTACC-3'). PCR program was set as follows: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 45 sec, 54°C (for Cdk2 gene). Samples were then separated on 1.5% agarose gel. Gel elution was performed to selectively isolate the desired bands followed by phenol: chloroform extraction. Ligation was then performed using InsTAclone PCR cloning kit by Fermentas followed by transformation into E. coli DH5α. Positive clones were obtained by ampicillin/X-gal/IPTG selection. Further confirmation was done by colony PCR. Positive clones were selected and plasmid isolation was done through alkaline lysis method (mini prep kit from Thermo Scientific US). 6-10 clones were sequenced by automated DNA sequencer (ABI 3130 genetic analyser) for each developmental stage. 50-100ng of plasmid DNA was used for cycle sequencing with The Big Dye® Direct Cycle Sequencing Kit as per manufacturer instructions.

RNA isolation and quantitative PCR analysis

Total RNA from normal, tumor tissues and blood was isolated using TRIzol reagent (Invitrogen) according to the protocol supplied by the manufacturer. DNaseI treatment was then given to digest any trace of DNA in the presence of RNase inhibitors and appropriate buffers for 30 min at 37°C followed by Phenol: chloroform extraction. RNA concentration was estimated by spectrophotometer (OD_{260}nm) before and after the DNAse treatment. Equal amount of the total RNA was reverse transcribed using oligodT primers and 200U of MMLV Reverse transcriptase (NEB USA).The reaction mix was incubated at 37°C for 60 min and then heat deactivation at 65° C for 10 min. PCR amplification was done with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific USA) in real time PCR machine (ABI-7500). Reactions were performed in triplicates for both GAPDH and Cdk2 for each tumor and normal sample. Average of these values was calculated and standard deviation was also calculated. Target mRNA amount was determined and normalized relative to the amount of GAPDH mRNA. ΔCt value was calculated by subtracting Ct value of reference gene (GAPDH) from Ct value of target gene (Sox-9 and Oct-4). For calculating ΔΔCt, ΔCt value of calibrator was subtracted from ΔCt value of each sample. Fold change in gene expression was calculated with the formula $2^{-\Delta\Delta Ct}$. The sequences of the primers are as follows (Cdk2 primers are FP:5′-TTAGTAAAGTTGTACCTCCCCTGG-3′and RP:5′-GACTAGAAGGTGAGTGTTTAAGGC-3′ and GAPDH primers are FP:5'-AAATCTGG-CACCACACCTTC-3' and RP: 5'-CTCCTT AATG TCACGCACGA-3'. PCR conditions were. 94°C for 4 min followed by 94°C for 1min, 60°C for 1min and 72°C for 1min with a final extension of 6 min for 35 cycles for GAPDH and Cdk2 both.

Results

Pictorial representation of location of CpGs in Cdk2 promoter region as well as primer sequences are shown in figure 1a. Sodium bisulfite reactions and real time PCR were done to evaluate the level of methylation and transcriptional activity of Cdk2 gene in different tumor and normal samples. Primers were designed for performing real time and the length of the product was 283 bp. GAPDH was PCR amplified as internal control (150 bp). Fourteen sites were targeted to interrogate the methylation level on promoter of Cdk2 by sodium bi-

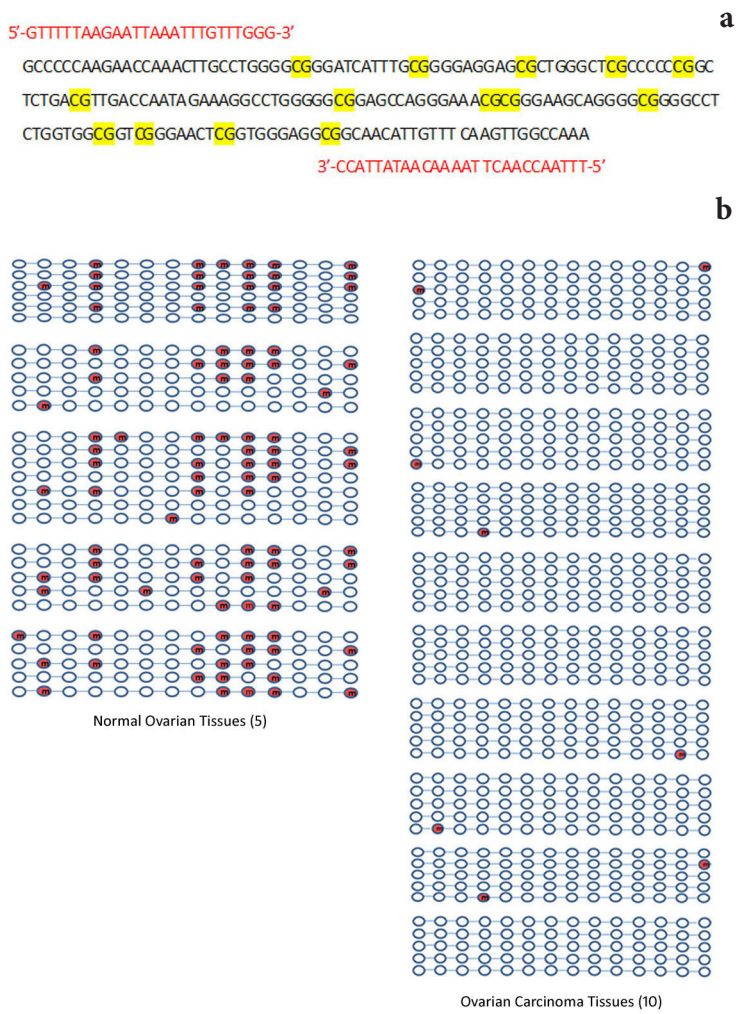

Figure 1. a: Nucleotide sequence of the Promoter region of Cdk2 gene. Sequences in red are the locations of primers. A 193bp region (-175 to +18bp relative to transcription start site) was chosen for methylation analysis in CDK2, covering a total of 14 CpG sites (highlighted in yellow). **b:** Methylation mapping of 14 CpGs was carried out by clonal bisulfite sequencing. Each line represents individual clone harboring 14 CpG sites. Methylated and unmethylated CpGs are represented by dark circle and white circles respectively.

sulfite conversion. Product length of bisulfite converted product was 193 bp. 6-10 clones were sequenced for each tumor and normal samples to check the level of methylation.

Expression analysis

Cdk2 expression was analyzed through quantitative real time PCR. Higher expression was observed in tumor tissues however normal tissues showed lower expression (results of 24 tumor samples and 5 normal tissue samples are shown in fig. 2). However variations in individual samples were observed. Eighteen tumor tissues out of 24 showed marked increase in expression level. Among them 14 tumor samples were showing notable enhanced expression.

Methylation profile

For Cdk2 gene, an average methylation of all the sites in all normal samples was found to be 30.92 % (fig 2) in contrast to 1.7% in tumor tissues, which is a significant finding. Pictorial representation of methylation pattern of 10 10 ovarian carcinoma samples is shown in fig 1b along with five normal ovarian tissues. Out of 14 CpG sites, significant methylation was pre-

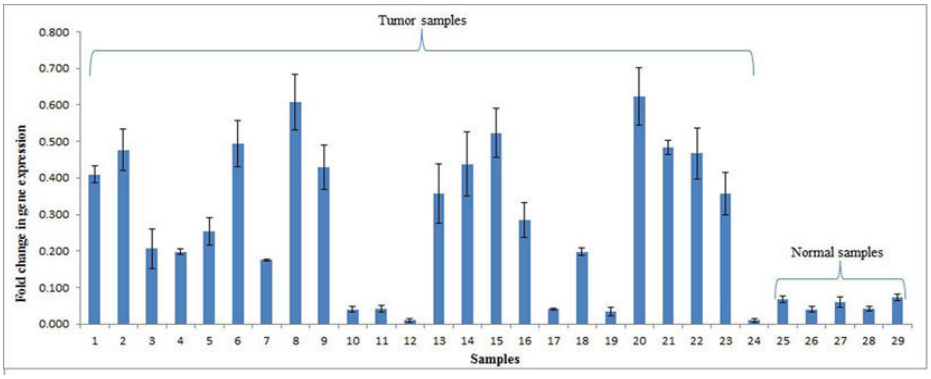

Figure 2. Real-time PCR expression analysis of Cdk2 in 24 tumor samples (T1-T24) and 5 normal ovarian samples (N1 and N5). GAPDH was taken as internal control. Reactions were performed in triplicates for both GAPDH and Cdk2 for each tumor and normal sample.

sent at four sites (#4, #8, #10 and #11). Furthermore, the frequency of methylation was found to be more at 4th CpG site (57%), 8th CpG site (48%), 10th site (76%) and 11th (65%) amongst the 14 CpG sites analyzed. 50% methylation level was considered as significant.

Comparing the quantitative gene expression analysis data with sequenced samples of bisulfite converted DNA indicated the inverse relationship between expression levels and methylation status of gene in Cdk2. Thus hypermethylation on these sites may have implications in down regulation of Cdk2 gene in normal ovarian tissues.

Transcription Factor binding sites analysis

Cdk2 promoter was also analysed for binding of transcription factors (methylation sensitive) through PROMO 3 software. Out of four methylated sites, 10th and 11th CpG sites showed binding with transcription factors like E2F-1, E2F-1: DP-1, E2 and c-Ets-1. Binding sites of E2F-1 (5′-GAGCGC GGG-3′) were methylation sensitive. The E2 family of transcription factors has be seen to affect differentiation, neurogenesis and in transcription regulation.

Discussion

A large number of studies have explicitly indicated inverse relationship between gene expression and promoter methylation (9). Similarly in this study, a comparison between the quantitative gene expression analysis data with methylation profile of tumor and normal DNA indicated the inverse relationship between expression levels at RNA status and in the methylation status at gene level for Cdk2. Expression study of the various genes and their correlation will surely help in a better understanding of the roles that these genes play in ovarian cancer development and progression.

Bisulfite sequencing analysis indicated that the Cdk2 promoter region was hypermethylated as it had more methylated CpG sites in normal ovarian samples than in tumor tissue. Thus one important observation of the study is that Cdk2, which acts at the G1-S transition checkpoint to promote the initiation of DNA synthesis and modulation of G2 phase progression, has important role to play in the development and progression of ovarian carcinoma. An inverse relationship was also found between methylation data and expression analysis which further supports this inference.

A significant hypermethylation pattern was observed in the studied Cdk2 promoter region at 4th, 8th, 10th and 11th CpG position. At 10th and 11th CpG site, E2F-1, E2F-1: DP-1, E2 and c-Ets-1 were found to bind. The E2 family of transcription factors has been seen to affect differentiation, neurogenesis and in transcription regulation (34). On the other hand, members of the Ets family of transcription factors have been implicated in the development of different tissues as well as cancer progression (35). These transcription factors bind to their specific sites when they are not methylated, and lead to higher expression in tumor tissues. However, in normal tissues, their binding sites are found to be completely/partially methylated. When the CpGs present in the consensus sequences for binding of TFs are methylated, these TFs may not be efficiently binding to the sequences resulting in slowing down of the transcription rate. It indicates that these transcription factors may speed-up the transcription rate after binding to their respective sequences. Thus, it can be inferred that due to methylation, these factors might not be able to bind and that can be one of the reasons effecting loss of Cdk2 gene expression in normal tissues. A greater understanding of the role of epigenetics in ovarian cancer will allow for improved interventions against this malignancy.

The expression of cyclin E and cdk2 gradually increased from benign to malignant tumors and suggested that loss of p27kip1 expression and over expression of cyclin E or cdk2 was significantly associated with malignancy in ovarian tumors (32). Our results suggest that site-specific hypermethylation might be the regulating factor for down regulation of Cdk2 in normal ovarian tissue. The progress of the G1 phase of the cell cycle is stimulated by G1 cyclins, such as cyclin D1 and cyclin E, as well as their partners, cdk4 and cdk2. These complexes play an important role in progression of cell cycle through the G1 checkpoint. Moreover, Cdk2 activity was higher in cancers than in benign tumors/normal tissue. It has also been reported that cyclin E and their partners Cdk2 and Cdk4 overexpression is related to deeply invasive tumors, lymph node metastasis, and advanced stage in gastric cancer and breast cancer (36). Thus, these studies suggest that cyclin E over expression may promote progressive characteristics of ovarian carcinoma. CDK2 inhibition sensitizes cyclin E1-driven ovarian cancer cells to platinum-based chemotherapy. This might be a logical approach for incorporating CDKi into treatment strategies for serous ovarian cancer. Cyclin E and cdk2 were overexpressed mostly in primary ovarian cancers compared to metastatic and recurrent diseases. Cyclin E RNA was overexpressed in 29.5% and cdk2 in 6.5% of ovarian tumors tested (37). In previous study, the genes (CDK1, CDK2 and CDK4) were dysregulated in breast cancer (38), ovarian cancer (39), colon cancer (40), hepatocellular carcinoma (41), thyroid carcinoma (42), and lung cancer (43). However higher expression of CDK1, CDK2 and CDK4, part of cyclin-dependent kinases, were related to tumorigenesis in Laryngeal Squamous Cell Carcinoma (39). Cdk2, which acts at the G1-S transition checkpoint to promote the initiation of DNA synthesis and modulation of G2 phase progression, may have important role to play in the development and progression of ovarian carcinoma as it was found to be methylated in normal ovarian samples but non-methylated in tumor tissues. An inverse relationship was also found between methylation data and expression analysis which further supports this inference. As there was a consistent hypermethylation at 4th, 8th, 10th and11th CpG sites, a set of such CpG sites in same gene may be looked further in large sample size for their methylation profile, their role in regulating the expression of Cdk2 and ovarian cancer manifestation and progression.

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