

RESEARCH COMMUNICATION

Association of Two Polymorphisms of DNA Polymerase Beta in Exon-9 and Exon-11 with Ovarian Carcinoma in India

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Abstract

Background: DNA polymerase beta (pol β) is a key enzyme in the base excision repair pathway. It is 39kDa protein, with two subunits, one large subunit of 31 kDa having catalytic activity between exon V to exon XIV, and an 8 kDa smaller subunit having single strand DNA binding activity. Exons V to VII have double strand DNA binding activity, whereas exons VIII to XI account for the nucleotidyl transferase activity and exons XII to XIV the dNTP selection activity. **Aim:** To examine the association between pol β polymorphisms and the risk of ovarian cancer, the present case control study was performed using 152 cancer samples and non-metastatic normal samples from the same patients. In this study, mutational analysis of pol β genomic DNA was undertaken using primers from exons IX to XIV - the portion having catalytic activity. **Results:** We detected alteration in DNA polymerase beta by SSCP. Two specific heterozygous point mutations of pol β were identified in Exon 9:486, A->C (polymorphism 1; 11.18%) and in Exon 11:676, A->C (polymorphism 2; 9.86%). The correlation study involving polymorphism 1 and 4 types of tissue showed a significant correlation between mucinous type with a Pearson correlation value of 4.03 (p=0.04). The association among polymorphism 2 with serous type and stage IV together have shown Pearson χ^2 value of 3.28 with likelihood ratio of 4.4 (p=0.07) with OR =2.08 (0.3-14.55). This indicates that there is a tendency of correlation among polymorphism 2, serous type and stage IV, indicating a risk factor for ovarian cancer. **Conclusion:** Hence, the results indicate that there is a tendency for pol β polymorphisms being a risk factor for ovarian carcinogenesis in India.

Keywords: DNA polymerase beta - DNA repair - somatic mutation - polymorphism - DNA-SSCP - India

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Introduction

Cancer is a result of a series of genetic alterations leading to a progressive disorder of the normal mechanisms of controlling growth, differentiation, cell death when genetic changes accumulate. DNA repair mechanisms are essential for the repair of such changes instantly thus preventing tumor initiation and progression. DNA polymerase beta (Pol β) is one of the important players of base excision repair (BER) pathway (Wilson et al., 2000). This gene is inducible under certain circumstances such as oxidative stress, or alkylating agent (Cabelof et al., 2002). Polymorphisms of several DNA repair-related genes have been found to be associated with different tumors (Wu et al., 2011). Single-nucleotide polymorphism (SNP) is the most frequent and most subtle genetic variation in the human genome and has great potential for application to association studies of complex diseases (Yen et al., 2007). Since Pol β is a key enzyme in DNA repair, any

perturbations in its expression or function can lead to increased mutation frequency and genomic instability (Cabelof et al., 2003). Pol β mutation has already been detected in 30% cases of cancer (Starcevic et al. 2004). The E295K DNA polymerase beta gastric cancer-associated variant interferes with base excision repair and induces cellular transformation (Lang et al., 2007). It has also been reported that Pol β splice variant, may be present with elevated frequency in tumor (Chen et al., 2002). An estimated 22,280 new cases of ovarian cancer are expected in the US in 2012. Ovarian cancer accounts for about 3% of all cancers among women. An estimated 15,500 deaths are expected in 2012 (American cancer society, 2012). Ovarian cancer causes more deaths than any other cancer of the female reproductive system. We had initiated the project to screen the mutation of pol β gene in the catalytic region of exon 9 to exon 11 in the ovarian carcinoma samples. Our aim of this study was to identify the association of any polymorphism of polymerase beta

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within exon 9-14 region with the risk of ovarian cancer in India which was not studied earlier.

Materials and Methods

Sample description

One hundred seventy five ovarian carcinoma samples along with corresponding distant non metastatic normal samples were collected from Haldia Seba Sadan and different Hospitals at Haldia. The samples were snap frozen in liquid N₂ immediately after collection and were given a number for identification and to protect the identity of the patients. Institutional Review Board approval for this study was received from the IRB of the Haldia Institute of Technology. The demographic data was collected from each patient with their consent, using a previously set questionnaire including age, stage, disease history, family history and other relevant details) as required. Written consents were collected after counseling them from each patient. Samples were characterized by their age, staging of cancer, immune-histological grading. After histological examination, 152 cancer samples were chosen for the present study.

Isolation of DNA

From the frozen tumor samples, powders were made individually. Genomic DNA was isolated from these powdered samples following DNA isolation protocol (Strauss, 2001). Purity and quantity of DNA was measured by spectrophotometry. DNA were diluted with TE buffer to a final concentration of 50 ng/ μ l for further analysis and rest of the DNAs was stored at -80°C.

Detection of mutation by SSCP

SSCP of PCR product of tumor sample was done by SYBR gold staining (David et al., 2004). The PCR reaction was performed in a volume of 30 μ L containing 500 nM unlabeled primers, 2 units of pfu polymerase (PE Applied Biosystems, NJ), 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 1 μ l of each primer (50 ng/ μ l), and 1 μ l of DNA (50 ng/ μ l). After an initial hot start at 95°C for 5 min, PCR was run for 30 cycles of 94°C for 30 seconds, the annealing temperature was set according to the T_m of the primer as shown in Table 1, and extension at 72°C for 30 seconds, and finally ending with an extension of 5 mins at 72°C. Samples of purified PCR product were diluted with equal volume of loading buffer (20 μ M EDTA, 0.05% bromophenol blue, 95% formamide and 0.05% xylene cyanol FF), heated at 95°C for 5 min and immediately cooled on ice. Aliquots were loaded on a pre-run gel. The electrophoresis was done in 0.5 x TBE buffer at 4°C for the time specified for each set of primer mentioned in Table 1. The percentage of acrylamide gel is given in Table 1. Patterns of resultant single stranded DNA mobility were visualized by staining with SYBR Gold Nucleic Acid gel stain (Molecular Probes). Any tumor sample showing extra band compared to normal sample are sequenced by direct sequencing of PCR product.

Statistical analysis

The mutation percentage, average age of patients at

Table 1. Sequences of the Primers used for DNA-SSCP, Conditions for PCR, and the Expected Size

Exon	Sense & antisense (5'-3')	T _m (°C)	Size
8+9	GCTGGTATGGCACGGACAA AACCCAAGATTAGGAAGTATG SSCP conditions* 14%,7.5w,6hrs	55	347
10	ATCTCATATGTGTCTTCTGTCA GTATTCAATGTTTTCTCTTTCAA SSCP conditions* 16%,7.5W,6 hrs	55	336
11	TTACTCTTTTTCTTATTCCCTAAT TGGATACTCTATTGGTGACTGTA SSCP conditions* 16%+10%GLY.,10W.,16.5 hrs	55	240
12	TGGCCTTGTGTTTTACTTGATTA TTGGCAAGAACATATGGCTCTT SSCP conditions* 16%,10w	58	280
13	TTGGGGTGATTTGAGCAGTCTACC TCTCCGTTGCCTGAAGGACTAAAA SSCP conditions* 10w, 12hrs	60	446
14	TCTTGAACCCTCTATACTAAACT GCAAAAGCATGATGAAGAAGAAACAAT SSCP conditions* 16%,10w,>4.5hrs	58	279

* 'W=Watt; GLY=Glycerol; %=% of polyacrylamide gel

diagnosis for the two polymorphisms were estimated. The maximum, minimum age, ranges were also calculated. All the statistical analysis was performed with Statistical Package of the Social Sciences (SPSS) for windows (version 10.0). Frequency of distributions of demographic variables and polymorphisms were evaluated by χ^2 test (for categorical variables). The polymorphism data were further stratified by subgroups of age, types, and stages. Odd ratio (OR) with 95% confidence interval (CI) were calculated to compute the strength of association between the polymorphism and the ovarian cancer risk. A probability level (P) of <0.05 was adopted as a criterion of significance.

Results

Pol β polymorphisms

Heterozygous mutation causing change of A to C was identified at nucleotide position 486 of exon 9 obtained from tumor DNA of some patients. This is a silent mutation (GTA to GTC) as there would be no change in the amino acid valine showing in Fig 1a. Tumors from different patients exhibited heterozygous mutation in exon 11 where A changed to C at nucleotide position 676 as shown in Fig 1b. Seventeen out of 152 samples showed synonymous mutation where change from amino acid valine to valine (GTA to GTC) took place in exon 9 (would be designated as Polymorphism 1). Fifteen samples showed an A->C transition at nucleotide position 676 of exon 11 substituting of threonine by proline at codon 226 (would be designated as Polymorphism 2).

Statistical analysis

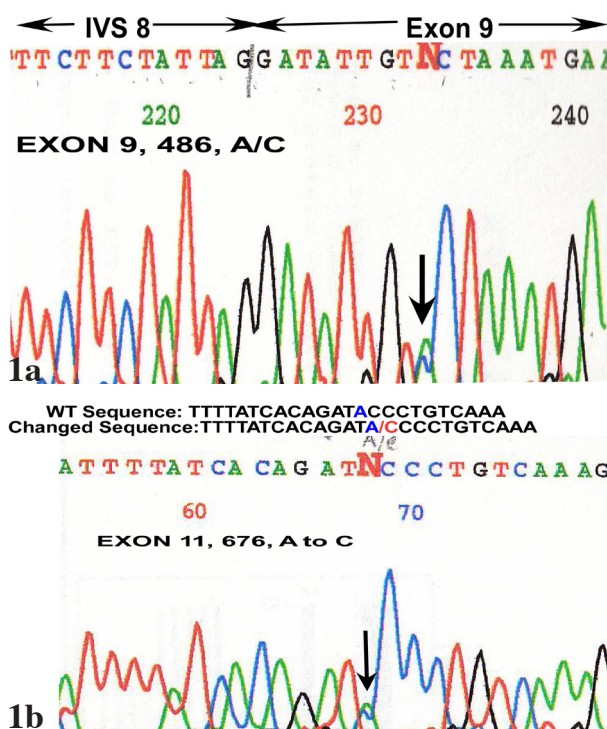
Descriptive data: The frequency distributions of patient characteristics are presented in Table 2. There was no significant difference in the frequency distributions of age, types, and stage (P=0.09, 0.1, 0.77). Seventeen patients (11.2%) were grouped in the age range of 21-30, 48 patients (31.6%) were grouped each in the age range of

Table 2. Patient Characteristics (n=152)

	No. of patients (%)	Chi-square	P
Age (yrs):	21 - 30	17 (11.2)	8.14
	31 - 40	48 (31.6)	
	41 - 50	48 (31.6)	
	51 - 60	32 (21.1)	
	61 - 70	7 (4.6)	
Histology:	Serous	96 (63.2)	6.16
	Mucinous	18 (11.8)	
	Endometrioid	19 (12.5)	
	Clear Cell Type	19 (12.5)	
Stage:	I	15 (9.9)	1.13
	II	44 (28.9)	
	III	40 (26.3)	
	IV	53 (34.9)	

Table 3. Association of Polymerase Beta Variants with Stage IV/Serous/Mucinous Types of Tumor Samples

Polymorphism	Pearson Chi-square	Like-lihood ratio	p	Odd ratio	95% Confidence Interval	
					Lower	Upper
Polymorphism 1/Serous/Stage IV	3.28	4.4	0.07	0.48	0.69	3.35
Polymorphism 2/Serous/Stage IV	3.28	4.4	0.07	2.08	0.3	14.55
Polymorphism 1/serous	2.58	2.66	0.1	0.28	0.06	1.37
Polymorphism 2/serous	2.58	2.66	0.1	3.56	0.73	17.32
Polymorphism 1/mucinous	4.03	5.56	0.04	1.3	1	1.7

**Figure 1. Two Types of Somatic Alterations in the Exon of Polb Gene of Ovarian Carcinomas are being Shown.**

a. depicts a heterozygous mutation at exon 9 at nucleotide position 486 where A has been changed to C; b. depicted a heterozygous mutation at exon 11 at nucleotide position 676 where A has been changed to C

31-40 and 41-50. Thirty two patients were grouped under the age range of 51-60 (21.1%) and seven patients were grouped under the age group of 61-70 (4.6%). Regarding histology, 96 samples belong to the serous type (63.2%), eighteen samples belong to mucinous type (11.8%), and 19 samples belong to the endometrioid and clear cell type each (12.5%). Fifteen (9.9%), 44 (28.9%), 40 (26.3%), and fifty three (34.9%) samples belong to stage I, II, III, and IV respectively.

Thirty two samples out of 152 samples showed two types of polymorphisms. Seventeen samples (53.1%) showed polymorphism 1 and fifteen samples (46.9%) showed polymorphism 2.

Association of clinical features with polymorphisms: The correlation study involving Polymorphism 1 and 4 types of tissue individually have shown a significant correlation between mucinous type with Pearson correlation value of 4.03 ($p=0.04$) (Table 3). The other types have insignificant correlation with both polymorphisms except serous type where there is a tendency of correlation (Pearson χ^2 value is 2.58) with likelihood ratio of 3.56 (CI: 0.73-17.32) for polymorphism 2 (Table 3). The association among polymorphism 2/serous/stage IV have shown Pearson χ^2 value of 3.28 ($p=0.07$) with likelihood ratio =4.42 and OR =2.08 (0.3-14.55). The other combinations did not show any significant correlation. Therefore, both these two results indicate that there is a tendency towards significant correlation as well as risk factor

Discussion

It is being reported that the E295K mutation in pol β which is a gastric cancer-associated variant interferes with base excision repair and induces cellular transformation (Lang et al., 2007). Another Leu22Pro tumor-associated variant of pol β is dRP lyase deficient (Dalal et al., 2008). Therefore, any kind of point mutation can change the conformation during the polymerase opening and thereby altering the DNA positioning in the active site (Yang et al., 2004). Polymorphism in exon 9 and exon 11 of genomic DNA and cDNA identified in this study may affect activity of protein.

The first mutation that is being reported here is the change of A to C at exon 9. As this mutation is located in the intron exon junction; there is a possibility of exon skipping during transcription which ultimately may have an effect on translated protein (Richard et al., 1993)

The second mutation is a missense mutation. There are several reports that missense mutation like A to C base substitution can be extremely pathogenic and led to different diseases such as dystrophin, cystic fibrosis, beta thalassaemia, hurler syndrome etc (Gomez et al., 1992). It has also been shown that missense and splice site mutations in hMSH2 and hMLH1 mismatch repair gene are pathogenic (Cravo et al., 2002). The aberrant transcripts of pol β may play a significant pathogenic role in ovarian carcinogenesis. A study consisting of 30 samples from China, have identified two hot spot region in pol β (454-466 and 648-670 nt) in esophageal squamous carcinoma (Zhao et al., 2005). The same group identified a 58 bp deletion (177-234 nt). An 87 bp deletion, deletion of

exon 11 in polymerase beta is common in lungs carcinoma may be results of alternative splicing (Bhattacharyya et al.,1999) which act as a dominant negative manner (Bhattacharyya et al.,1997). The altered form of polymerase beta have impaired activity (Bhattacharyya et al., 2001). In addition, several studies have shown that alternative RNA splicing and aberrant transcripts occur more frequently in human cancer cells than in normal tissues (Weinhouse et al., 1982).

Regarding the stages of patients at diagnosis, patients of stage IV have more mutation than stage II and III. Any kind of mutation in the exon 9 to exon 11 may adversely affect polymerizing activity and also nucleotide selection at the time of repair. It has been shown in the literature that almost 30% of the tumor samples studied so far have pol β mutation and these mutant proteins are being expressed in these tumor samples (Starcevic et al., 2004). Almost 44% of these tumors have single amino acid substitution. Therefore, studying pol β and its variants can lead to a deeper understanding of the association of mutation of polymerase beta with ovarian tumor.

In conclusion, a clear cut significant relationship has been established with mucinous type is associated with polymorphism 1, where 4 samples belonging to this type have this alteration. It was also noticed that level of tendencies of association between the polymorphisms/serous type/stage IV and polymorphism/serous, the previous ones is greater (p=0.07 rather than 0.1 in case of 2nd combination). However, this study involves only 152 samples and after validation by larger studies, may help to identify at-risk populations for primary cancer prevention. Therefore, larger sample size along with detailed environmental exposure data, and detailed clinical information of tumors are required.

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