RESEARCH COMMUNICATION

Association of a Newly Identified Variant of DNA Polymerase Beta (polβΔ$_{63-123, 208-304}$) with the Risk Factor of Ovarian Carcinoma in India

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Abstract

    <u>Background</u>: DNA polymerase is a single-copy gene that is considered to be part of the DNA repair machinery in mammalian cells. The encoded enzyme is a key to the base excision repair (BER) pathway. It is evident that pol beta has mutations in various cancer samples, but little is known about ovarian cancer. <u>Aim</u>: Identification of any variant form of polβ cDNA in ovarian carcinoma and determination of association between the polymorphism and ovarian cancer risk in Indian patients. We used 152 samples to isolate and perform RT-PCR and sequencing. <u>Results</u>: A variant of polymerase beta (deletion of exon 4-6 and 11-13, comprising of amino acid 63-123, and 208-304) is detected in heterozygous condition. The product size of this variant is 532 bp while wild type pol beta is 1 kb. Our study of association between the variant and the endometrioid type shows that it is a statistically significant factor for ovarian cancer [OR=31.9 (4.12-246.25) with p<0.001]. The association between variant and stage IV patients further indicated risk (χ^2 value of 29.7, and OR value 6.77 with 95% CI values 3.3-13.86). The correlation study also confirms the association data (Pearson correlation values for variant/stage IV and variant/endometrioid of 0.44 and 0.39). <u>Conclusion</u>: Individuals from this part of India with this type of variant may be at risk of stage IV, endometrioid type ovarian carcinoma.

Keywords: DNA polymerase beta - DNA repair - cDNA - polymorphism - RT-PCR - India

Introduction

Ovarian cancer is one of the most frequent gynecological cancers and the leading cause of death among malignancies in most women in the world (American Cancer Society, 2012) Almost 70% of the patients diagnosed with ovarian cancer will decease this year (American Cancer Society, 2012). The overall 5 year survival rate in most of the countries is generally more than 90% if the cancer is confined to ovary only (Buys et al., 2011). Nevertheless, when the disease is diagnosed at advanced stage, the survival rate decreases dramatically to less than 30% (Buys et al., 2011). The treatment includes surgery followed by chemotherapy. Initially the response rate is high (almost 76%), but when cancer relapses the response rate dramatically reduced to 20% (Kigawa et al., 1993; Kigawa et al., 1999). One of the reasons for the chemo-resistance is due to overexpression of the proteins of base excision repair (BER) pathway, particularly DNA polymerase beta (polβ). Hence, it is logical to sensitize the cancer cells by suppressing the polβ protein and then treat the patients with chemo; thereby the therapy would be effective (Liu et al., 2004; Bulgar et al., 2010). If the polβ protein is being mutated then the function will be altered. It has been noted that more than 30% of the tumor samples studied so far have polβ mutation (Starcevic et al., 2004; Trivedi et al., 2008). Pol β variants, K289M and I260M have functional phenotypes that could be related to the etiology of human cancer (Lang et al., 2003; Dalal et al., 2005). The Val246 variant was shown to mis-incorporate nucleotides through altered DNA positioning in the active site (Dalal et al., 2008). It was evidenced that expression of the E295K gastric carcinoma-associated pol beta variant (Lang et al., 2007) in mouse cells interferes with BER and induces sister chromatid exchanges and cellular transformation (Lang et al., 2007). An 87-bp deletion variant of polβ that has been found to be associated with breast, lung (Bhattacharyya and Banerjee 1997; Bhattacharyya et al., 1999a), colon, kidney, prostate cancer, has been shown to act as a dominant negative mutant (Bhattacharyya et al, 1999; Chen et al., 2000; Bhattacharyya et al, 2001). There is no report of any mutation of polβ in ovarian cancer so far.

Therefore, the objective of this study is to screen and identify any alteration of polβ in ovarian cancer and then to determine the association between the variant and the risk factor of the patients with ovarian cancer from India. The proposed study may have an implication in the chemotherapy of ovarian patients.

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Materials and Methods

Study subjects

The 152 ovarian cancer samples were included previously in a study that investigated the screening of genomic DNA for the presence of polβ mutation. The same samples were used for this study of screening of cDNA for the presence of polβ alteration. The informed consents were obtained from each patient. The non-metastatic tissues from the same patients were collected as normal. All the samples were given a code to protect the identity of the patients. Samples were snap frozen in liquid N2. Patients with the history of cancer in the family were excluded from this study. The design of the study was approved by the Ethics Review Committee of the Institute. Samples were characterized depending upon their age, stage, and type of tissue.

RNA preparation and RT-PCR

RNAs were isolated from tumor tissue samples and its normal counter part by using Trisure (BIOLINE USA Inc, MA) following the manufacturers instruction. Tumor samples were grinded in liquid nitrogen. One ml of Trisure was used per 100 mg of tissue sample. Then the samples were incubated at room temperature for 5 minutes and 0.2 ml chloroform was added per sample. Samples were shaken vigorously for 1 min and then incubated further for 5 min at room temperature. Samples were then centrifuged at 10000xg for 20 min at 4 °C. The upper aqueous phase was collected into separate tube and added 0.5 volume of iso-propanol to precipitate RNA. The RNA was precipitated by centrifuging at 10,000xg for 20 min at 4°C. The RNA was then washed with 70% ethanol, and then dried and dissolved in DEPC treated water and stored at -80°C. RNA isolated from ovarian tissues was reverse-transcribed for first strand cDNA synthesis using Velocity DNA polymerase (Bioline). For cDNA synthesis 1 μl of RNA (5 μg), 1 μl of Oligo(dT)$_{22}$ (0.5 μg/μl) , 1 μl of Hexamer (0.2 μg/ml) are mixed and incubated at 700 C for 10 mins then cooled at room temperature and add 4ul of 5xRT-PCR buffer, 1 μl of 200 mM dNTPs and 1μl of 100 U Reversed transcriptase enzyme in reaction tube. The reverse transcription reaction was set at 450C for 1 hr. To eliminate the possibility of genomic DNA contamination, reaction was also set up without reversed transcriptase enzyme.

Detection of mutation by PCR and sequencing

RT-PCR product were diluted ten times (100 ng of cDNA) to set up PCR reaction. For 50 μl of reaction,

reaction mixture was as follows: 5 μl of 10xPCR buffer (Fermentas), 1.5 μl of 25 mM MgCl$_2$, 1 μl 0.2 mM dNTPs, 1 U Pfu polymerase and 200 nM of each primer. PCR was done using polβ cDNA forward primer: 5'-ATATGAGCTCAGGAGACTCTCAACG-3', and polβ cDNA reverse Primer: 5'-ATATCTGCAGATGTCTTTTTCACT-3'. After heating denaturation at 95 °C for 2 min, 30 cycles was performed as follows: 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, extension at 72 ^{0}C for 90 sec, final extension was 5 mins at 72 ^{0}C.

Statistical data

The mutation percentage, average age of patients at diagnosis for the polymorphism was estimated. 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 polymorphism 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

Descriptive data

The histopathological data showed that out of 96 serous sample (63.2%), 43 (65.15%) have pol beta alteration. Nineteen samples each belong to Endometrioid, and Clear Cell Type (12.5%). Eighteen samples from Endometrioid (27.27%) and only 2 samples (3%) from clear cell type have polβ alteration. Eighteen samples were of Mucinous type (11.8%) out of which 3 samples (4.5%) exhibited polβ alteration (Table 1).

Thirty three samples each belong to stage I & II (21.7%). Thirty four samples belong to stage III (22.4%), whereas 70 samples belong to stage IV (46.1%) (Table 1). Eight samples belonging to stage II and III exhibited alteration (12.12%). Only three samples of stage I (4.54%) showed polβ alteration. Forty seven stage IV samples (71.2%) showed the polβ alteration (Table 1). The mean age of the samples was 43.24.

PCR and sequencing

PCR analysis showed an extra band of 515 bp along with wild type 1 kb band (Figure 1A) .This band was excised from the gel and sequenced directly. Sequence

Table 1. Relationship Between Clinical Features and polβ Mutations (n=66)

Correlation analyses		No. of patients with polb mutation (%)	Pearson chi-square value	Likelihood Ratio	OR (95%CI) in multivariate analysis	P
Histology:	Variant/Serous	43 (65.15)	0.20	0.20	1.16 (0.60-02.26)	0.650
	Variant/Mucinous	3 (04.50)	5.95	6.57	0.22 (0.06-00.81)	0.015
	Variant/Endometrioid	18 (27.27)	23.27	26.30	31.87 (4.12-246.3)	<0.001
	Variant/Clear Cell Type	2 (03.00)	9.56	11.10	0.12 (0.02-00.57)	0.000
Stage:	Variant/I	3 (04.54)	3.71	4.00	0.29 (0.08-01.08)	0.050
	Variant/II	8 (12.12)	6.31	6.63	0.33 (0.14-00.80)	0.012
	Variant/III	8 (12.12)	7.05	7.42	0.32 (0.13-00.76)	0.008
	Variant/IV	47 (71.21)	100.72	30.65	6.77 (3.30-13.86)	<0.001
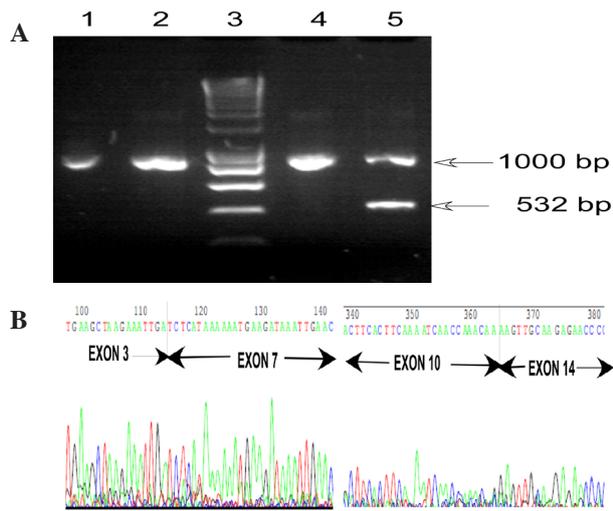
Figure 1. Variant of polβ Coding Sequence in Ovarian Carcinomas is Shown Here. A. RT-PCR products from tumor samples along with the corresponding normal tissue were separated on agarose gel. Lane 1,2 denotes RT-PCR product from normal & Tumor tissue#4; Lane 3 denotes DNA molecular weight marker; Lane 4, 5 depicts RT-PCR products from Normal & tumor sample #6. B. The 532 bp RT-PCR product was sequenced. The sequence of this product depicting the loss of exon 4 to 6 and exon 11 to 13.

analysis showed deletion of exon 4-6 and 11-13 (Figure 1B).

Statistical data analysis

Association study between the variant and the types of tissues individually revealed that although the Pearson chi-square value for variant/ mucinous, variant/ endometrioid, and variant/clear cell type is 5.95, 23.27, and 9.56 respectively with p value within statistically significant range (0.01, 0, and 0.002), but the OR value of only variant/endometrioid exceeded >1.0 [OR=31.87 (4.12-246.25) with p=0]. Therefore, the association between the polβ variant and the endometrioid patients from India is statistically significant. On the other hand, the p value of variant/serous is not statistically significant though the OR value was slightly >1 (OR=1.16).

Association study between the variant and the different stages revealed that although the p value for all the 4 stages are within the statistically significant range but the OR value of variant/stage IV is greater than 1 (6.77, 3.3-13.86) alone. The Pearson chi square value is 29.72 which confirm the previous result. This suggest that the stage IV Indian patients are at higher risk for predisposed of ovarian cancer if they have been identified with this variant for polβ.

The correlation study between any of the four stages or types of tissues with the variant showed the Pearson correlation value of variant/stage IV and variant/ endometrioid are 0.44 and 0.39 which were positively. The correlation is significant at the 0.01 level (1-tailed).

Discussion

There are several reports of association between polymorphisms of different DNA repair genes (e.g. XRCC1, p53) with the cancer risk factors from different countries including India (Huang et al., 2011; Pan et al., 2011; Zhao et al., 2011; Vijayraman et al., 2012; Wang & Zheng, 2012). But no work has been reported about the association of polβ alteration and the risk for ovarian cancer. Recently, we have reported (Khanra et al., 2012) the association between the polβ polymorphism and the ovarian cancer risk in genomic DNA from Indian patients. In this paper, we are reporting the association between polβ cDNA polymorphism and the ovarian cancer risk in Indian patients. DNA polymerase is a 1kb gene with 14 exons (Chyan et al., 1994). Exon 1 to 4 have single stranded DNA binding activity and dRP lyase activity, 5 to 7 have double strand binding activity, exon 8-11 carrying nucleotidyl transferase activity and 12 to 14 have dNTP selection activity (Idriss et al., 2002). The present newly identified pol beta variant lacks exon 4 to 6 and 11 to13. Therefore, the variant may lose single strand, double strand binding activity, and dNTP selection activity. Amino acid 63-123 and 208-304 is missing in the new variant. The deletion may introduce a stop codon at aa 71 causing a truncated protein. If this is true then the double strand binding domain, nucleotidyl transferase domain, and dNTP selection domain would not be effective. Only the dRP lyase activity and the single strand activity would be retained. As the alteration is heterozygous in nature, there is a possibility of dominant negative action of this variant on wild type polβ as shown previously (Bhattacharyya & Banerjee, 1997).

In 30% cases of cancer, pol beta has mutation (Chen et al 2002; Starcevic et al 2004). Leu22Pro tumor-associated variant of polβ is dRP lyase deficient (Dalal et al., 2008). It is being reported that the E295K mutation in polβ which is a gastric cancer-associated variant interferes with base excision repair and induces cellular transformation (Lang et al., 2007). Therefore, any kind of point mutation or large deletion can change the conformation during the polymerase opening and thereby altering the DNA positioning in the active site (Yang et al., 2004). Deletion of exon 4-6 and exon 11-13 of cDNA of pol beta may be non functional protein or it may hinder the function of wild type pol beta. The hindrance of the function of wild-type polβ may sensitize the cancer cells to the alkylating agent and thus may the target of an effective therapy.

In conclusion, our findings indicate that the association of the variant with ovarian cancer in the present study may indicate that the patients at stage IV and with endometrioid types of tissue may be predisposed to ovarian cancer. 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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