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Original Article

Identification of mitochondrial DNA profiling (COX – 2) in ovarian cancer patients – A population-based study in South India

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ABSTRACT: Objectives: Ovarian cancer (OC) is a most dangerous gynecological cancer affecting women aged mostly in 50s and above due to its poor prognosis. mtDNA has been emerging as a prime hotspot candidate for the progression of OC. Thus, the objective of the present study was to investigate polymorphisms in COX – 2 gene in mtDNA by using PCR – RFLP. **Methods**: In the present study, detailed questionnaire and consent forms have been obtained from the OC patients and the age – matched controls. Blood samples from OC patients (n = 72) were collected from oncological clinics, and by population-based survey in South India. Control subjects (n=72) who had no history of tumors were selected and they were matched for age, sex and race. Peripheral blood was collected to detect polymorphism in the COX – 2 gene using PCR – RFLP. **Result**: In the present study, we found that OC patients with COX – 2 CC homozygous genotype showed higher risk for OC progression, whereas, the GG genotype in controls revealed its protection against the OC risk. **Conclusion**: In conclusion, our results suggests that, COX – 2 CC genotype may contribute to the development of OC pathogenesis. Though genetic polymorphism investigation was very limited to modulate the OC risks, the outcome of this study may help in future genotypic analysis. Thus, in future more genetic studies are warranted to prove that genotypic variation, mutations in COX – 2 would be a prime factor in Ovarian Carcinogenesis, and it can be used as candidate biomarker in treatment strategies.

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INTRODUCTION

Ovarian cancer (OC) is the most pathogenic form with invasion to the surrounding stroma, distant metastasis, with anticancer drug resistance and angiogenesis in females [1]. OC symptoms are commonly elusive and appear only in the advanced metastatic stage of the disease. To name a few abdominal bloating and swelling, pelvic pain and frequent urination are most frequently observed symptoms in OC [2]. OC has been ranked in the seventh position for cancer mortality among the women throughout the world [3]. Acc ording to the age – standardized incidence rate of OC registries in India fluctuates from 0.9 to 8.0 per 100,000 populations were observed [4]. Genetic studies are the best tool to implicate certain genomic regions or a particular gene by associating a link between the mutations in the gene and the progression in OC. There is an instantaneous need for the development of novel genetic assays and approaches to apprehend the mechanism behind the tumorigenesis of OC. In a recent review, it was studied that mutations in the mtDNA and transmission of this mutated mtDNA through maternal lineages is the reason for the gynaecological cancers like OC were running in the family [5].

Thus, mutations in the mtDNA and its associated genes are a prime risk for women with OC. Cyclooxygenase (COX) alias prostaglandin – endoperoxide synthase consist of two isozymes like COX – 1 and COX – 2. COX – 2 is the major enzyme for the production of prostaglandins, which is an important molecule in OC development. It has been reported that COX – 2 is overexpressed in OC cells [2].

However, COX – 2 is highly associated with chemo-resistant and poor prognosis of the OC, but the mechanism of COX – 2 in increasing the risk of OC progression remains poorly understood. Thus, in this study, we aim to observe for any possible association between COX – 2 polymorphisms with the risk of developing OC.

METHODOLOGY

Study Site

We collected the data, which covered the geographical areas including Coimbatore, Nilgiri, Dharmapuri Chennai and Kozhikode districts from South India.

Study Population

A total of (n = 72) OC patients were recruited from various oncology clinics, and through population-based survey from South India. Age, sex and race matched equal number of controls (n = 72) were selected for the present study. In our study, the following primary inclusion criteria were included as a part of the analysis: age, lifestyle (alcohol consumption, smoking and family history with cancer). All patients who were terminally ill, OC associated with other disease, autoimmune diseases, immunology and genetic disease were excluded from the study. Informed consent form was collected and information concerning age, marital status and past history were obtained from each patient through interview and archive of the patients. The Institutional Review Board approval from Avinashilingam Institute for Home Science and Higher Education for Women - Coimbatore (Ref No. AUW/IHEC-17-18/ZOO/FHP-10), Balaji Medical College - Chennai were obtained for our study. The Helsinki (1966) declaration was followed throughout the study.

mtDNA Isolation

The post nuclear supernatant fraction will be collected in a centrifuge tube and centrifuged at 15000g/ 4˚C for 30 min to sediment the mitochondrial pellet. This pellet will be washed thrice with low salt buffer (TKM1) ensuring complete elimination of lysed erythrocytes. The mitochondrial pellet will then be transferred to a 2ml eppendorf tube, suspended in 480µl of high salt buffer (TKM2: Tris HCl 10mM pH 7.6, 10mM KCl, 10mM MgCl2, 0.4M NaCl and 2mM EDTA). Then 75 µl of 10% SDS will be added and incubated at 55˚C in heating block for complete denaturation and solubilization of protein. Salting out of protein will be done by adding 200µl of 6M NaCl and centrifugation at 11300 g for 20 min in microcentrifuge. The supernatant will be transferred to a 2mL tube and twice the volume of 100% ethanol will be added for complete precipitation of mtDNA pellet. The mtDNA pellet will be washed with 70% ethanol, dried and finally dissolved in 200 µl of sterile water.

Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP)

The isolated genomic DNA was then amplified using Polymerase chain reaction by Applied Biosystem Verti PCR. The primer for COX - 2 gene amplification was designed based on Primer 3 and UCSC in silico PCR analyzing bioinformatics tool. The primer for amplifying the polymorphism region was FP: 5′-GTGAGCTAGCATGCTCGCCCGCGCCCTGC-3′ and RP: 5′-CTGAGAATTCCTACAGTTCAGTCGAACGTTC-3′. A 0.5µl of DNA template of each sample was added to the PCR reaction solution (Forward Primer: 0.5 µl, Reverse Primer: 0.5 µl, Sigma-Aldrich Ready Mix: 4.5 µl, deionized Water: 19 µl). The PCR condition was 2 min at 94 ℃, followed by 35 cycles of 30 s at 94 ℃, 30 s at 60 ℃, and 30 s at 72 ℃, and with a final extension at 72 ℃ for 7 min. The amplified PCR products were electrophoresed on 1.5% agarose gel containing EtBr and viewed under Gel documentation UV transilluminator.

Further, the PCR products were digested by AciI restriction enzyme and the reaction mix was incubated at 37◦C for 8 to 12h. the digestion products were visualized on 4% Metaphor agarose gel containing EtBr.

DNA Sequencing of Amplicons

All the PCR products of samples were subjected for DNA sequencing. For the reading accuracy, all the PCR products were sequenced with forward and reverse primers. PCR amplified product will be analyzed and the chromatograms were checked by DNA baser version and aligned by BLAST [www.ncbi.nlm.nih.gov/blast] for identification of the products.

Data Analysis

Statistical analysis was performed using SPSS 13.0 Windows Software. All values were considered to be significant at $p < 0.05$ throughout the study.

RESULT

In the present study, 72 women were diagnosed with OC and 72 age – matched controls were recruited who met the study inclusion criteria such as age, lifestyle (alcohol consumption, smoking and family history with cancer. OC patients and controls were grouped based on their age, as group I ≤55 and group II ≥56, in which the group I consist of (n = 29) and group II (n = 43) in both the OC patients and control. Table 1 shows, the general characteristics such as (educational qualification, food habit, marital status, smoking and family history with previous tumors) of OC patients and controls. From the present study it has been observed that OC patients were having high number of family history with gynecological cancer when compared to their controls.

Table 2 represents the frequencies of COX – 2 genotype distribution in OC patients and controls. The individuals who had COX – 2 CC genotype were at a high risk for OC progression and showed significant difference ($p < 0.0004$) when compared to other genotypes.

The control group had genotype GG, which depicts that GG genotype shows protective role from OC. COX – 2 gene is the important gene in mtDNA, which is the precursor for the production of prostaglandins for normal process of ovaries.

Thus, from the present study, we suggest that mutations in COX – 2 with CC genotype will disturb the levels of prostaglandins causing OC development.

Table 1: General Characteristics of OC patients and Control

General Characteristics		Groups based on age Group I ≤55* and Group II - ≥56* and Percentage (%)						
		Group I			Group II			Total
		OC	Control	Percentage (%)	OC	Control	Percentage (%)	
Educational Qualification	Literate	18	18	25%	15	15	20.8%	33
	Illiterate	08	08	11.1%	30	30	41.6%	38
Food Habits	Vegetarian	05	05	6.94%	10	10	13.8%	15
	Non vegetarian	22	22	30.5%	35	35	48.6%	57
Marital Status	Married	24	24	33.3%	29	29	40.2%	53
	Unmarried	00	00	00	00	00	00	00
	(W/S/D)*	03	03	4.16%	16	16	22.2%	19
Smoking Status	Smoker	02	02	2.77%	01	01	1.38%	03
	Non-Smoker	10	10	13.8%	18	18	25%	28
	Passive Smoker	15	15	20.8%	24	24	33.3%	39
Family History	Yes	22	22	30.5%	31	31	43.0%	53
	No	07	07	9.72%	12	12	16.6%	19

OC - Ovarian Cancer, W/S/D – Widow/ Separate/ Divorced

Table 2: Frequencies of COX – 2 genotype distribution in OC patients and controls

COX – 2 genotype	Ovarian cancer (n = 72)		Control (n = 72)		P – value
	Group – I	Group - II	Group – I	Group - II	
GG	04 (5.55%)	12 (16.6%)	09 (12.5%)	10 (13.8%)	0.126
CC	12 (16.6%)	05 (6.94%)	02 (2.77%)	04 (5.55%)	**0.0004***
CG	07 (9.72%)	25 (34.7%)	08 (11.1%)	07 (9.72%)	0.427

***** Values significant at $p < 0.05$ level

DISCUSSION

OC is the second most frequent women associated cancer throughout the world. Most of the OC patients are diagnosed at the later stage of metastasis due to improper diagnosis. OC is classified into three different categories based on its origin such as epithelial, stromal and germ cell tumours. Among these three types, epithelial OC is the most frequently observed, almost 1 in 70 women with OC [6]. In India, the incidence rate of OC is mainly dependent on the age and varies from 5.4 to 8.0 per 100,000 populations [7]. Thus, novel techniques are needed to detect OC in their early stages.

It has been reported that various characteristics factor such as age, sex, socio – economic status, educational qualification, past family history with cancer, smoking an update about these information's will be very useful to detect the OC in the early stages of the disease [8]. Similar to the previous study, we found that frequent characteristics found in the OC patients are illiteracy rate, non – vegetarian food intake, passive smoking and high number with a family history of cancer [9 – 11].

In present study, the OC patients and controls were differentiated into two groups as Group I ≤55 (n = 29) and Group II ≥56 (n = 43).

COX – 2 is one of the important genes which regulates the functions of mtDNA. In review, we discussed that mutations in the mtDNA is the main reason of gynaecological cancers running in families, because mtDNA is only transferred from mother to her offspring 5. In a previous study, it was observed that individuals with COX – 2 -756 C allele are at higher risk for the progression of OC. Similar to the previous study, in our study we found that OC patients with COX – 2 CC genotype were at higher risk for OC progression. While the control group individuals revealed that, the GG genotype protects from OC risk. This could be due to the over – expression of the COX – 2 gene in the mtDNA. Several studies have reported that over – expressed COX – 2 in cancer cells can induce the cancer growth by promoting angiogenesis, inhibit apoptosis, enable tumour vascular invasion and enhance the cancer cell survival from chemotherapeutic drugs [12 – 14].

In conclusion, the present study indicates that gene polymorphism of COX – 2 CC genotype in OC patients have been observed in the South Indian population. Hence, more novel molecular genetic assays have to be carried out to analyze the whole mtDNA associated gene for risk assessment of OC patients. However, the small sample size stands as a limitation to end up into judgement regarding the results obtained and further studies are needed to confirm our findings.

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