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ORIGINAL ARTICLE

Genomic DNA based characterization of *C-MYC* gene mutations in ovarian carcinoma patients.

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ABSTRACT
C-MYC has been a central role in orchestrating biological functions such as apoptosis, cell proliferation, differentiation and cell metabolism. The present study provides the prediction method for the early detection of ovarian cancer on the basis of DNA based PCR method. The study was intended to investigate the involvement of exon 3 mutation in ovarian cancer and its association with positive family history of cancer. The cases and control group recruited for the study had a positive history of cancer. Sanger method of DNA sequencing was deducted for the small stretch of 165 nucleotide sequence of C-MYC gene exon 3 that provides a large open reading, linked to be associated in mRNA stability and translational control in cancer. The results were analyzed with the bioinformatics approach and indicate that C-MYC exon 3 plays a crucial role in ovarian cancer and could be used as the prediction method for the screening at the earliest stage.
Keywords: Apoptosis, *C-MYC* gene, gene mutation, malignancy, ovarian cancer

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INTRODUCTION

Proto-oncogenes are the normal genes involved in cell growth and proliferation, which stimulate the cell proliferation, inhibit the cell differentiation and prevent the apoptosis through a cascade of biochemical events within a cell [1]. Though an advantageous role of the proto-oncogenes was not apparent initially and was considered to be non-expressed or "silent" until being "switched on". Mutations in proto-oncogenes turn them into oncogenes that lead to a protein hyperactivity, increased level of expression and/or loss of regulation [2]. The proto-oncogenes transform into oncogenes by diverse processes that include point and insertion mutations, base substitution mutations, retroviral integration or transduction, chromosomal translocation, gene amplification, and/or protein–protein interactions that ultimately lead to an uncontrolled cell growth and proliferation and act in concert to cause the cell to be cancerous [3]. Vast number of proto-oncogene studies has explained an immense oncogenic activity in the progression of various types of cancers. Among women ovarian cancer is the most deadly gynecological malignancy and the second leading cause of cancer-related death in women all over the world [4,5]. Ovarian cancer being a complex heterogeneous neoplasmic group accounts for 2.5% of the female cancer cases [6]. The ovarian cancer patients diagnosed with advanced stage *i.e.* metastasis of the disease that spread all through the abdominal cavity to penetrate nearby tissues and/or organs [7]. Due to the inadequate molecular diagnostic markers and scarce therapeutic targets restricts the understanding of ovarian cancer causes and specificity. Therefore, a few known diagnostic markers are the main reasons behind the lack of success in treating this fatal disease [8,9]. Approximately 20 to 25% of ovarian cancer females have been seen to have a hereditary predisposition to develop ovarian cancer. Some of the genes discovered to be linked to ovarian cancer are: *BRACA1*, *BRACA2*, *MYC*, *p53* etc.[10]. The *MYC* family of proto-oncogenes constitutes *C-MYC*, *N-MYC* and *L-MYC* and associated in few of the major prime cellular

processes, such as proliferation, differentiation, apoptosis and neoplastic phenomena. In >50% of human cancers the *MYC* family oncogenes are deregulated, which leads to poor prognosis and adverse patient survival [11]. In the MYC family, *C-MYC* gene has been widely studied in human ovarian carcinomas from tissue samples [12]. *C-MYC* gene is the first member of *MYC* family genes, present in chromosome number 8 at q2l in humans. It was first reported in colonic carcinoma in which 10-fold elevation in expression of the gene shown in murine, while in human colon tumor the overexpression of the c-myc gene was detected [13]. The *C-MYC* gene is 14518 bp long and encodes a nuclear phosphoprotein that forms a heterodimer with the related transcription factor MAX [14]. This complex binds to the [CAC(G/A)TG] E box DNA core sequence and regulates the transcription of specific target genes [15]. Amplification of this gene is frequently observed in other numerous human cancers [16]. Translocations involving this gene are associated with Burkitt lymphoma and multiple myeloma in human patients. There is evidence to show that translation initiates both from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site, resulting in the production of two isoforms with distinct N-termini [15,17]. As *MYC* genes are located within the nucleus, it has proved impractical for decade to target the nuclear *MYC* genes with some specific monoclonal antibodies for the treatment and screening of ovarian cancer [18]. *C-MYC* comprises of three exons that span over 4 kb including exon 3 encoding most *MYC* protein. Exon 3 of *C-MYC* provides the regions (sequences) that have some hotspots for mutations [19].

Therefore, the present study investigated the amplification of small region of exon 3 because it was difficult to sequence the whole exon 3 (9963-12518 bases) of the particular gene since the exon 3 of *C-MYC* bears total of 2555 bases. Due to the financial constraint, the present pilot study was designed to investigate the *C-MYC* gene which will help in the early prediction and screening marker for the ovarian cancer. The current pioneer study involved the genomic DNA amplification from blood samples to analyze the mutation in *C-MYC* exon 3 gene in ovarian cancer.

MATERIALS AND METHODS

Permission

The study was approved by the Doctoral Advisory Committee, Institutional Research Committee and Institutional Ethics Committee of Adesh University, Bathinda. Permission was granted by the competent authority of Sher-e-Kashmir Institute of Medical Sciences, Soura, Srinagar, for 50 blood samples from ovarian cancer patients.

Inclusion criteria for the study were: (a) patients with complete histological documentation; (b) known surgery information; (c) both operated and non-operated; (d) patients without any cardiac complications; (e) eligible for neo-adjuvant treatment or inoperable; (f) metastatic at presentation; (g) mentally fit to give informed consent.

Same in-person information was collected from control group excluding ovarian cancer stage, treatment, surgical information, histological type, known surgery information, metastatic at presentation etc.

Blood specimens

1 ml of blood was drawn from fifty ovarian carcinoma patients and fifty control group in EDTA-vials. The in-patient information collected includes demographics, biochemical investigations, familial predisposition, ovarian cancer stage, treatment, surgical information, histological type, etc. after receiving written informed consent.

Genomic DNA extraction and PCR assays

Genomic DNA was isolated from peripheral blood leukocytes by using CTAB method with some modifications [20]. The purity and concentration of the extracted DNA was determined using Cary 60 UV-Visible Spectrophotometer (Agilent Technologies). The extracted DNA was diluted to a final stock concentration of 50μg/μl with sterilized MilliQ water and stored at −20 °C for further analysis. PCR reaction was performed in BIO-RAD T100 Thermo cycler. The conserved primers used for the amplification of *C-MYC*-EXON 3 were selected from the previous studies [21] with primer sequences were: Forward 5'CTCGGAAGGACTATCCTGCTGCCAA3' and Reverse 5'GGCGCTCCAAGACG TTGTGTGTTCG3'. The sensitivity of the above primers was checked by primer stat, primer 3 and *in-Silico* PCR software to search a sequence database with a pair of PCR primers by using an indexing strategy for fast performance. The primers resulted in the amplification of sequences of a length of 165 bases for *C-MYC* gene. The primers were synthesized from Biokart Private Limited, Bangalore, India. Amplification was done by T100™ Thermal Cycler BIORAD. The ready-to-use PCR master mix from BIORAD was used. Thermal Cycler settings for initial denaturation at 94ºC for 5 mins, followed by 34 cycles of denaturation at 94ºC for 1 min , 61ºC for 1 min. to anneal the primers, extension at72ºC for 2 min. and 72ºC for 8 min. for final chain elongation.

Agarose gel electrophoresis

After the PCR amplification, the 8µl of each reaction product was electrophoresed (8V/cm) with 100 bp molecular marker (BIORAD) directly on 1.2% of agarose gel stained with 10µl of ethidium bromide. After electrophoresis, the gels were visualized under Gel Doc™ EZ imager (BIO-RAD).

Strategy for Sanger sequencing

Out of 50 cases and 50 controls, only six cases and four controls were selected on the basis of some characteristics and desirable parameters including band expression pattern and family history. The desired base pair bands of the *C-MYC* genes were cut and eluted from an agarose gel and sent for customized sequencing from Biokart Private Limited, Bangalore.

Bioinformatics techniques in mutation analysis

After sequencing, bioinformatics approach was used for alignment to the reference sequence (hg19) (Accession number: >NG_007161) for mutational analysis in control and cases through online BioEdit Sequence Alignment software (version 7.2).

After BioEdit sequence alignment software, Mutation Taster software version 2.0 was used to evaluate DNA sequence variants for their disease-causing potential. The software performed a battery of *in-silico* tests to estimate the impact of the variant on the *C-MYC* gene product/ protein.

Statistical analysis:

All the necessary analyses were done using MS-excel 2007. The means or frequencies of patient demographics were interpreted among subgroups. Pearson's coefficient correlation was used for correlation assay. Chi-square test was used for categorical variables. The differences between experimental and control groups were analyzed by using two-tailed unpaired t test. Two-sided $p \leq 0.5$ were considered statistically significant.

RESULTS AND DISCUSSION

The present study recruited fifty ovarian cancer patients registered at Sher-e-Kashmir Institute of Medical Sciences, Soura, Srinagar. According to the age interval, the ovarian cancer patients were divided into five groups (15-29, 30-44, 45-59, 60-74, and 75^{+}). Among them most of the affected age group of the patients was 44-59 years with mean and standard deviation as 47.56 and ±15.07 respectively (Table 1). This outcome is in corroboration with the study led at tertiary care hospital Ghana in which authors reported 49 years as the mean age of ovarian cancer [9], whereas Murthy *et al*. [6] concluded that the incidence of ovarian cancer begins to emerge from 35 years of age and maximum at the age between 55-64 years. In the current study two patients with age 15 and 16 years also reported ovarian cancer at a very young age. The cases had their menarche at very early age and had a family history of ovarian and breast cancer in primary relatives. The results were similar to the data presented by Reid *et al*. [22] that reported ovarian cancer with ≤ 14 and ≤15 years of age with positive history of cancers in the primary relatives. While Gong *et al*. [23] conducted meta-analysis and reported that menarcheal age was inversely associated with the risk of ovarian cancer and stated that more number of studies are required to differentiate the results by different histotype and cancer grading in ovarian cancer. In general population awareness of the ovarian cancer risks and symptoms amongst females is very low. Additionally, the ovarian cancer presenting symptoms overlaps with those of more common gastrointestinal and abdominal diseases. Therefore, present study reported the comparison of symptoms with cases and control. The abdominal distention, ascities and leg edema presented to be highly significantly ($p<0.01$) reported by the ovarian cancer patients (Table 2), while pelvic pain presented to be significant in comparison to control group whereas vaginal bleeding, urinary symptoms and gastro intestinal symptoms were not significant (Table 2). Similarly, Gajjar *et al*. [24] reported the highest scored symptoms were abdominal swelling followed by abdominal bloating and pelvic pain. Further, in the current study mean value of *CA125*U/ml was included with standard deviation (Table 3). The analysis of *CA125* has been widely used in the ovarian cancer follow up, but at present some researchers reported that serum *CA125* has no significance value for the monitoring of the recurrence for postoperative patients with epithelial ovarian cancer, but in another clinical follow-up found that when the serum *CA125* value was <35 U/ml, postoperative patients of epithelial ovarian carcinoma had already showed recurrent lesions in some ecological and imaging examinations and biopsy, therefore, given the patients timely treatment, the prognosis were improved [25]. Previously, Wilder *et al*. [26] reported eleven epithelial ovarian cancer patients which displayed recurrence with radiological or histological analysis and their serum *CA125* constantly enhanced three times from the normal range within one to 3 months. In the current study 14 cases and 03 controls showed the familial predisposition of various cancers including ovarian cancer (Table 3). As reported earlier about 23% of ovarian carcinomas have been associated with hereditary conditions [27].

Among gynecological malignancies the epithelial ovarian cancer is a leading cause of morbidity and mortality. In the current study majority of the cases (48.81%) had malignant serous epithelial tumors followed by 34.90% mucinous epithelial tumors, only 4.65% endometroid carcinoma and 2.33% ovarian clear cell carcinoma (Table 4). All the subtypes of epithelial ovarian cancer differ in their molecular signatures, site of origin, response to therapy and prognosis but all the subtypes are still receiving front-line platinum-based therapy [28]. Further, in current study, only seven cases belong to non-epithelial tumor type (Table 5). Nonepithelial cancers of the ovary are very rare and account for 10% only, which include malignancies of sex cord-stromal tumor, germ cell tumor, metastatic carcinomas to the ovary and exceptionally uncommon ovarian cancers, such as lipoid and sarcomas cell tumors [29].

The *C-MYC* gene bands were expressed at 165 base pair both in cases and controls after PCR amplification (Fig.1). Out of fifty cases and fifty controls, only five cases and three controls of *C-MYC* gene were selected for the gene mutational analysis with online BioEdit Sequence Alignment software (version 7.2) after sequencing. The pilot study conducted on the basis of characteristic band expression pattern in the agarose gel electrophoresis and specific parameter of family history of cancers upto tertiary relatives (Fig.1 & Fig. 2). Similar studies were reported by Ismaeel and Ablahad, [30] in which various types of mutations were detected in *BRAC1* and *BRACA* 2 genes by the use of various bioinformatics approach. The cases selected belonged to stage I, II, III and stage IV respectively. Out of five cases, two cases belonged to stage IV. Stage I case belonged to 25 years age group with 65 U/ml as the level of *CA125* U/ml. The stage II case belonged to 35 years age group with 160U/ml as the level of *CA125 U*/ml. Stage III case belonged to 40 years age group with 405 U/ml as the level of *CA125* U/ml marker. While as one of the two stage 1Vcase was 45 years with 995 U/ml as the level of *CA125U*/ml. The other stage IV case belonged to 60 year age group with 1001 U/ml as *CA125 U*/ml of marker respectively. Additionally, the other case belonging to stage IV was diagnosed with mucinous epithelial ovarian cancer group. All the cases selected for the mutational study were diagnosed with serous epithelial ovarian cancer and had a positive familial predisposition of cancers. In the current study, the control group was of the same age to remove any inconsistency. Two controls selected had positive familial history while another one with no familial history. Similarly, Tsaousis *et al.* [31] analyzed all the genes in the panel and reported mutation, pathogenic variant in 264 of 1197 total individuals (cases and controls) with positive family history of various types of cancers. In our study, the control with no family history of cancer reported no mutation in the particular DNA stretch (10176-10341 bp) within *C-MYC* gene exon 3 (9963-12518 bases). The proto-oncogene *C-MYC* regulates a large set of genes which display significant roles in growth regulation and metabolism and dysregulated in more than 70% of human cancers. In the present study transversion, transition and deletion mutation was observed in *C-MYC* gene in cases while no insertion mutation was observed (Table 7). Whereas transversion and transition was the only type of mutations observed in control group (Table 8). The transverse mutation was the most prevalent type of mutation observed in both cases and control. All the cases show mutation at the following loci of *C-MYC* gene located on chromosome 8. The 100% mutation was observed at 10303-10308, 10311, 10313-10315, 10317-10320, 10322-10325, 10330-10334, 10336, 10339 and 10340 locus of *C-MYC* chromosome 8 respectively (Table 6). Minimum mutation i.e., 20% was observed at 10267 and 10274 loci. Moreover, in the two controls, 66.67% was the maximum mutation observed with 33.33% minimum mutation while as some loci showed no mutation at all. The total mutations in 165 bases of exon 3 of *C-MYC* gene were 188 in cases, while as 56 were investigated in controls. The results showed the significant difference among cases and controls. The statistical analysis showed the significant difference with p-value < 0.001 (Table 6). Osman *et al.* [32] compiled review article on *BRCA1* and *BRCA2* mutation in ovarian cancers and reported that mutation in the gene causes high-grade serous adenocarcinomas in ovarian cancer. Several ***BRCA1***-mutated tumors support a *C-MYC* overexpression, mutant ***p53*** gene and overexpression of epidermal growth factor receptor. Similarly, Li *et al.* [5] reported that in maximum cases the somatic and germline mutations were found in TP53 (86%) and *BRCA1/2* (21%) genes, respectively. Previously reported that *C-MYC* gene family in different cancers known to be controlled at the transcriptional and post-transcriptional levels [33]. Another study revealed that *C-MYC* expression was significantly enhanced in a cisplatin-resistant cell line [34]. In addition, Tashiro *et al.* [35] determined that *MYC* gene was overexpressed in ovarian tumor tissues approximately 37.3% and serous adenocarcinoma expressed in 63.5% tissues, respectively. Researchers demonstrated that *C-MYC* gene dramatically overexpressed in stage III of ovarian cancer as compared to the other stages of ovarian cancer. On the contrary, in the present study the expression of *C-MYC* in blood was under expressed as compared to the control samples.

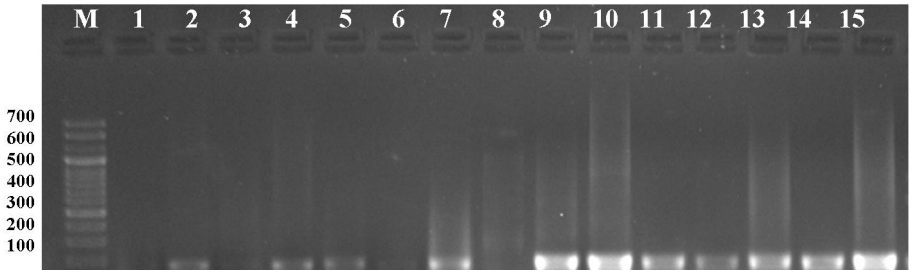

Figure 1. *C-MYC* exon 3 gene amplification Lane 1-8 ovarian cancer patient samples of 165 bp; Lane 9-15 control group; Lane M: 100 bp molecular weight marker.

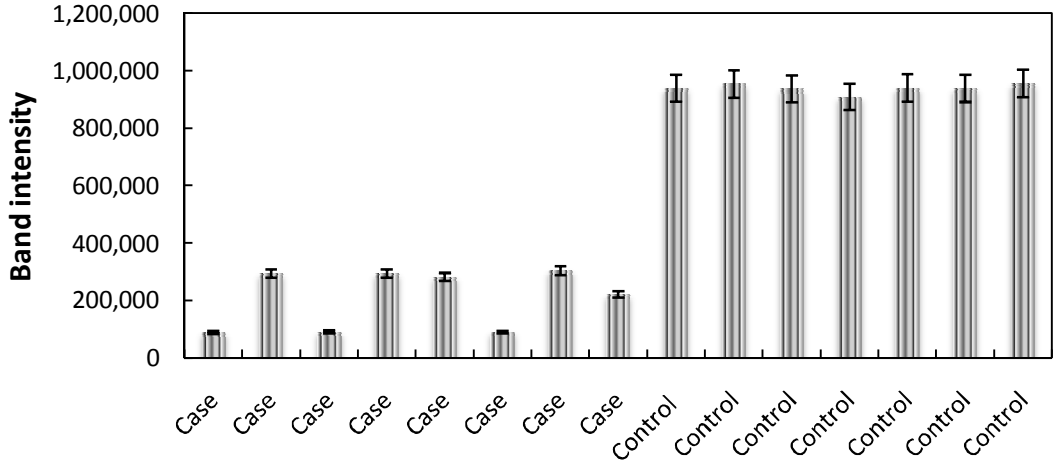

Figure 2. Relative band intensities of cases and controls in *C-MYC* gene

Table 1. Demographics

Age interval (years)	Cases		Control		Mean	±S.D	P-value
	n=50	Percentage	n=50	Percentage			
15-29	06	12%	07	14%	47.56	±15.07	0.941
30-44	11	22%	10	20%			
45-59	24	48%	21	42%			
60-74	07	14%	09	18%			
75+	02	4%	03	06%			

Table 2. Distribution of ovarian cancer

Signs and Symptoms	Number (cases)	Percentage (cases)	Number (controls)	Percentage (controls)	Chi-square X^2	P-value
Pelvic pain	22	44%	11	22%	5.4726	.019
Abdominal distention	23	46%	04	08%	18.315	.000
Vaginal bleeding	07	14%	03	06%	1.7778	.182
Urinary symptoms	03	6%	02	04%	0.2105	.646
GI symptoms	10	20%	06	12%	1.1905	.275
Ascities	17	34%	02	04%	14.6199	.000
Leg edema	12	24%	01	02%	10.6985	.001

Table 3. Basis of familial disposition and *CA125.*

Familial predisposition	No of cases	No. of control	Total participants	Chi-square value	P-value
	14	03	17	10.714	0.01

CA125 marker	No. of cases	CA125 U/ml Mean±S.D	P-value
0 to >35 U/ml	04	29.75±01.71	0.16
35-100 U/ml	06	77.33±15.43	0.05
101-299 U/ml	01	295±92.16	0.00
300-499 U/ml	04	420±63.10	0.16
≥500 U/ml	35	3780.2±1249	0.00

Table 4. Epithelial tumors

Surface epithelial Tumor	Type of surface epithelial Tumor	No. of patient diagnosed	Percentage of patients.
Serous epithelial tumor	Benign	01	02.33%
	Malignant	21	48.81%
Mucinous epithelial tumor	Benign	02	04.65%
	Borderline	01	02.33%
	Malignant	15	34.90%
Endometroid carcinoma	-	02	04.65%
Ovarian Clear cell carcinoma	-	01	02.33%
	Total	**43**	**100%**

Table 5. Non-epithelial tumors

Non-epithelial Tumor	Type of non-epithelial Tumor	No. of patient diagnosed	Percentage of patients.
Sex cord stromal tumor	Granulosa cell tumor	1	14.2%
	Sertoli leydig cell tumor	2	28.6%
Germ cell tumor	Fibroma	2	28.6%
	Dysgerminoma	2	28.6%
Total		**7**	**100%**

Table 6. Gene mutations

Gene Base	Ref Seq Nucleotide bases	CA	CB	CC	CD	CE	Total mutations at base	Percentage of mutations	Ca	Cb	Cc	Total mutations at locus	Percentage of mutations
10267	C	C	C	G	C	C	1	20%	C	C	C	0	0%
10274	C	C	C	G	C	C	1	20%	C	C	C	0	0%
10300	G	G	T	G	A	-	3	60%	A	A	G	2	66.67%
10301	A	T	T	A	A	T	3	60%	T	T	A	2	66.67%
10302	G	-	-	T	G	C	4	80%	A	G	G	1	33.33%
10303	G	-	-	-	-	C	5	100%	C	C	G	2	66.67%
10304	A	-	-	-	-	T	5	100%	T	T	A	2	66.67%
10305	G	-	-	-	-	T	5	100%	T	T	G	2	66.67%
10306	G	-	-	-	-	-	5	100%	T	T	G	2	66.67%
10307	A	-	-	-	-	-	5	100%	C	C	A	2	66.67%
10308	A	-	-	-	-	T	5	100%	T	T	A	2	66.67%
10309	C	-	-	-	-	C	4	80%	G	C	C	1	33.33%
10310	G	-	-	-	-	G	4	80%	G	G	G	0	0%
10311	`A	-	-	-	-	T	5	100%	T	T	A	2	66.67%
10312	G	-	-	-	-	G	4	80%	G	G	G	0	0%
10313	C	-	-	-	-	T	5	100%	T	T	C	2	66.67%
10314	T	-	-	-	-	C	5	100%	C	C	T	2	66.67%
10315	A	-	-	-	-	G	5	100%	G	G	A	2	66.67%
10316	A	-	-	-	-	A	4	80%	A	A	A	0	0%
10317	A	-	-	-	-	G	5	100%	G	G	A	2	66.67%
10318	A	-	-	-	-	C	5	100%	A	A	A	0	0%
10319	C	-	-	-	-	G	5	100%	C	C	C	0	0%
10320	G	-	-	-	-	T	5	100%	T	T	G	2	66.67%

10321	**G**	-	-	-	-	G	4	80%	G	G	G	0	0%
10322	**A**	-	-	-	-	G	5	100%	G	G	A	2	66.67%
10323	**G**	-	-	-	-	G	4	80%	G	G	G	0	0%
10324	**C**	-	-	-	-	G	4	80%	G	G	C	2	66.67%
10325	**T**	-	-	-	-	-	5	100%	C	C	T	2	66.67%
10326	**T**	-	-	-	-	T	4	80%	T	T	T	0	0%
10327	**T**	-	-	-	-	G	4	80%	G	G	T	2	66.67%
10328	**T**	-	-	-	-	G	4	80%	G	G	T	2	66.67%
10329	**T**	-	-	-	-	T	4	80%	T	T	T	0	0%
10330	**T**	-	-	-	-	G	5	100%	G	G	T	2	66.67%
10331	**G**	-	-	-	-	C	5	100%	C	C	G	2	66.67%
10332	**C**	-	-	-	-	A	5	100%	A	A	C	2	66.67%
10333	**C**	-	-	-	-	T	5	100%	T	T	C	2	66.67%
10334	**C**	-	-	-	-	T	5	100%	T	T	C	2	66.67%
10335	**T**	-	-	-	-	T	4	80%	T	T	T	0	0%
10336	**G**	-	-	-	-	T	5	100%	T	T	G	2	66.67%
10337	**C**	-	-	-	-	C	4	80%	C	C	C	0	0%
10338	**G**	-	-	-	-	G	4	80%	G	G	G	0	0%
10339	**T**	-	-	-	-	-	5	100%	G	G	T	2	66.67%
10340	**G**	-	-	-	-	T	5	100%	T	T	G	2	66.67%
Total							188					56	
X^2	**159.05**												
p-value	**0.00**												

Table 7. Types of mutations in cases

Case	Transition mutation	Transverse mutation	Insertion	Deletion	Total mutation	Total percentage
1	0	1	0	4	5	3.03%
2	0	2	0	3	5	3.03%
3	0	3	0	3	6	3.636%
4	1	0	0	3	4	2.424%
5	8	13	0	3	24	14.54%
Total	**9**	**19**	**0**	**16**	**44**	**26.66%**

Table 8. Types of mutations in control

Control	Transition mutation	Transverse mutation	Insertion	Deletion	Total mutation	Total percentage
1	11	15	00	00	26	15.76%
2	08	16	00	00	24	14.54%
3	00	00	00	00	00	00%
Total	**19**	**31**	**00**	**00**	**50**	**30.30%**

CONCLUSION

The present pilot study concluded that the mutation analysis provided a strong predictor of ***C-MYC*** mutation status that may serve as early screening diagnostic marker in diagnosis of ovarian cancer from blood sample only. The current study illustrates the scope of the disease and exemplifies the necessary characteristics of lifestyle genetics and the environment in ovarain cancer. Furthermore, greater awareness among the population is required to decrease the mortality as well as morbidity associated with the ovarian carcinoma.

Henceforth, the tissue samples should be taken into consideration for the comparison of the expression level of *C-MYC* gene. The whole exon 3 should be sequenced from both cases and controls to determined the efficacy and validate the method for screening or clinical procedures in future to detect ovarian cancer at the earliest possible stage.

DISCLOSURE

There are no conflicts of interest.

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