ASSOCIATION BETWEEN ESTROGEN RECEPTOR GENE VARIATIONS AND RISK OF OVARIAN CANCER IN PAKISTANI POPULATION

By

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FINAL APPROVAL

It is certified that we have read and evaluated the thesis "Association Between Estrogen Receptor Gene Variations And Risk Of Ovarian Cancer In Pakistani Population" submitted by Ms Aisha Arshad and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M S Degree in Biotechnology

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A thesis submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree Master of Science in Biotechnology.

DEDICATION

I dedicate this dissertation, with all my heart, to my beloved parents and to my supervisor. Without their assistance, bunch of sincere prayers and sacrifices it would not have been possible for me to accomplish my work I also dedicate my thesis to all those students who could get maximum benefit from my work.

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

Date 30-12-2016

Aisha Arshad

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May Allah bless you all with eternal happiness and success! Ameen

Aisha Arshad

LIST OF ABBREVIATIONS

HRT	Hormone Replacement Therapy
ASRs	Age Standardized Rates
ASIR	Age Specific Incidence Rate
CDC	Centre for Disease Control
KIRAN	Karachi Institute of Radiotherapy and Nuclear Medicine
NOS	Not Otherwise Specified
DMPA	Depot Medroxyprogesterone Acetate
KRAS	Kirsten RAS Oncogene Homolog
BRCA	Breast Cancer Susceptibility Gene
HNPCC	Hereditary Non-Polyposis Colon Cancer
HR	Homologous Recombination
NHEJ	Non-Homologous End Joining
BARD1	BRCA1 Associated RING Domain
PTEN	Phosphatase and Tensin Homolog
MLH1	Mutl Homolog 1
MLH3	MutL Homolog 3
MSH2	MutS Protein Homolog 2
MSH6	Muts Homolog 6
TGFBR2	Transforming Growth Factor-Beta Receptor, Type II
PMS	Postmeiotic Segregation
STK11	Serine/Threonine Kinase 11

MUTYH	MutY DNA Glycosylase, previously known as MutY Homolog
CTNNB1	Catenin Beta 1
PIK3CA	Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
HER-2/neu	Human Epidermal Growth Factor Receptor 2
HRE	Hormone Response Element
PI3K/Akt	Phosphatidylinositol-3-Kinase and Protein Kinase B
TGF-α	Transforming Growth Factor Alpha
EGF	Epidermal Growth Factor
IL-6	Interleukin-6
c-myc	Avian Myelocytomatosis Virus Oncogene Cellular Homolog
IGF-I	Insulin-Like Growth Factor I
TVUS	Transvaginal Ultrasound
FFPE	Formalin Fixed Paraffin Embedded
PIMS	Pakistan Institute of Medical Sciences
IB&GE	Institute of Biomedical and Genetic Engineering
TE	Tris-EDTA
ODs	Optical Densities
SNPs	Single Nucleotide Polymorphisms
RFLP	Restriction Fragment Length Polymorphism

ESR1	Estrogen Receptor Gene
TBE	Tris Boric EDTA
EDTA	Ethylene Diamine Tetra Acetic Acid
rpm	Revolution per Minute
ml	Milliliter
µl	Microliter
mM	Millimolar
nm	Nanometer
ng	Nanogram
DNA	Deoxyribonucleic Acid
ERA	Estrogen Receptor Alpha
bp	Base Pair
rs	Reference Sequence
RM	Recessive Model
DM	Dominant Model
OR	Odds Ratio
CI	Confidence Interval

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ABSTRACT

Ovarian cancer is the most frequent cause of death among all cancers of female genital tract In Pakistan it accounts for 3 9% after breast and uterus cancers in adult females Scarce data is available regarding epidemiology, clinical presentation and genetic risk factors of ovarian cancer in Pakistan The aim of the present study was to assess the relative frequencies of major classes and histological subtypes of ovarian cancer and to evaluate the association between risk of ovarian cancer and 397T>C (rs2234693) and 351A>G (rs9340799) single nucleotide polymorphisms in Pakistani population We analyzed the relative frequencies and patterns of ovarian cancer through clinical information of 186 patients The relative frequencies of major ovarian cancer classes were surface epithelial tumors (82 3%, 153/186) followed by germ cell tumors (13 4%, 25/186) and sex-cord stromal tumors (4 3%, 8/186) The most frequent malignant subtype evaluated was 'serous cystadenocarcinoma' (41 9%, 31/74) followed by 'mucinous cystadenocarcinoma' (17 6% 13/74) and 'germ cell tumors' (8 1%, 6/74) The most common benign tumor was 'serous cystadenoma' (47 06%, 48/102) followed by 'mucinous cystadenoma' (22 55%, 23/102) and mature cystic teratoma (18 63%, 19/102) We analyzed the distribution of genotypes and frequency of alleles of the *ESR1* polymorphisms in 79 women with ovarian cancer and 46 negative controls Both polymorphisms were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) We didn't find any significant difference in the distribution of genotypes and allele frequencies of 397T>C and 351A>G single nucleotide polymorphisms between cases and negative controls (P>0 05) Frequency of the homozygous polymorphic genotype 'GG' of 351A>G polymorphism has observed to be very less in our population In the present study we demonstrated an insignificant association between the *ESR1* gene *Pvu*II and *Xba*I polymorphisms and risk of ovarian cancer in Pakistani population However, a study needs to be performed on large sample size and further investigations are required for the confirmation and to determine whether the findings are generalizable to other populations

CHAPTER 1

INTRODUCTION

1.1. Ovarian Cancer

Ovarian cancer ranks as the seventh most frequently occurring cancer among women worldwide (18th most common cancer on the whole) and eighth common cause of death from cancer (Ferlay *et al*., 2014). The etiology of ovarian cancer is still unknown but there are several risk factors that can lead to ovarian cancer, age, family history, genetic predisposition, hormone replacement therapy (HRT), early menarche, late menopause, obesity and infertility. A woman's lifetime risk of developing ovarian cancer in the general population is 1 3% affecting 1 in 75 women and her lifetime chance of dying from it is 1 in 100 (American Cancer Society, 2016) Women aged 55 to 64 are found to be at an increased risk of developing the disease and it has also been found that half of all the ovarian cancer cases involve women aged 63 or older (SEER Cancer Statistics Review, 2016)

1.1.1. Global Perspective

About 239,000 new cases were diagnosed with ovarian cancer in 2012 worldwide and 152,000 died from it The highest incidence of ovarian cancer was observed in Europe and Northern America i.e 9 9 and 8 1 (Age standardized rates, ASRs) per 100,000 respectively, and the lowest incidence was in Africa and Asia i e 4 8 and 5 0 (ASRs) respectively (Ferlay *et al* , 2014)

In the United States, ovarian cancer is designated as the ninth most common cancer and the fifth leading cause of death from cancers among adult females after lung and bronchus, breast, colorectal, and pancreatic cancers Each year about 20,000 women per 100,000 women in the United States get ovarian cancer and more than 14,000 die from it In 2013 (the most recent data available) 20,927 per 100,000 women in the United States were diagnosed with ovarian cancer and 14,276 died from it (Centre for Disease Control [CDC], 2016) In 2013, an estimated 195,767 women were suffering from ovarian cancer in the United States (SEER Cancer Statistics Review, 2016) The American Cancer Society has estimated that in the current year 2016 about 22,280 will be the new cases of ovarian cancer and about 14,240 women will die from it (American Cancer Society, 2016).

Ovarian cancer is the fifth most common cancer in Europe among adult females In 2012, about 65,538 new cases were diagnosed and about 42,716 females died In Europe (2012), the highest World age-standardized incidence rate for ovarian cancer was in Latvia, the lowest was in Albania i e 18 9 and 4 2 respectively (Ferlay *et al*, 2013)

1.1.2. Regional Perspective

Ovarian cancer has appeared as one of the most common malignancies and emerged as the common cause of increased rate of mortality in India In India, the age-standardized incidence rates (ASR) for ovarian cancer deviated from 0 9 to 8 4 per 100,000 women during the period of 2001-2006 among different registries The highest incidence was observed in Pune and Delhi registries i e 8 4 and 8 3 (ASRs) respectively The Age Specific Incidence Rate (ASIR) for ovarian cancer disclosed that the disease increases from 35 years of age and culminate between the ages 55-64 (Murthy *et al*, 2009)

1.1.3. National Perspective

Among South Asian countries including Srilanka, India, Bhutan, Bangladesh and Nepal, ovarian cancer is relatively frequent in Pakistan (Moore *et al*, 2009) According to Punjab Cancer Registry Report of 2014 by Shaukat Khanum Memorial Cancer Hospital & Research Centre Lahore about 3, 147 women were diagnosed with cancers during the time period of January 01, 2014-December 31, 2014 (1-year) out of which 124 women were diagnosed with ovarian cancer, representing 3 9% among ten most frequently reported cancers in adult females According to this report ovarian cancer is the 3rd most common cancer among women in Pakistan after breast and uterine cancers

A retrospective nine year data analysis done by Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN), a tertiary care cancer institution during the period from 1st January 2000 to 31 December 2008 showed that ovarian cancer is the 4th common cancer among women in Pakistan after breast, head and neck and cervical cancers,

accounting for 4 9% among common malignancies in Pakistani adult females (Hanif *et al* , 2009)

1.2. Classification of Ovarian Tumors

Ovarian tumors are classified into three major categories based on the type of tissues in which they occur surface epithelial-stromal tumors, sex cord-stromal tumors, and germ cell tumors Each category is further classified into different histologic subtypes and each subtype includes benign, borderline and malignant ovarian neoplasms (Table 1 1)

1.2.1. Surface Epithelial-Stromal Tumors

These tumors originate from the epithelium of the ovarian surface and constitute about 75% of all ovarian tumors and 90-95% of ovarian malignancies These tumors affect women in their reproductive ages and usually found in post-menopausal women (mean age of appearance is 56 years) They rarely occur in young females particularly before menarche (Jones, 2004) Surface epithelial-stromal tumors are further classified into five major subtypes including serous, mucinous, endometrioid, clear cell, and transitional cell (or Brenner type) Some surface epithelial-stromal tumors are not classified further into any specific subtype and are therefore known as adenocarcinomas not otherwise specified (NOS)

1.2.1.1. Serous Tumors

These surface epithelial-stromal tumors are formed by the increased proliferation of those epithelial cells that smack of the cells of the internal lining of the fallopian tube (Chen *et al* , 2003)

1.2.1.2. Mucinous Tumors

Mucinous tumors are those surface epithelial-stromal tumors that can be either of endocervical or müllerian type formed by those cells that resemble the cells of endocervical epithelium or they can be of intestinal type formed by the cells that smack of the intestinal epithelium (Tavassoli and Devilee, 2003)

1.2.1.3. Endometrioid Tumors

Endometrioid surface epithelial stromal tumors of the ovary are formed from an escalation of those epithelial cells that are quite similar to the cells of endometrium (Chen *et al* , 2003)

1.2.1.4. Clear Cell Tumors

These surface epithelial tumors are formed from epithelial component that contains clear, peg-like or hobnail-like cells. Most of the clear cell epithelial tumors are malignant whereas benign and borderline clear cell neoplasms occur infrequently 60% of the cases are at Stage I when diagnosed with clear cell malignancy, hence representing good prognosis About two-thirds of all females with diagnosed epithelial clear cell carcinoma are nulliparous and 50-70% have endometriosis (Chen *et al* , 2003)

1.2.1.5. Transitional Cell (Brenner) Tumors

Transitional cell tumors occur infrequently and are formed by the ovarian epithelial component that histologically resembles to the urothelium (internal lining of urinary bladder also known as transitional epithelium) Transitional cell malignant ovarian tumors are grouped into malignant Brenner tumors and transitional cell carcinomas (Table 1 1) They are called as malignant Brenner tumors when benign Brenner tumor component is associated with ebullient proliferative but non- invasive urothelium and are called as transitional cell carcinomas when benign or borderline Brenner tumor component is absent (Tavassoli and Devilee, 2003)

1.2.1.6. Squamous Cell Tumors

Squamous cell tumors are those ovarian neoplasms which are comprised of squamous epithelial cells that are not of germ cell origin Epidermoid cysts are considered as benign squamous cell epithelial tumors if they are of surface epithelial origin and do not contain teratomatous component but if teratoma element is present in the cyst they will be classified into the category of germ cell tumors

Most ovarian malignant squamous cell carcinomas stand in the category of germ cell tumors and emerge in dermoid cysts Squamous cell carcinomas of surface epithelial-

stromal tumor type may develop either in pure form or in combination with Brenner tumor or ovarian endometriosis (Scully and Sobin, 1999)

1.2.1.7. Mixed Epithelial Tumors

These are the surface epithelial ovarian tumors which are comprised of the mixture of two or more than two major cell types (serous, mucinous, endometrioid, clear cell or transitional/Brenner) The second or third cell types must constitute at least 10% of the tumor in order to qualify for the diagnosis of a mixed epithelial tumor If second or third cell types are present <10% then the tumors are systematized according to the element that is preponderated An example is of Endometrioid carcinomas which mostly do not fall in this category of tumors, as usually they contain foci of glands which are lined by epithelial cells filled with mucin but their concentration is not sufficient to be qualified for the diagnosis of mixed epithelial tumors (Tavassoli and Devilee, 2003)

1.2.1.8. Undifferentiated Carcinomas

Surface epithelial ovarian tumors that are formed of highly poorly differentiated cells or by the cells that exhibit highly malignant attributes such as high nuclear grade and no differentiation of cytoplasm are referred to as undifferentiated carcinomas (Chen *et al* , 2003)

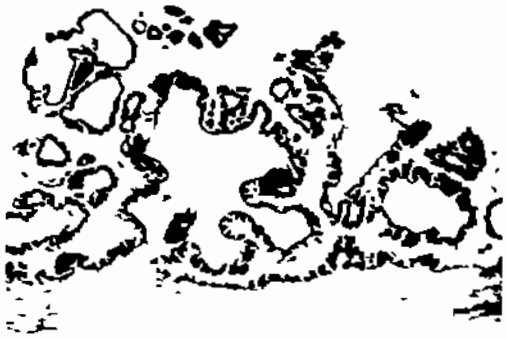
Table 1.1 WHO Histologic Classification of Epithelial Ovarian Tumors

Types and Histologic Subtypes of Ovarian Tumors	Composition	Diagnosed Age	Laterality	References
1 Surface Epithelial-Stromal Tumors	about 75% of all ovarian tumors and 90-95% of ovarian malignancies			(Jones, 2004)
1.1.Serous Tumors				
➢ Benign	two-thirds of all ovarian serous tumors	between the 4th and 5th decades	Often are bilateral	(Chen *et al*., 2003)
➢ Borderline	accounts for 10-15% of all ovarian serous tumors.	fifth decade of life	one-third are bilateral	(Chen *et al* 2003)
➢ Malignant	one-third of all ovarian serous tumors and half of all ovarian malignancies	sixth decade of life	Almost two-thirds are bilateral	(Chen *et al*., 2003)
1.2 Mucinous Tumors				
➢ Benign	about 75-85% of all ovarian mucinous tumors and one-fourth of all benign ovarian tumors	between 3rd and 5th decades of life	Often unilateral	(Chen *et al* 2003)
➢ Borderline	10-14% of all ovarian mucinous neoplasms	occur between 4th and 6th decades of life	40% of endocervical type are bilateral and <10% of intestinal type are bilateral	(Chen *et al* 2003)
➢ Malignant	5-10% of all ovarian malignancies	diagnosed at sixth decade of life	about 6-20% of these tumors are bilateral	(Chen *et al* 2003)

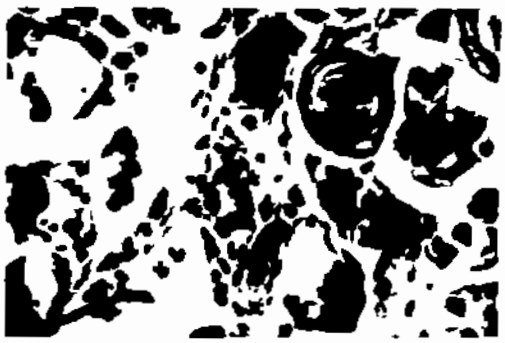
Types and Histologic Subtypes of Ovarian Tumors	Composition	Diagnosed Age	Laterality	References
1.3.Endometrioid Tumors				
➢ Benign	rare tumors	Diagnosed at 6th decade of life	unilateral	(Tavassoli and Devilee, 2003)
➢ Borderline	occur infrequently	Diagnosed at 6th decade of life	unilateral	(Tavassoli and Devilee, 2003)
➢ Malignant	2nd most common malignancy, represent 80% of all endometrioid tumors and 10-25% of all ovarian malignancies	Identified in the sixth decade of life	13-28% of endometroid malignant tumors are bilateral	(Tavassoli and Devilee, 2003)
1.4.Clear Cell Tumors				
➢ Benign	occur rarely			
➢ Borderline	rare tumors			
➢ Malignant	represents 4-5% of all epithelial ovarian malignancies	Fifth decade of life	15-20%of these tumors are bilateral	(Chen *et al* 2003)
1.5.Squamous Cell Tumors				
➢ Benign Epidermoid Cysts	rare tumors			
➢ Malignant Carcinomas	occur infrequently	Occur in between 23-90 years		(Tavassoli and Devilee, 2003)

Types and Histologic Subtypes of Ovarian Tumors	Composition	Diagnosed Age	Laterality	References
1.6.Transitional Cell Brenner Tumors				
➢ Brenner tumor (Benign)		affect women between 5th and 6th decades of life	unilateral	(Tavassoli and Devilee 2003)
➢ Brenner tumor of borderline malignancy		Occur between 6th and 7th decades of life	unilateral	(Tavassoli and Devilee, 2003)
➢ Malignant (malignant Brenner tumor, transitional cell carcinoma)	infrequent occurring tumors		one-tenth of malignant transitional cell tumors are bilateral	(Tavassoli and Devilee, 2003)
1.7 Mixed Epithelial Tumors	0 5-4% of surface epithelial tumors are mixed epithelial tumors			(Tavassoli and Devilee 2003)
➢ Benign				
➢ Borderline			22% of mixed epithelial borderline tumors are bilateral	(Tavassoli and Devilee 2003)
➢ Malignant				
1.8. Undifferentiated Carcinomas	Covers almost 5% of all ovarian malignancies and 14% of all surface epithelial tumors	Diagnosis occurs in the sixth decade of life	Half of these tumors are bilateral	(Chen *et al* 2003)

(a) Benign Serous Cystadenoma Ciliated epithelium of cyst adjacent to stromal component



(b) Serous papillary cystic tumor of borderline malignancy Stratification with cellular budding of epithelial cells and absence of invasion



(c) Serous papillary carcinoma Characteristic lamination of psammoma body

(d) Mucinous Cystadenoma Cyst lined by benign endocervical like epithelium

(e) Mucinous papillary cystic tumor of borderline malignancy of endocervical type

(f) Mucinous papillary cystic tumor of borderline malignancy of intestinal type

Figure 1.1(a): Surface Epithelial-Stromal tumors.

(Source Scully and Sobin, 1999)

(g) Mucinous adenocarcinoma Mucinous glands and cysts lined by mucinous and mucin-free cells and large cyst filled with "dirty" necrotic material

(h) Endometrioid adenocarcinoma Tubular glands lined by stratified non mucin containing epithelium and solid areas of carcinoma

(i) Clear cell carcinoma. Diffuse arrangement of polyhedral clear cells with ec-centric nuclei

(j) Malignant Brenner Tumor Two cystic Benign Brenner nests and single irregular carcinomatous aggregate lying in fibromatous stromal component

(k) Transitional Cell Carcinoma Cyst lined by malignant transitional epithelium invading stroma in irregular rounded nests

Figure 1.1(b): Surface Epithelial-Stromal tumors.

(Source Scully and Sobin, 1999)

1.3. Risk Factors of Ovarian Cancer

The etiology of ovarian cancer is still not completely understood but some reproductive, environmental and genetic factors are known that can increase the risk of developing ovarian cancer

1.3.1. Reproductive Factors

The risk of getting ovarian cancer increases with the age Fertility is an important reproductive factor that can affect a woman's chance of developing the disease Women who can conceive have a 50% decreased risk of developing ovarian cancer compared with those who are infertile or nulliparous The use of fertility drugs such as clomiphene citrate (clomid) for more than one year is also a risk factor for developing ovarian cancer and the risk is even higher in those women who do not get pregnant even after using this fertility drug. The use of hormone replacement therapy after menopause is another reproductive risk factor for developing ovarian cancer and the risk is higher in those women who take only estrogen and it is less in those women who take estrogen and progesterone both (Salehi *et al* , 2008)

On the other hand use of oral contraceptives reduces the risk of ovarian cancer Recently, a study has found that the women who use depot medroxyprogesterone acetate (DMPA), an inoculating hormonal contraceptive, have a lower risk of ovarian cancer (American Cancer Society, 2016) Furthermore, tubal ligation and hysterectomy lowers the risk of developing ovarian cancer by up to two-thirds and one third respectively (Salehi *et al* , 2008)

1.3.2. Environmental Factors

The exposure to some of the environmental elements may become the basis of getting the disease The use of products directly in the genital area can be harmful as they might contain some contaminants which can enter into the surface of the ovaries as female genital tract permit the passage of fine particle pollution and provides the route to the ovarian surface from the outer vagina through the uterus and fallopian tubes (Cramer and Xu, 1995) Some studies suggested that the use of cosmetic talcum

powder in the genital area can be the risk factor for developing epithelial ovarian cancer A research was conducted for the confirmation and founded the presence of talcum powder particles in ovarian cancerous tissues with the reported information that the women suffering from ovarian cancer were in more use of talcum powder in their genital area as compared to the healthy women, suggesting that the talcum powder use might increases the risk of ovarian cancer (Chang and Risch, 1997) Similarly, an occupational exposure to pesticides, insecticides and herbicides specifically triazine herbicides make some women more vulnerable to develop ovarian cancer (Young *et al* , 2005)

Life style factors such as obesity, imbalanced diet including excessive use of saturated fats or red meat and restricted or no use of fruits and vegetables, and cigarette smoking can be the risk factors of ovarian cancer (Salehi *et al* , 2008)

1.3.3. Family History

Another pre-eminent risk factor that can affect a woman's chance of developing the ovarian cancer is the family history of this disease Studies have shown that 10 to 15% incidences of ovarian cancer are attributable to hereditary genetic factors (Christie and Oehler, 2006) If any woman has one first-degree relative (mother, sister or daughter) suffering from an ovarian cancer then she has a 5% lifetime risk of ovarian cancer and a woman having two or more first-degree relative with ovarian cancer has a 7% risk of getting the disease in her lifetime and women with no family history of ovarian cancer have 1 6% lifetime risk of developing ovarian cancer (Cook 2002) Furthermore, a woman having family history of some other types of cancer such as colorectal and breast cancers is also at an increased risk of ovarian cancer (American Cancer Society, 2016)

1.3.4. Genetic Factors

1.3.4.1. Inherited Mutations

Genetic factors play an important role in the pathogenesis of ovarian cancer As already mentioned, 10 to 15% of ovarian cancer cases are linked to inheritance Some

studies have shown that 90% of hereditary ovarian cancer is related to an inherited mutation in *BRCA1* (located on 17q21) or *BRCA2* (located on 13q12-13) tumor suppressor genes (Prat *et al*, 2005) and 10% of hereditary ovarian cancer is associated with hereditary non-polyposis colon cancer (HNPCC) syndrome In the general population, an estimated 1 in 800 women may carry *BRCA1* or *BRCA2* genes mutations but the frequency is much higher in Ashkenazi Jewish women 1 in every 50 Ashkenazi Jewish women may carry *BRCA1* and *BRCA2* mutations (Roa *et al* 1996) Women with *BRCA1* mutation have an estimated lifetime risk of 40-50% to get ovarian cancer and for those who are *BRCA2* mutation carriers, an estimated lifetime risk is 20-30% to get the disease (Ricciardelli and Oehler, 2009) The carriers may be suggested by the consultants to go for a prophylactic salpingo-oophorectomy as a risk minimizing procedure for mutation carriers

Under normal conditions *BRCA1* and *BRCA2* act as tumor suppressors in the ovarian and breast cancer development The proteins encoded by these two genes are synergistically involved in two fundamental cellular processes DNA damage repair either by homologous recombination (HR) or by non-homologous end joining (NHEJ), and transcriptional regulation (Roy *et al*, 2012) DNA repair by these two proteins is carried out through coalition with other proteins including RAD51 (Welcsh and King, 2001), RAD50 (Zhong *et al*, 1999), and BARD1 (Bennett *et al*, 2000)

Mutations of *BRCA1/2* are associated with increased vulnerability for ovarian cancer Researchers have identified *BRCA1/2* loss-of-function mutations in approximately 84% of ovarian carcinomas, including germ-line cells and somatic cells (Hilton *et al*, 2002) Cells that carry mutations in *BRCA1* and *BRCA2* genes may accumulate chromosomal abnormalities including chromosomal breaks, grievous aneuploidy and centrosome amplification This chromosomal instability due to mutations in these DNA repair genes may be the pathogenic basis of ovarian cancer

Similarly women with inherited mutations in the genes that are allied to other family cancer syndromes are also at an increased risk of ovarian cancer, such as *PTEN* gene (PTEN tumor hamartoma syndrome also called Cowden disease), *MLH1, MLH3, MSH2, MSH6, TGFBR2 PMS1, and PMS2* DNA repair genes (causing hereditary nonpolyposis colon cancer (HNPCC), previously known as Lynch syndrome), *STK11* gene (Peutz-Jeghers syndrome) and an inherited mutation in *MUTYH* gene (causing MUTYH-associated polyposis) (American Cancer Society, 2016)

1.3.4.2. Acquired Mutations

Almost 85% of ovarian carcinomas are believed to be sporadic that occur because of acquired mutations and are not associated with inheritance (Christie and Oehler, 2006) On the basis of development, ovarian carcinomas are divided into two main groups Type I and Type II ovarian tumors (Willner *et al* , 2007) These tumors arise through two distinct pathways and are referred to as low-grade and high-grade ovarian carcinomas, respectively Type I tumors (low grade) arise from endometriotic lesions or from an ovarian inclusion cyts and develop slowly from benign cyst adenomas and borderline lesions to malignant tumors while Type II tumors grow de novo' from the surface epithelium and grow rapidly, lacking any identifiable precursor lesions (Singer *et al* , 2005)

Type I tumors account for 25% of ovarian cancers which include low grade serous low grade endometrioid, mucinous and clear cell ovarian carcinomas and contain mutations in *KRAS* and *BRAF* oncogenes and *CTNNB1* and *PTEN* tumor suppressor genes (Ricciardelli and Oehler, 2009) *KRAS* point mutations occur most frequently in mucinous ovarian carcinomas, having the highest rate (50%) of *KRAS* mutations (Gemignani *et al* , 2003) They also occur commonly in about 35% of low-grade serous carcinomas (Singer *et al* , 2003) Somatic point mutations of *BRAF* oncogene occur commonly in almost 30% of low-grade ovarian serous cancers (Singer *et al* , 2003) On the other hand, mutations in the beta-catenin tumor suppressor gene *CTNNB1* and mutations in the *PTEN* tumor suppressor gene are more common in

endometrioid ovarian carcinomas but are infrequent in other tumor types (Palacios and Gamallo, 1998, Obata *et al* , 1998)

Type II tumors constitute 75% of ovarian carcinomas and include high grade serous, high grade endometrioid, undifferentiated and clear cell carcinomas These tumors are characterized by mutations or overexpression of *p53* tumor suppressor gene, mutations in *BRCA1* or *BRCA2* tumor suppressor genes and mutations in Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA* also known as *PI3K*) (Ricciardelli and Oehler, 2009) Mutation in *p53* is most frequently present in up to 70% of high-grade ovarian serous carcinomas It also occurs in almost 8% of clear cell ovarian carcinomas and in most high-grade endometrioid carcinomas but is rare in low-grade serous and mucinous carcinomas (Willner *et al* , 2007). *BRCA1/2* loss of function mutation occur more frequently in the sporadic and hereditary high-grade serous carcinomas as compared to the low-grade serous carcinomas (Hilton *et al*., 2002) while *PIK3CA* mutations are reported exclusively in high-grade endometrioid or high-grade clear cell carcinomas (Willner *et al* , 2007)

Furthermore, overexpression of the HER-2/neu oncogene which codes for a growth factor receptor that is similar in structure to epidermal growth factor receptor has been identified in human ovarian carcinomas Researchers have identified two fold to forty fold increased levels of HER-2/neu protein in approximately one third of ovarian carcinomas using immunohistochemical techniques, and found that overexpression of this oncogene is associated with poor prognosis

In summary, molecular and genetic studies have revealed that high-grade ovarian carcinomas carry a high frequency of *p53* and *BRCA* tumor suppressor genes mutations and do not contain mutations in *KRAS* or *BRAF* oncogenes, in contrast, low-grade ovarian cancer has a high frequency of *KRAS* or *BRAF* mutations but possess mutations in *p53* or *BRCA1/2* infrequently

1.4. Signs and Symptoms of Ovarian Cancer

Signs and symptoms that are associated independently with ovarian cancer include gastrointestinal symptoms such as severe and persistent (if occur more than 12 times per month) pelvic/abdominal pain, bloating, difficulty in eating, nausea, vomiting, constipation, diarrhea and gynecologic symptoms such as severe and persistent dyspareunia, increased urination and menstrual changes (polymenorrhagia, dysmenorrhea or amenorrhea) (Goff *et al*, 2007) Gastrointestinal symptoms are usually associated with late-stage ovarian cancer while gynecologic symptoms are associated with early stage disease (Ryerson *et al*, 2007)

1.5. Diagnosis of Ovarian Cancer

It is difficult to detect the ovarian cancer at initial stage Early ovarian cancer stage causes no severe symptoms. Early symptoms caused by the cancer in ovaries tend to be caused by other complaints Abdominal swelling/pain, difficulty in eating, feeling full with less food intake, pelvic pressure and urinary symptoms anticipate the ovarian cancer but it is not the case However, the earlier mentioned complaints should be addressed with some gynecologist By the time ovarian cancer is diagnosed, it has spread to the other organs of the body, thus, prompt action is required if symptoms found

Most often, screening tests are used to detect the cancer, for example, mammogram for breast cancer detection However, there has not been much success in the development of screening test method in ovarian cancer detection The two tests which are used to screen are transvaginal ultrasound (TVUS) and the CA-125 blood test An ultrasound test is also used to produce the image of the size and texture of the ovaries and any possible abnormal growth such as cysts or swellings present, there If the patient is diagnosed, further tests are recommended to know the stage of the cancer that includes a chest X-ray, CT-scan, MRI scan, abdominal fluid aspiration, and laparoscopy

1.6. Treatment of Ovarian Cancer

After the diagnosis of ovarian cancer is made, the treatment process follows The main methods for the treatment of the ovarian cancer are surgery, chemotherapy, hormone therapy, targeted therapy and radiation therapy One method is not adequate to fight the disease Different methods listed above are used to treat the disease However, it does not entail that all methods are used for the treatment Most often, two methods are used for the treatment, surgery and chemotherapy

The treatment may differ from one patient to the other because the types and stages may not be the same Many factors are kept in front before deciding the treatment procedure, for example whether the patient is planning a child, or age factor Furthermore, the new innovative model of allocating ovarian epithelial carcinomas into two groups requires a drastic change in the current treatment procedures Type I and II ovarian carcinomas entail different diagnostic and therapeutic methods The cumulative understanding of the molecular pathology of all subtypes of ovarian carcinoma will be helpful in the development of more rational, individualized targeted therapies The knowledge of targeted therapy is scarce in some countries especially in under developing countries If the knowledge about targeted therapy treatment increases then it would definitely lead to a substantial improvement in the clinical outcome of ovarian cancer patients in the near future

1.7. Survival Rates of Ovarian Cancer Patients

The cancer patients survive at least for 5 years after their cancer is diagnosed Certain factors are there which may affect the 5-year survival rate of ovarian cancer such as overall health of patient, grade of the ovarian cancer, treatment received by the patient and how well the patient responds to treatment If general health of the patient is good, cancer is diagnosed at its early stage and if patient responds well to the treatment then the patient may survive much longer than relative 5-years and even in

some cases it may be cured but only 15-20% of ovarian cancers are diagnosed at its localized stage (stage I) (American Cancer Society, 2016)

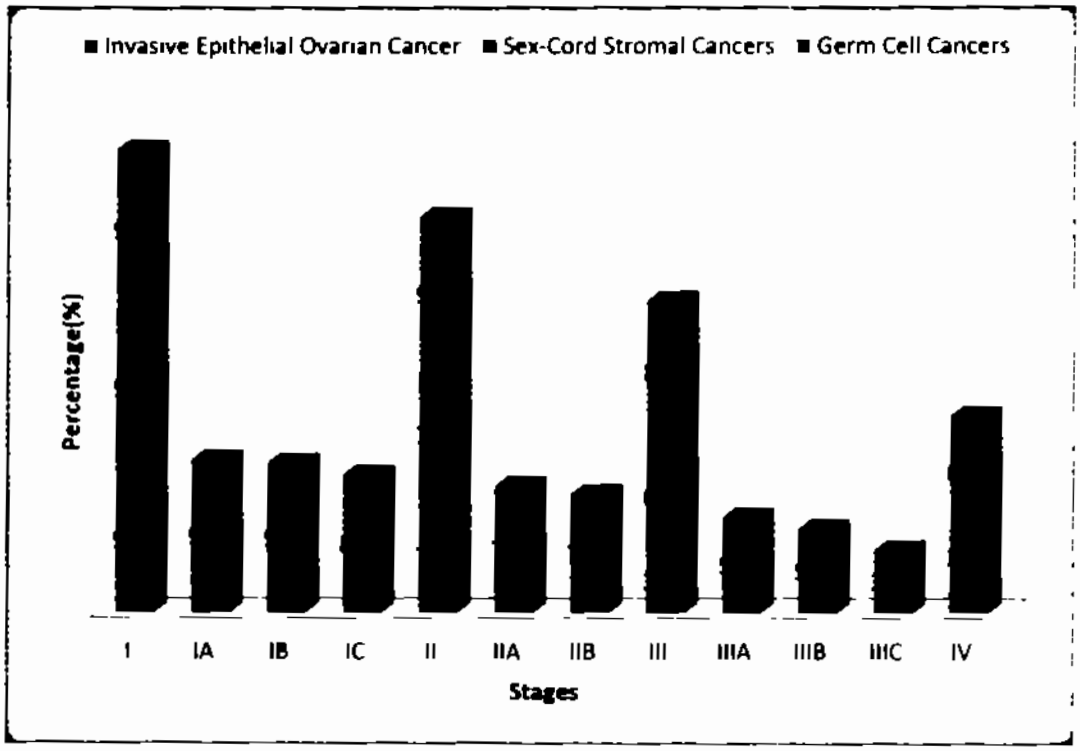


Figure 1.2: A graph representing relative 5-years survival rate of different types of ovarian cancers with different stages and sub-stages (Source American Cancer Society, 2016).

1.8. Estrogen Receptors and Ovarian Cancer

Estrogen receptors are a group of proteins that are activated by the estrogen hormone (17β-estradiol) They exist in two different forms, ERα and ERβ encoded by *ESR1* and *ESR2* genes respectively Once the receptors are activated by estrogen, the hormone binding dislocates heat-shock proteins (chaperones), after that receptor dimerization (homodimers or heterodimers) occurs and translocate into the nucleus Inside the nucleus the hormone receptor complex binds to a hormone response element (HRE) on the DNA and regulates the transcription of an enormous number of genes (Segnitz and Gehring, 1995) In an ovarian carcinogenesis estrogen receptor alpha regulate the expression of a variety of genes that encourage cell proliferation such as PI3K/Akt, TGF-α, EGF, cytokine IL-6, c-myc and IGF-I (Leung and Choi, 2007). Hence estrogen receptors act as a ligand responsive transcription factors and may interact with co-activators and proteins in order to regulate the activity of different genes One of the interacting proteins is BRCA1 with which it interacts at N-terminus, 1–300 amino acid residues (Welcsh and King, 2001) Variations within estrogen receptor genes (*ESR1* and *ESR2*) may have an impact on estrogen receptor and thus interrupts its normal functioning which in turn might induce mutations in *BRCA1* gene, suggesting its possible role in the occurrence of ovarian carcinoma

Similarly, loss of BRCA1 function in granulosa cells of the ovary has reported to cause the upregulation of an enzyme aromatase An increased expression of this enzyme leads to the increased production of estrogen from androgens (Hu *et al*, 2005) This proposes that *BRCA1* mutation does not only affect DNA damage repair but also increase the risk of ovarian cancer by producing increased levels of estradiol

1.9. Literature Review

Estrogen mediates its mode of action through two receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) that are ligand responsive transcription factors ERα is encoded by *ESR1* gene located on chromosome 6q25 1 while ERβ is

encoded by ESR2 gene located on chromosome 14q22-24 (Lindgren *et al*., 2004) The human ERα protein consists of 595 amino acids and possesses a molecular weight of 66kDa while ERβ comprises of 534 amino acids and has molecular weight of 54kDa

Like other nuclear receptors, estrogen receptors have five functional domains with different functions The A/B domains in the N-terminal contain activation function 1 (AF1) which is important for interaction with other proteins and co-activators and involves in the transcriptional activity of estrogen receptors This domain also encompasses amino acids that undergo post transcriptional modifications in order to stimulate the activity of AF1 The C domain contains a DNA binding domain (DBD) that binds to the estrogen responsive element (ERE) on the DNA with the consensus sequence of 5'-GGTCANNNTGACC-3' in order to regulate the expression of target genes The D domain is a hinge region that connects the DBD and LBD together and involves in the stimulation of estrogen receptor signaling in cells after facilitating post-translational modifications of ERs The last E domain in the C-terminal, known as the activation function 2 (AF-2) contains a ligand-binding domain (LBD) that assists in an interaction with estrogen hormone The resulting hormone-receptor complex binds to the ERE on the DNA and activates transcription through AF-1 and AF-2 The last F-domain is the C-terminal elongation part of LBD (Lee *et al*., 2012) (Figure 1 3)

ERα and ERβ differ greatly in their functional domains The N-terminal A/B domains and C-terminal F-domain are the least conserved regions in both of them The A/B domains of both receptors exhibit only 15% identity while F-domains of both receptors exhibit only 18% identity The E-domain (ligand binding domain) is also moderately conserved and depicts only 59% amino acid similarity between both receptors, suggesting that both have different modes of action (Lazennec, 2006)

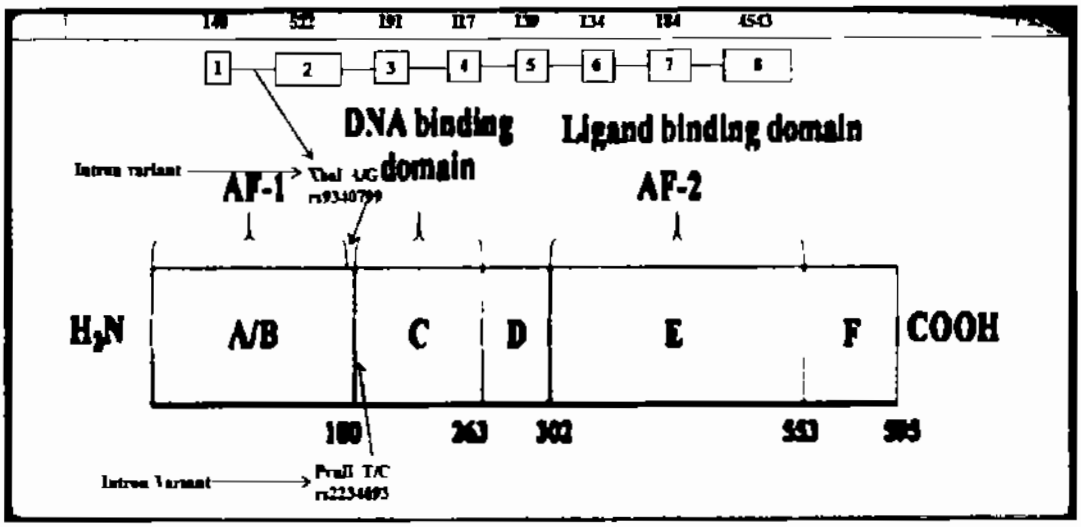


Figure 1.3: Functional domains of estrogen receptor alpha (ERα)
(Source: Ratanaphan, 2012)

There are also main differences between ERα and ERβ regarding their tissue distribution ERα is vastly expressed in the uterus, ovarian theca cells, Leydig cells in testes, prostate stroma, liver, breast and epididymis (Lane, 2008) ERβ is greatly expressed in testes, brain, ovarian granulosa cells, prostate epithelium and bone marrow (Weiser *et al* , 2008)

Several studies have recommended that estrogens may play an imperative role in ovarian carcinogenesis, through their receptors In a study the concentration of estrogen receptors in cytosol of malignant ovarian tissues was observed to be significantly dissimilar from those of normal ovaries and benign tumor tissues (P< 0 01) 22% of normal ovarian tissues were containing relative low concentrations of estrogen receptors ranging from 2 to 9 fmol/mg cytosol protein The estrogen receptors distribution was similar in benign tumors as well However, in malignant ovarian tissues, ERs were present in 57% of cases in concentrations ranging from 1 to 132 fmol/mg cytosol protein (Willcocks *et al* , 1983)

Sun *et al* (2005) conducted a study to examine the part of human estrogen receptor-related receptors (ERRs) in ovarian cancer cell lines They found that the expressions of the ERα were elevated in 58% of ovarian cancer cases as compared to the normal ovarian tissues ($P < 0.05$) While ERβ was inadequately expressed in the ovarian cancer tissues as well as in the normal ovarian tissues Their survival analysis indicated that ERα positive group had a worse overall survival (19 0 months vs 31 5 months, $P < 0.05$) Hence, their study indicated that ER alpha might play a significant role in the development of ovarian cancer

Pujol *et al* (1998) suggested that ER-alpha may be a marker of ovarian cancer after they revealed an increase in the expression of ERα mRNA in ovarian cancer relative to ERβ while comparing with normal ovarian tissue Similarly, another study has revealed that ERβ is chiefly expressed in normal epithelial ovarian tissues or benign ovarian tumors but ERα is highly expressed in malignant ovarian cancer (Cunat *et al.*, 2004)

Li *et al* (2003) recognized a two-fold up-regulation of ERα protein relative to ERβ in primary cells culture obtained from epithelial ovarian cancer while he was doing researches on ovarian cell cultures These all above findings favors an association between the up-regulation of ERα and occurrence of ovarian carcinoma

Polymorphism is defined as the presence of more than one allele at a given gene locus with a frequency greater than 1% and single nucleotide polymorphism (SNP) occurs when a single nucleotide change takes place at the level of DNA sequence Many variants of estrogen receptor alpha gene have been reported and it is assumed that these genetic variations may interrupts with estrogen actions on physiological processes Among all the polymorphisms in *ESR1* gene, 397T>C and −351A>G are the most widely studied They are also known as *Pvu*II and *Xba*I respectively because of the presence of restriction sites for these restriction enzymes

These polymorphisms are intron variants and located at the first intron of *ESR1* gene Their association with other diseases have already been reported, implantation failure in infertile women (Mirzapour *et al* , 2014), risk of non-small cell lung cancer (Chang *et al*., 2012), prostate cancer (Gu *et al* , 2014), idiopathic premature ovarian failure (Liu *et al* , 2013), infertility (Liaqat *et al* , 2015), endometrial cancer risk (Wedren *et al* , 2008), breast cancer (Boyapati *et al* , 2005), osteoporosis (Massart, 2005), and Alzheimer's disease (den Heijer *et al* , 2004)

Summarizing all the above literature it was concluded that estrogen receptors seem to play a significant role in the development of ovarian carcinoma, particularly ERα Association of its genetic variants rs2234693 and rs9340799 with ovarian cancer has not been documented yet in any population and it needs to be investigated Looking at the reported association of these variations with other above mentioned diseases, it seemed significant to investigate their association with risk of ovarian cancer, keeping in view the fact that association may vary from population to population

1.10. Statement of the Problem

In Pakistan, scarce data is available on ovarian cancer Very limited information is available on epidemiology, clinical presentation and genetic causes of ovarian cancer in Pakistani population Therefore, abundant work is required to investigate the genetic risk factors of ovarian cancer in Pakistan Furthermore, in Pakistan cancer treatment is limited to surgery, chemotherapeutic and radiotherapeutic drugs and most of the patients fail to respond them when their cancer is diagnosed at late stage, resulting into an increased rate of mortality due to ovarian cancer

1.11. Purpose of the Study

Considering the statement of the problem the purpose of the study was to investigate the association between genetic polymorphisms in estrogen receptor alpha gene with ovarian cancer in Pakistani population and to identify genetic risk factors of ovarian cancer with the aim of introducing targeted therapy for ovarian cancer in Pakistan

1.12. Objectives of the Study

The objectives of the study were

- To analyze the collected clinical information of patients and negative controls in order to assess the relative frequencies of histological types of ovarian tumors
- To examine the association of estrogen hormone receptor alpha gene single nucleotide polymorphisms (rs2234693 and rs9340799) with risk of ovarian cancer in Pakistani Population
- To assess the role of estrogen receptor gene variations with disease presentation

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1. Study Subjects

A total of 193 cases (including 184 Formalin Fixed Paraffin Embedded Tissues and 09 fresh tumor samples preserved in formalin) and 177 age & sex matched negative control tissue samples (FFPE) were collected in a period of 7 months Formalin Fixed Paraffin Embedded Tissues of 2012 to 2016 were collected from the tissue repositories of Histopathology Department of Islamabad Hospital and Pakistan Institute of Medical Sciences (PIMS) Islamabad and fresh tumor samples preserved in formalin were collected routinely from the Gynecology Department of PIMS Hospital, Islamabad Women presented with malignant, benign or borderline ovarian neoplasm were taken as cases and women presented with unremarkable ovary, polycystic ovary, corpus luteal or corpus albicans were taken as negative controls

2.2. Sampling Technique

Consecutive sampling technique was used to recruit the participants from the study population of laboratory in both groups cases and controls The study included surgical specimens of Ovarian Pathology received in the department of Histopathology All the surgical specimens were preserved in 10% formalin Their emblematic sections taken during gross examination after which they were processed regularly in automatic tissue processor The paraffin embedded blocks were then cut into thin slices through microtome and stained with haematoxylin/eosin for pathological examination Thin slices of about 8-10μ of paraffin embedded tissues were collected in 2ml eppendorf tubes in order to extract the DNA for genetic analysis

2.3. Data Resources

Primary data (clinical information) was collected from laboratories records, from medical records and files Principle investigator was engaged in all data collection activities

2.4. Settings

The study was conducted at Institute of Biomedical and Genetic Engineering (IB&GE) Islamabad.

2.5. Inclusion and Exclusion Criteria

All cases and controls with clinical information and consent (for fresh tumor samples) available were included in the study and all those cases and control samples were excluded from the study for which the consent was not taken and clinical information was not available Therefore, after applying the exclusion criteria a total of 7 samples (cases) were excluded and the study was performed on 186 cases and 177 negative control samples

2.6. Ethical Consideration

- The study was approved by the Ethics Committee of Pakistan Institute of Medical Sciences, Islamabad and Institute of Biomedical and Genetic Engineering, Islamabad and was in consonant to the Helsinki declaration
- Informed consent was taken from the patients at the time of surgeries for collecting fresh tumor samples

2.7. Techniques Used in the Study

Following techniques were used in order to investigate the association of *ESR1* gene's A351G (rs9340799) and T397C (rs2234693) single nucleotide polymorphisms with ovarian cancer

2.7.1. DNA Extraction

DNA from fresh tumor samples was extracted directly by adding the Lysis Buffer (Table 2 1) while the DNA extraction from Formalin Fixed Paraffin Embedded Tissues (FFPE) for genetic analysis was a three days process consisted of an additional step of adding xylene in order to remove paraffin

2.7.1.1. DNA Extraction Steps

Day 1: 500µl xylene was added in each sample in order to remove paraffin The samples were then mixed vigorously through vortex and incubated at 55°C in water bath for 10 min After incubation, the samples were centrifuged for 5 minutes at maximum speed (14000rpm) and supernatant was discarded The same steps were repeated one or two times if needed, in order to remove the paraffin completely Once the paraffin was removed perfectly, the samples were washed with 500µl absolute ethanol in order to remove xylene The samples were then centrifuged for 5 minutes at maximum speed (14000rpm) and supernatant was discarded In the next step, 1ml of deionized water was added in each sample and incubated at room temperature for 2-3 minutes after which the samples were again centrifuged for 5 minutes at maximum speed of 14000rpm In the last step of day 1, 500µl of Lysis Buffer (Table 2 1) and 20µl of Proteinase K were added in the samples and the samples were then incubated in water bath at 55°C overnight

Day 2: The samples were taken out from the water bath and 500µl of 2 5M NaCl was added in each sample after which each tube was shaken vigorously The samples were then incubated on ice for 10 minutes After incubation, the samples were centrifuged at 6000rpm for ten minutes and the supernatant containing the DNA pipetted out in new tubes In the last step of day 2, 1ml of isopropanol was added in each sample that facilitated the precipitation of DNA After this the samples were refrigerated at -20°C overnight

Day 3: On day 3, the samples were taken out from -20°C and centrifuged at 14000rpm for 20 minutes Supernatant was discarded After this step, 500µl of 70% ethanol was added in each tube and the tubes were tapped slightly for few seconds after which they were centrifuged again at 14000rpm for 15 minutes Supernatant was discarded and the pellet was left to dry until it turns semitransparent Once the pellets dried, they were resuspended in 50µl to 200µl of Tris-EDTA (TE) buffer (pH 8 0) After tapping the tubes well the DNA quantity and optical densities (ODs) of the

samples were measured by spectrophotometer (Nanodrop 2000c, Thermo Scientific, USA) at 260nm and 280nm considering ratio 1 7-2 0 as ideal whereas ratio 1 8 the most ideal (Senguven *et al*., 2014) After taking the ODs, all samples were relocated in 1 5ml labeled eppendorf tubes and were stored at 4°C till use For PCR amplification, 100ng dilutions of stock DNA were made and unless PCR amplification was not carried out all the dilutions were stored at -20°C

2.7.2. Primer Designing

Primers are single stranded chains of oligonucleotides that bind to a particular complementary sequence of DNA The exact order of nucleotides of primers should be known The primers for *ESR1* were designed by primer 3 free online primer design tool and then purchased commercially The primers ERA 93F and ERA 93R were used to amplify a 451bp region in estrogen receptor gene (*ESR1*) The primers LRA 99F and ERA 99R were used to amplify 524bp region in *ESR1* gene (Table 2 3)

2.7.3. Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP)

The 451bp and 524bp regions in *ERS1* gene to detect rs2234693 and rs9340799 SNPs, respectively were amplified using polymerase chain reaction (PCR) and were further used for genotyping by restriction fragment length polymorphism technique (RFLP)

The polymerase chain reaction is used to amplify our target gene This technique requires a template molecule DNA and two primers (forward and reverse) in order to initiate the process of amplification The PCR reactions were performed in 20µl of reaction volume Details of the reagents used in the PCR and their volumes are given in the (Table 2 2)

The thermal cycler program for the two polymorphic sites of *ER1* gene consisted of an initial melting step at 94°C for 3 minutes, followed by 30 cycles encompassing three steps each, denaturation at 94°C for 1 min, primer annealing at 62°C for 30 sec,

and primer extension at 72°C for 1 min and final extension temperature of 72°C for 5 min

The amplified products were then subjected to restriction fragment length polymorphism technique (RFLP) It is a most common technique used for analysis of known mutations The digestion reaction was performed in a 15µl of reaction volume Twelve microliters of PCR product, 0 5µl of enzyme, 1 5µl of 1X buffer and 1 5µl of water were used for restriction digestion of c 454-351 A>G and c 454-397 T>C 0 5 units of XbaI and PvuII restriction enzymes were used separately for digestion The amplified products were digested overnight at 37°C

2.7.4. Agarose Gel Electrophoresis

After digestion, the PCR amplified fragments were electrophoretically separated for about 60 min at a voltage of 100V on a 2% agarose gel 100bp ladder was used as a marker The 2% agarose gel was prepared by melting 2g of Agarose in 100ml TBE buffer in a microwave oven A small amount of ethidium bromide was also added during the preparation of a gel which aided in the visualization of DNA bands upon UV transillumination After 60 min an image of the gel was captured by gel documentation system (Uvitec Cambridge, UK)

2.7.5. Statistical Analysis

Data was analyzed using statistical approach Percentages were calculated of the genotype and allele frequencies in benign, borderline and malignant ovarian cancer cases and in negative controls and statistical significance of the differences in allelic frequencies and distribution of genotypes between cases and control groups were analyzed by logistic regression Statistical significance was fixed at p values < 0 05

Table 2.1 Required reagents for the composition of lysis buffer (50ml)

S.No	Reagents Required	Amount
1	1M Tris pH 8.0	500µl
2	5M NaCl	1ml
3	0 5M EDTA pH 8.0	1ml
4	10% SDS	2 5ml
5	Deionized H_2O	45ml

Table 2.2 Details of reagents used in polymerase chain reaction

S.No	Reagents	Stock Concentration	Final Volume
1	Taq buffer	10X	2µl
2	$MgCl_2$	25mM	2µl
3	dNTPs	50mM	1µl
4	Forward Primer	10uM	1µl
5	Reverse Primer	10uM	1µul
6	Taq DNA Polymerase	5U	0 25µl
7	DNA	100ng	1µl
8	dH_2O		11 75µl
	Total Volume		20µl

Table 2.3 Primer sequence of *ESR1* gene polymorphisms

Primers	Sequence 5'—3'	Annealing Temp.	Amplicon Length
ESR1_rs9340799_XbaI_F	GGGCTTAAACAATTCTC CTGCT	62°C	524bp
ESR1_rs9340799_XbaI_R	CATTACCTCTTGCCGTC TGTTG		
ESR1_rs2234693_PvuII_F	GATTCTCCCACCTCAGC CTTAC	62°C	451bp
ESR1_rs2234693_PvuII_R	ACCAATGCTCATCCCAA CTCTA		

CHAPTER 3

RESULTS

3.1. Epidemiological Aspects and Characteristics of Ovarian Cancer Patients

The collected clinical information of 186 ovarian cancer patients and 177 negative control samples was analyzed thoroughly in order to evaluate the frequency and patterns of ovarian lesions, to assess the laterality and grading of ovarian cancer patients and to find out the prevalence of disease in different age groups

All the ovarian cancer patients were categorized into three major categories of ovarian cancer with the highest percentage evaluated was of surface epithelial tumors (Table 3 1) Next all patients were divided into malignant, benign and borderline groups, malignant (74), benign (102) and borderline (10) patients (Table 3 2) Out of 74 malignant cases, FFPE blocks of 55 cases were available in the repository of histopathology department and were collected, 101 blocks of benign patients were available and all blocks of borderline cases were available and collected (Table 3 3)

All malignant cancer patients were subdivided into histologic malignant subtypes with the most common subtype evaluated was 'serous adenocarcinoma' (41 9%) (Table 3 5) Grading of malignant ovarian cancer patients was also assessed (Table 3 6) Similarly, all benign patients were subdivided into different histologic subtypes with the most frequent subtype calculated was 'serous cystadenoma' (47 06%) (Table 3 7) 'Borderline serous tumor' was calculated as the most common borderline ovarian tumor (Table 3 8)

All patients were also divided into different age groups in order to evaluate the most susceptible age for the diagnosis of benign and malignant ovarian cancer The diagnosis of benign tumor was observed to be highest between the second and third decade of life while the diagnosis of malignant ovarian cancer was assessed to be highest between the fifth and sixth decade of life (Figure 3 7) While evaluating the laterality of ovarian cancer patients, right ovary was observed to be more susceptible to the disease in all cases including benign, borderline and malignant (Figure 3 8)

Table 3.1 Categories of ovarian cancer and number of patients reside within each category

Category	No. of Patients	Percentage (%)
Surface Epithelial Tumors	153	82 3
Sex Cord Stromal Tumors	8	4 3
Germ Cell Tumors	25	13 4
Total	186	100

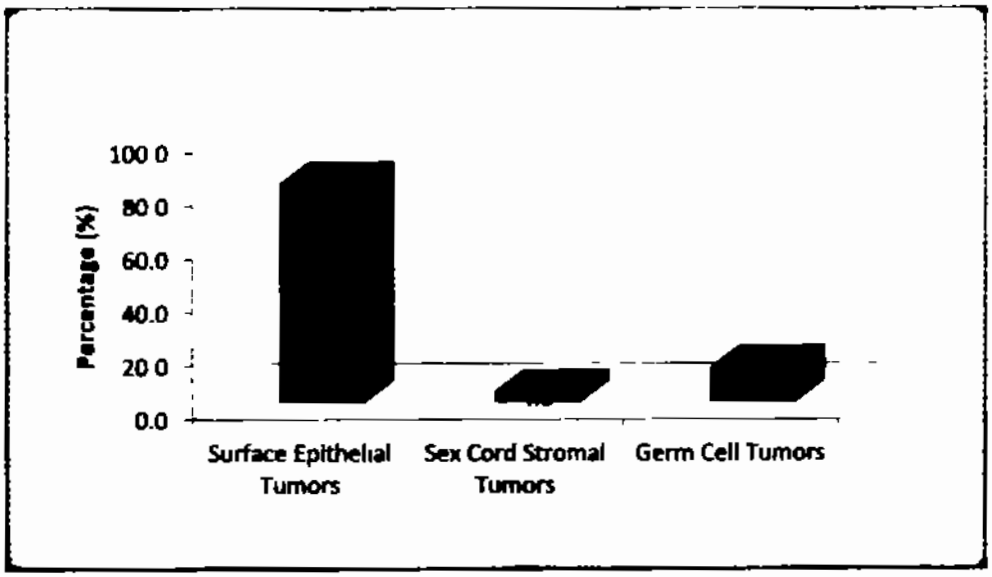


Figure 3.1: Graphical representation of ovarian cancer categories

Table 3.2 Distribution of Malignant, Benign and Borderline ovarian cancer patients

Ovarian Cancer	No. of Patients	Percentage (%)
Malignant	74	39 78
Benign	102	54.84
Borderline	10	5 38
Total	186	100

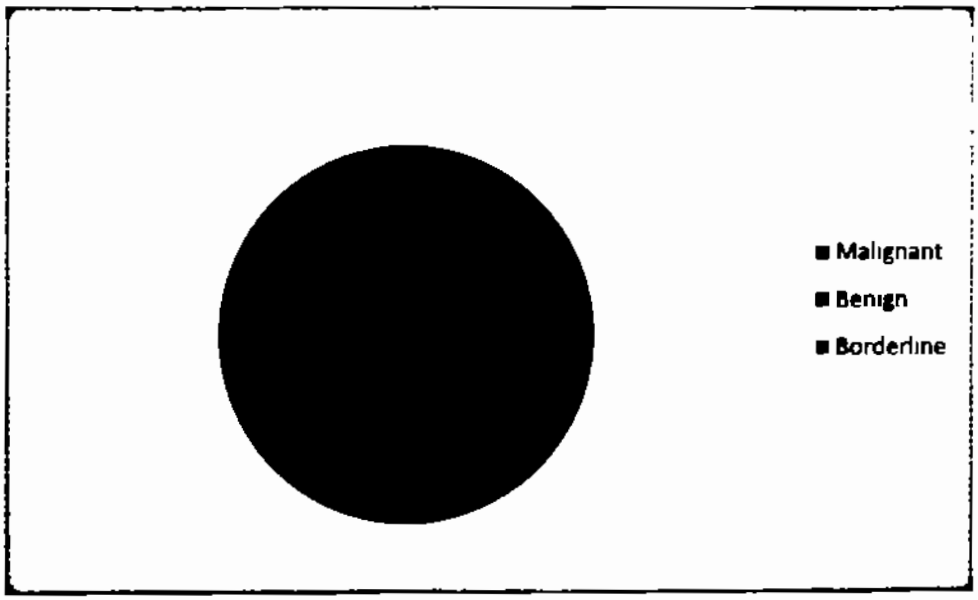


Figure 3.2: Pie diagram representing percentages of malignant, benign and borderline ovarian cancer cases.

Table 3.3 Represents the percentage of blocks available and the percentage of blocks not available of ovarian cancer patients

Ovarian Cancer	Cases with Available Blocks	Percentage (%)	Cases with Not Available Blocks	Percentage2 (%)	Total
Malignant	55	74	19	26	74
Benign	101	99	1	1	102
Borderline	10	100	0	0	10
Total	166		20		186

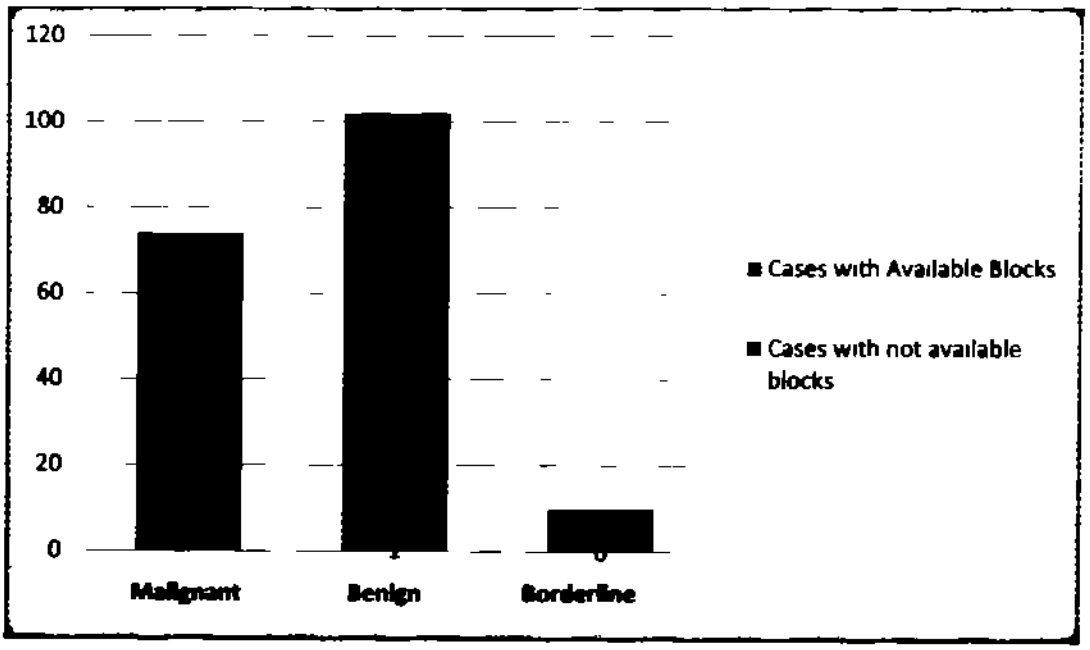


Figure 3.3: Column chart representing the number of blocks available and the number of blocks unavailable of malignant, benign and borderline cases.

Table 3.4 Details of collected negative control samples

Negative Control Samples	Number of Samples	Percentage (%)
Patients with no cancer history	129	72.9
Patients with uterine malignancy	1	0 5
Normal ovary of ovarian cancer patients	47	26 6
Total	177	100

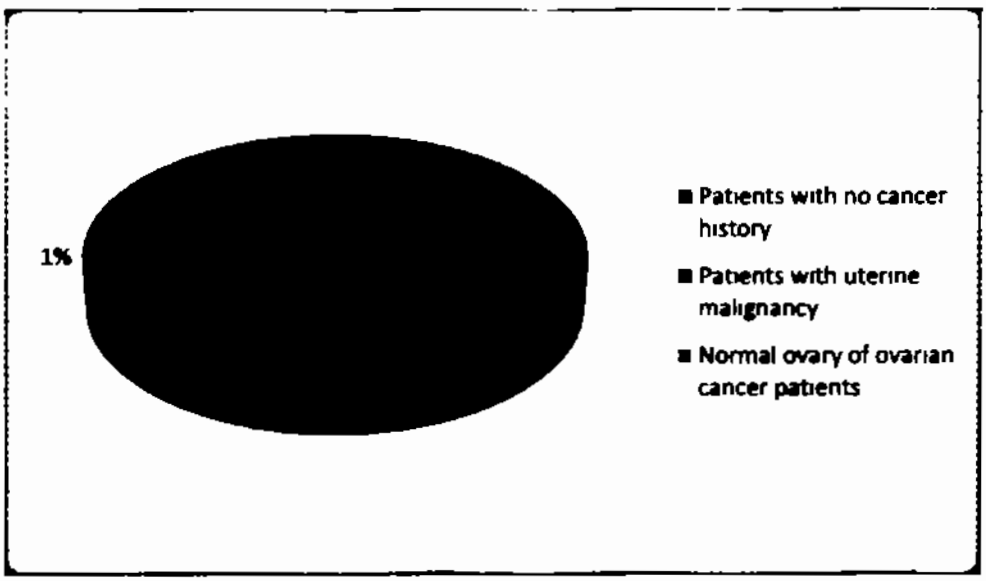


Figure 3.4: Pie chart illustrating categories of negative control samples

Table 3.5 Histologic subtypes of malignant ovarian cancer and the number of patients reside within each subtype

Histologic Sub Type of Cancer	No. of Patients	Percentage (%)
Serous Adenocarcinoma	31	41.9
Mucinous Adenocarcinoma	13	17 6
Endometrioid Adenocarcinoma	5	6 8
Clear Cell Adenocarcinoma	2	2 70
Carcinosarcoma	1	1 3
Granulosa Cell Tumor	4	5 4
Sertoli Leydig Cell Tumor	1	1 3
Germ Cell Tumor	6	8 1
Subtypes Unidentified	11	14 9
Total	74	100

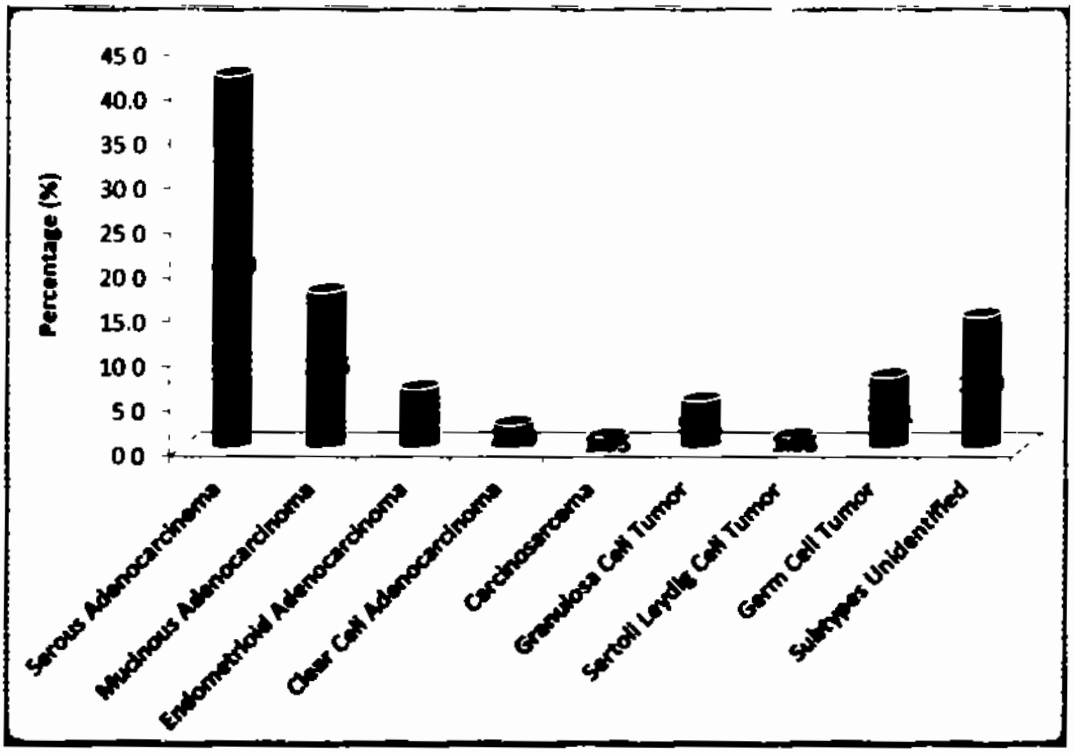


Figure 3.5: Graphical representation of histologic subtypes of malignant ovarian cancer. The most frequent type observed was 'Serous Adenocarcinoma'.

Table 3.6 Grading of malignant ovarian cancer

Grade	No. of Patients
Low Grade (G1)- Well differentiated	11
Intermediate Grade (G2)- Moderately differentiated	10
High Grade (G3)- Poorly differentiated	28
Grading Unidentified	25
Total	74

Table 3.7 Histologic subtypes of benign ovarian tumors and the number of patients lie within each subtype

Histologic Sub Type of Benign Tumor	No. of Patients	Percentage (%)
Serous Cystadenoma	48	47.06
Serous Cystadenofibroma	9	8 82
Mucinous Cystadenoma	23	22 55
Mature Cystic Teratoma (Dermoid Cyst)	19	18 63
Fibrothecoma	1	0 98
Fibroma	1	0 98
Seromucinous Cystadenoma	1	0.98
Total	102	100

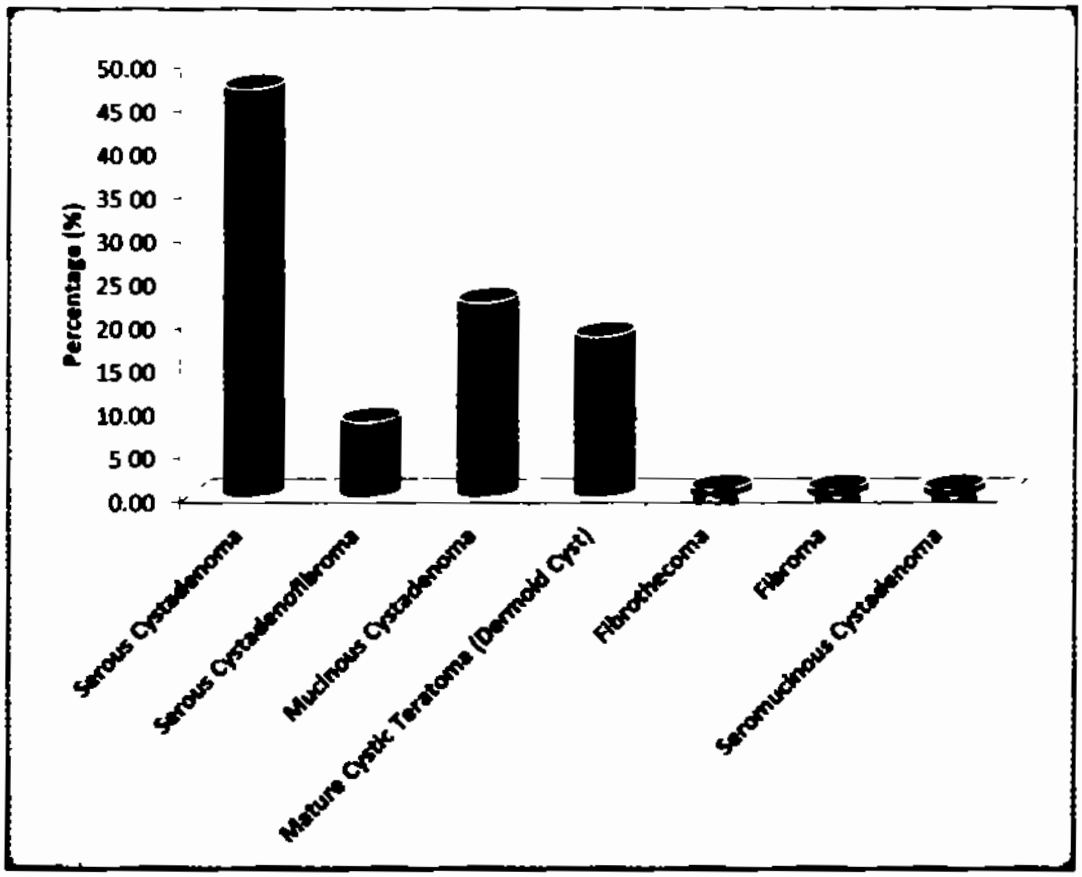


Figure 3.6: Graphical representation of histologic subtypes of benign tumors. The most frequent type observed was of Serous Cystadenoma.

Table 3.8 Histologic subtypes of borderline ovarian tumors

Histologic Sub Type of Tumor	No. of Patients
Borderline Seromucinous Tumor	1
Borderline Serous Tumor	6
Borderline Mucinous Tumor	3
Total	10

Table 3.9 Distribution of malignant, benign and borderline ovarian cancer patients according to the age groups

Age Groups	Malignant	Benign	Borderline
<20	6	8	1
20-34	10	41	5
35-44	18	24	0
45-54	17	18	1
55-64	16	9	2
65-74	7	2	0
>74	0	0	1
Total	74	102	10

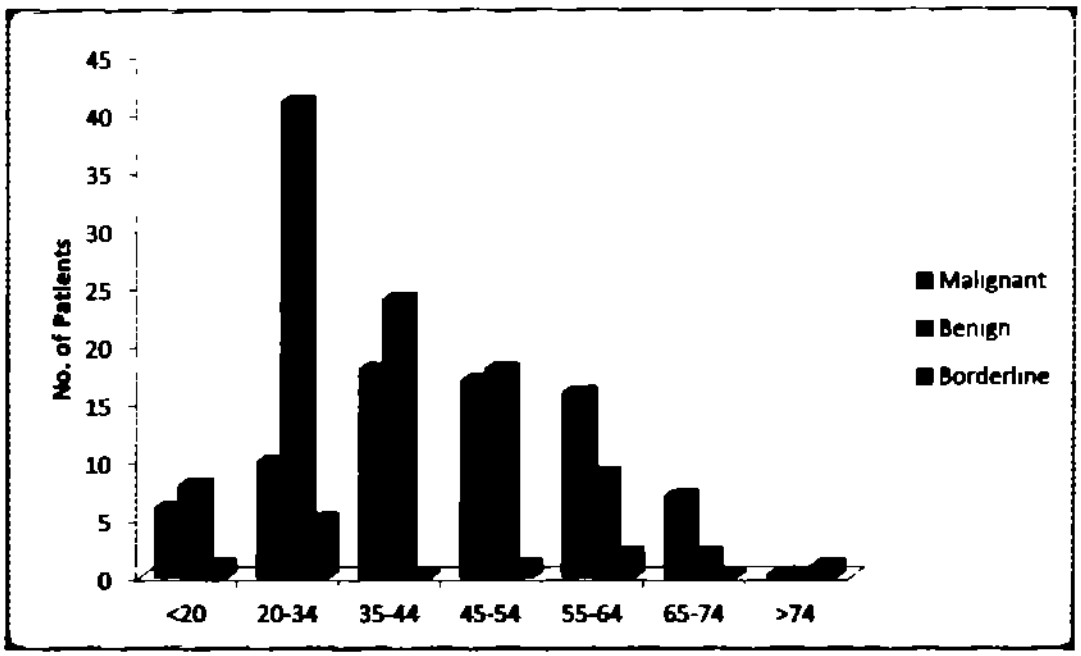


Figure 3.7: Graph representing distribution of ovarian cancer patients according to the age groups

Table 3.10 Laterality information of ovarian cancer patients

Laterality	Malignant cases	Benign cases	Borderline cases	Total	Percentage (%)
Right Ovary	20	39	4	63	34
Left Ovary	17	29	4	50	27
Bilateral	18	7	1	26	14 0
Not Specified	19	27	1	47	25
Total	74	102	10	186	100

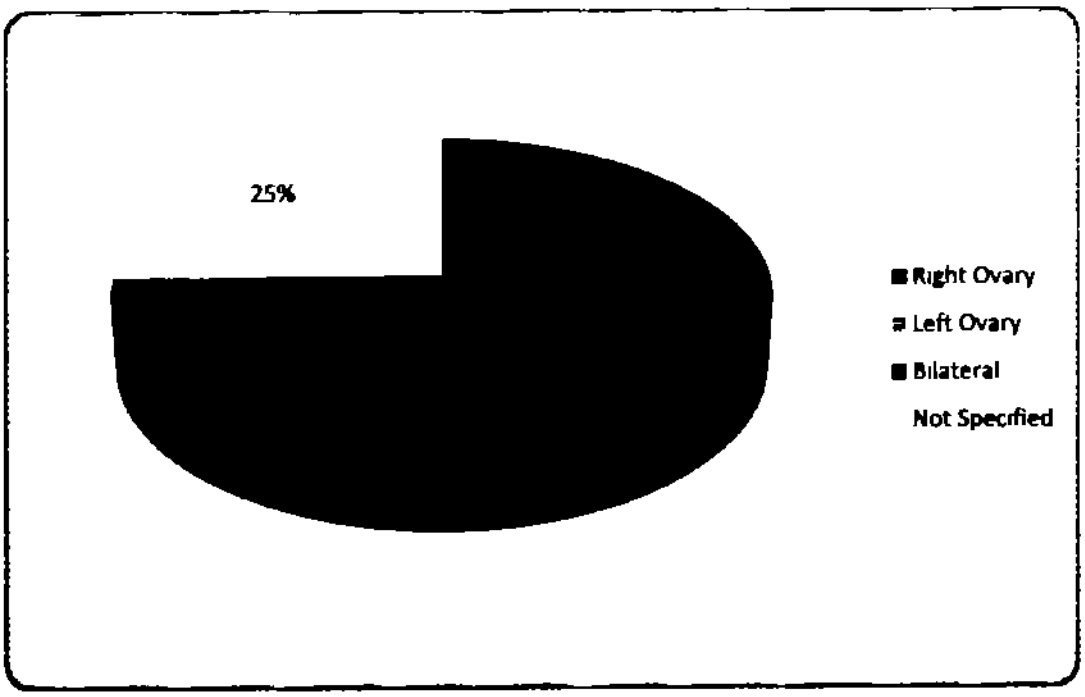


Figure 3.8: Pie chart representing laterality of ovarian cancer patients

3.2. Identification of Genetic Polymorphisms in *ESR1*

In order to assess genetic polymorphisms of *ESR1*, 2 SNPs were selected The detection of SNPs was performed by RFLP technique In order to detect rs9340799 polymorphism, 524bp region of *ESR1* gene was amplified first and then the PCR product was digested with *Xba*I restriction enzyme The digested fragments were then separated through gel electrophoresis and three different genotypes were obtained i e AA (Homozygous, wild type), AG (Heterozygous), GG (Homozygous, polymorphic), digestion through restriction enzyme yielded two fragments of 227bp and 297bp for wild type A allele and G allele yielded only one fragment of 524bp (Figure 3 9)

In order to detect rs2234693 polymorphism, a 451bp region of *ESR1* gene containing the polymorphism was amplified and then restriction digestion was performed by *Pvu*II enzyme Three genotypes were obtained when the digested fragments run on 2% agarose gel i e TT (Homozygous, for major allele), TC (Heterozygous), CC (Homozygous, for minor allele), restriction digestion yielded two fragments of 385bp and 66bp for T allele and only one fragment of 451bp for C allele (Figure 3 10)

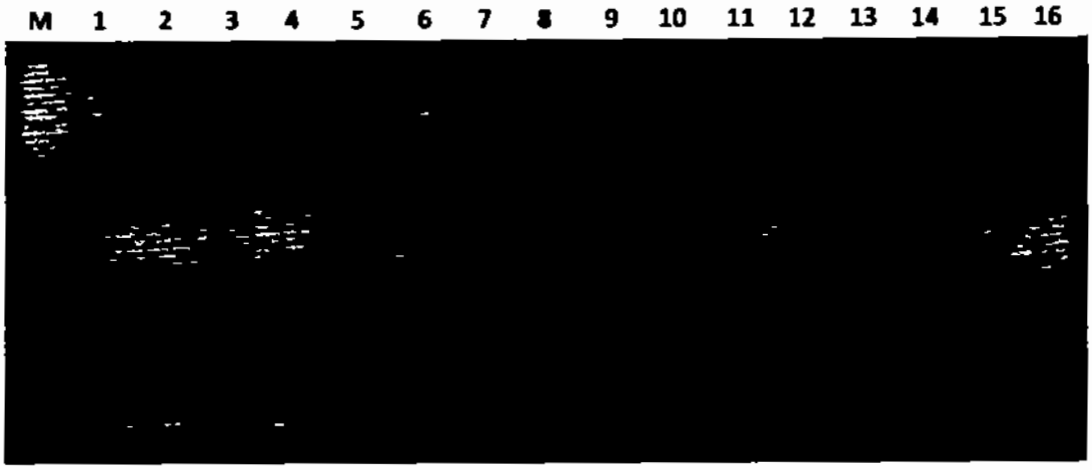


Figure 3.9: The restriction profile of ESR1 gene *XbaI* (c454-351). Lane M: DNA marker (100bp, gene ruler), lane 12: undigested PCR product, GG genotype (homozygous, polymorphic), lanes 3, 4, 8, & 11: AG genotype (heterozygous), lanes 1, 2, 5, 6, 7, 9, 10, 13, 14, 15, & 16: AA genotype (homozygous, wild type).

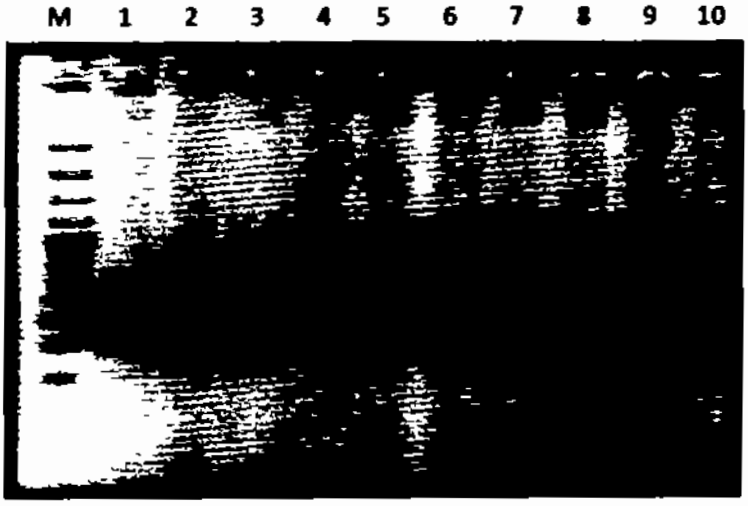


Figure 3.10: The restriction profile of ESR1 gene *PvuII* (c454-397). Lane M: DNA ladder (100bp), lanes 3, 4, 9, & 10: undigested PCR product, CC genotype (homozygous, polymorphic), lanes 2, 5, & 6: TC genotype (heterozygous), lanes 1, 7, & 8 TT genotype (homozygous, wild type).

3.3. Association between rs2234693 and Ovarian Cancer

Out of 177 negative control samples and 186 cases, 46 negative control samples and 79 samples of patients were typed for *ESR1* gene polymorphisms, rs2234693 and rs9340799 DNA of other samples was degraded and in some samples DNA was present in minute quantity which couldn't be amplified

3.3.1. Overall Analysis

In Controls, the genotype frequency distribution of homozygous ancestral genotype 'TT' was 26 087%, heterozygous genotype 'TC' was 50 0% whereas 23 913% of females were homozygous variant with genotype 'CC' and allele frequency of the wild type allele 'T' was 51 087% and that of the risk allele 'C' was 48 913% Among all cases (n=79), 24 1% were homozygous wild type 'TT', 50 6% of patients were heterozygous 'TC' and 25 3% were homozygous variant CC' In all cases, allele frequency distribution of the wild type allele 'T' was 49 4% and that of risk allele 'C was 50 6%. In Benign patients (n=53), 26 4% were homozygous wild type 'TT' 49 1% were heterozygous 'TC' and 24 5% were homozygous variant 'CC while the allele frequencies of wild type 'T' and risk allele 'C' were 50 9% and 49 1% respectively In Malignant patients (n=23), the genotype frequency distributions of 'TT', 'TC', 'CC' were 21 7%, 47 8%, and 30 4% respectively while the allele frequency of wild type 'T' and variant 'C' was 45 7% and 54 3% respectively Only 2 Borderline samples were amplified and in those patients (n=2), the genotypic frequencies of 'TT', 'TC' and 'CC' were 0%, 100% and 0% respectively and allele frequency of ancestral allele 'T' was 50% and that of risk allele 'C' was 50%

3.3.2. Comparison between all Cases and Controls

The data of all ovarian cancer patients and negative controls were compared statistically (Table 3 11) Logistic regression analysis did not reveal any significant variance between the genotype frequencies of cases and negative controls neither under the DM (OR=1 09, [95% CI=0.45-2 66], p=0 83) nor under the RM (OR=1 14, [95% CI=0 40-3 22], p=0 79)

Similarly, no significant difference was observed between the allele frequencies of all cases and negative controls (OR=1 07, [95% CI=0.64-1 79], p=0 79)

3.3.3. Comparison between Benign Group and Controls

No significant difference was observed between the genotype frequencies of benign samples and negative controls when the data was analyzed through logistic regression analysis neither under the DM (OR=0 96, [95% CI=0 37-2 51], p=0 94) nor under the RM (OR=1 01, [95% CI=0 33-3 08], p=0 98) (Table 3 12)

Univariate logistic regression analysis also did not reveal any significant difference between the allele frequencies of benign cases and negative controls (OR=1 00, [95% CI=0 57-1 75], p=0 98)

3.3.4. Comparison between Malignant Group and Controls

When statistically analyzed through logistic regression analysis, no significant difference was perceived between the genotype frequencies of malignant samples and negative controls neither under the DM (OR=1 14, [95% CI=0 32-4 07], p=0 83) nor under the RM (OR=1 52, [95% CI=0 37-6 25], p=0 55) (Table 3 12)

Similarly, univariate logistic regression analysis also did not reveal any significant difference between the allele frequencies of malignant samples and negative controls (OR=1.24, [95% CI=0 61-2 52], p=0 54)

3.3.5. Comparison between Benign and Malignant Groups

The data of the benign patients was also compared with malignant patients statistically (Table 3 12) No significant difference was detected between the genotype frequencies of benign patients and malignant patients when the data was analyzed through logistic regression; neither under the DM (OR=1 18, [95% CI=0 34-4 09], p=0 78) nor under the RM (OR=1 50, [95% CI=0 38-5 95], p=0 55)

Logistic regression analysis also did not divulge any significant difference between the allele frequencies of benign cases and malignant cases (OR=1 23, [95% CI=0 61-2 47], p=0 54).

Table 3.11 Genotype and allele frequency distribution of *ESR1* gene rs2234693 polymorphism among Total Cases and Controls

Genotype	Controls (N=46)	Total Cases (N=79)	Controls vs Total Cases OR (95%CI) (p-value)
TT	12 (26.087%)	19 (24.1%)	1
TC	23 (50%)	40 (50.6%)	DM 1 09(0 45-2 66) (0.83)
CC	11 (23 913%)	20 (25.3%)	RM.1 14(0.40-3 22) (0 79)
Allele Frequency	**Controls (N=92))**	**Total Cases (N=158)**	**Controls vs Total Cases OR (95%CI) (p-value)**
T	47 (51 087%)	78 (49 4%)	1
C	45 (48 913%)	80 (50.6%)	1 07(0 64-1 79) (0 79)

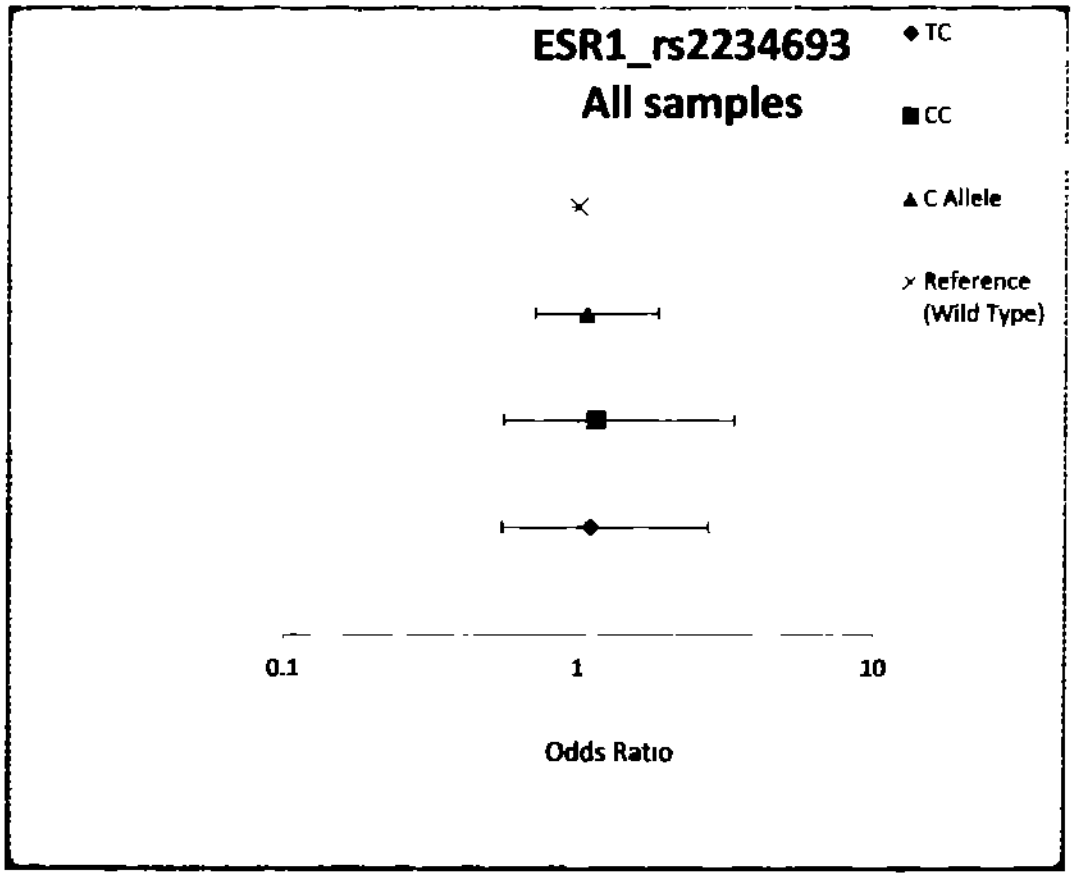


Figure 3.11: Graphical representation of non-significant difference in the genotype and allele frequencies of ERA 93 between all cases and negative controls

Table 3.12 Genotype and allele frequency distribution of *ESR1* gene rs2234693 polymorphism among Benign cases, Malignant cases and Controls

Genotype	Controls (N=46)	Benign (N=53)	Malignant (N=23)	Controls vs Benign OR (95%CI) (p-value)	Controls vs Malignant OR (95%CI) (p-value)	Benign vs Malignant OR (95%CI) (p-value)
TT	12 (26 087%)	14 (26 4%)	5 (21 7%)	1	1	1
TC	23 (50%)	26 (49 1%)	11 (47 8%)	DM 0 96(0 37-2 51) (0 94)	DM 1 14(0 32-4 07) (0 83)	DM 1 18(0 34-4 09) (0 78)
CC	11 (23 913%)	13 (24 5%)	7 (30 4%)	RM 1 01(0 33-3 08) (0 98)	RM 1 52(0 37-6 25) (0 55)	RM 1 50(0 38-5 95) (0 55)
Allele Frequency	**Controls (N=92)**	**Benign (N=106)**	**Malignant (N=46)**	**Controls vs Benign OR (95%CI) (p-value)**	**Controls vs Malignant OR (95%CI) (p-value)**	**Benign vs Malignant OR (95%CI) (p-value)**
T	47 (51 087%)	54 (50 9%)	21 (45 7%)	1	1	1
C	45 (48 913%)	52 (49 1%)	25 (54 3%)	1 00(0 57-1 75) (0 98)	1 24(0 61-2 52) (0 54)	1 23 (0 61-2 47) (0 54)

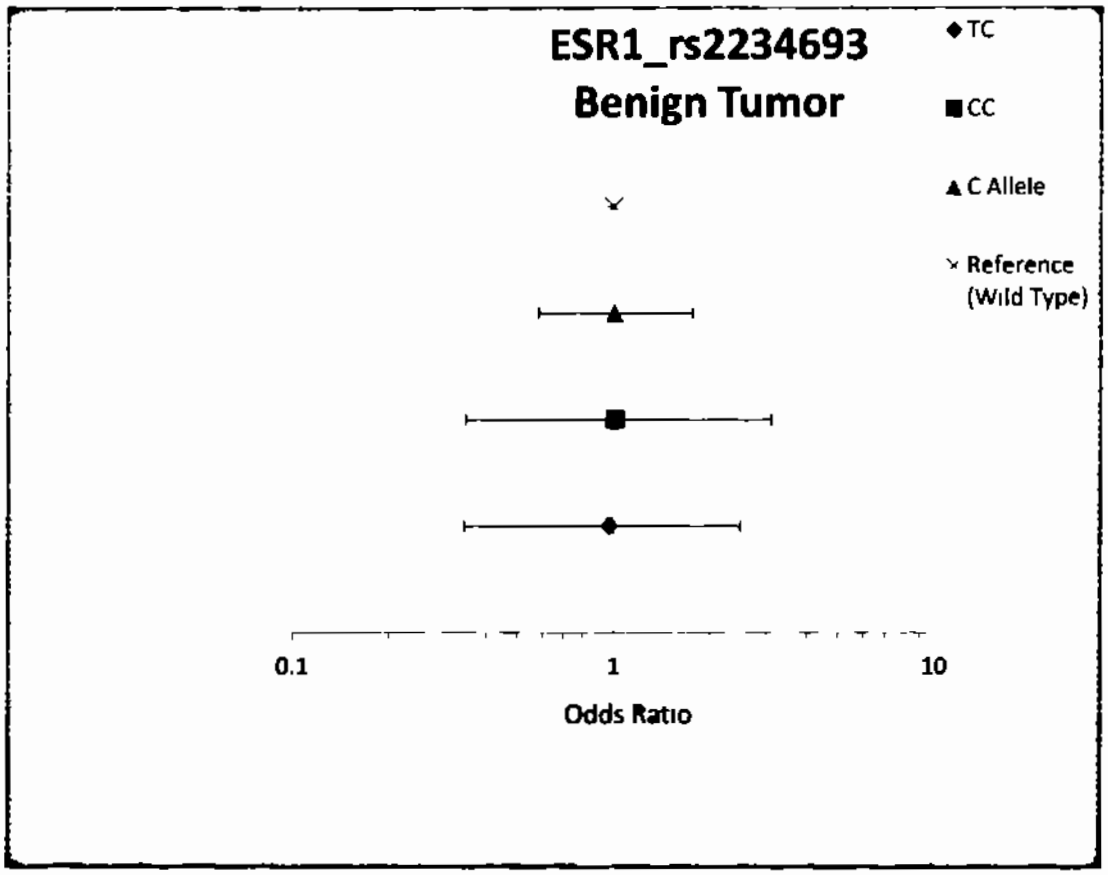


Figure 3.12: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 93 between benign group and control group.

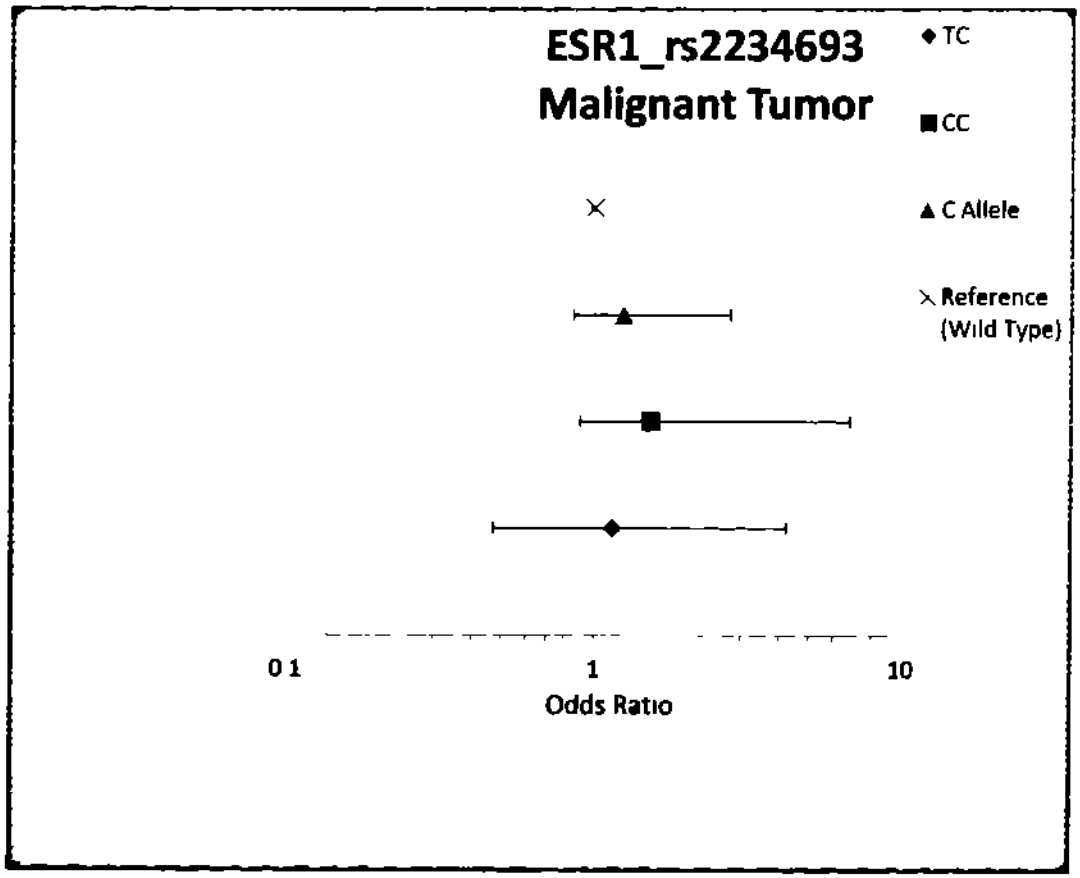


Figure 3.13: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 93 between malignant group and control group.

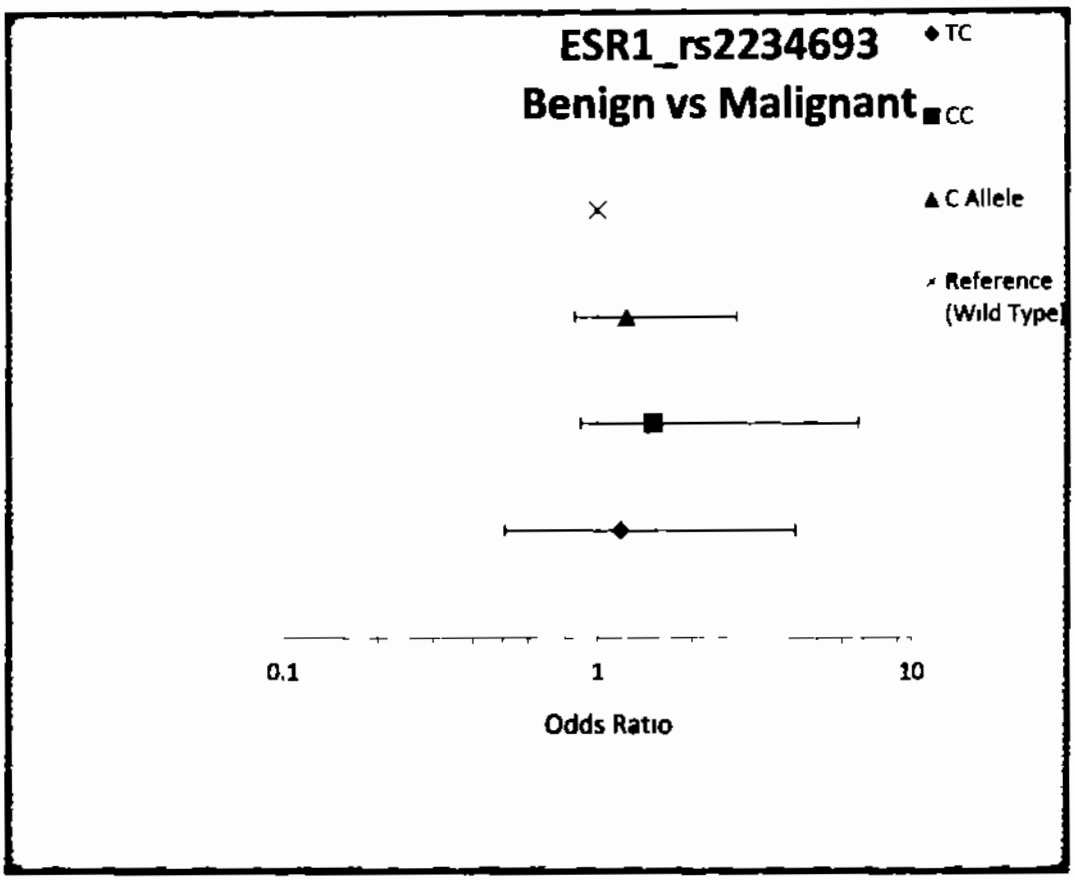


Figure 3.14: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 93 between benign group and malignant group.

3.4. Association between rs9340799 and Ovarian Cancer

3.4.1. Overall Analysis

In Controls, the genotype frequency distribution of homozygous ancestral genotype 'AA' was 71 739%, heterozygous genotype 'AG' was 26 087% whereas 2 1739% of females were homozygous variant with genotype 'GG' and allele frequency of the wild type allele 'A' was 84 783% and that of the risk allele 'G' was 15 217% Among all cases (n=79), 70 9% were homozygous wild type 'AA', 27 8% of patients were heterozygous 'AG' and 1 3% were homozygous variant 'GG' In all cases, allele frequency distribution of the wild type allele 'A' was 84 8% and that of risk allele 'G' was 15 2% In Benign patients (n=53), 69 8% were homozygous wild type 'AA', 28 3% were heterozygous 'AG' and 1 9% were homozygous variant 'GG' while the allele frequencies of wild type 'A' and risk allele 'G' were 83 96% and 16 04% respectively In Malignant patients (n=23), the genotype frequency distributions of 'AA', 'AG', 'GG' were 69 6%, 30 4%, and 0% respectively while the allele frequency of wild type 'A' and variant 'G' was 84 8% and 15 2% respectively Only 2 Borderline samples were amplified and in those patients (n=2), the genotypic frequencies of 'AA', 'AG' and 'GG' were 100%, 0% and 0% respectively and allele frequency of ancestral allele 'A' was 100% and that of risk allele 'G' was 0%

3.4.2. Comparison between all Cases and Controls

The data of all ovarian cancer patients and negative controls were compared statistically (Table 3 13) Logistic regression analysis did not reveal any significant variance between the genotype frequencies of cases and negative controls under the DM (OR=1 08, [95% CI=0.47-2 46], p=0 85) Significant variance between the genotype frequencies of cases and negative controls under the RM could not be assessed as the number of samples amplified with the genotype homozygous variant was only one

Similarly, no significant difference was observed between the allele frequencies of all cases and negative controls (OR=0 99, [95% CI=0 48-2 04], p=0 99)

3.4.3. Comparison between Benign Group and Controls

No significant difference was observed between the genotype frequencies of benign samples and negative controls when the data was analyzed through logistic regression analysis under the DM (OR=1 11, [95% CI=0 45-2 72], p=0 81) (Table 3 14) Significant difference between the genotype frequencies of benign cases and negative controls under the RM could not be evaluated as the number of samples amplified with genotype homozygous polymorphic was only one

Univariate logistic regression analysis also did not reveal any significant difference between the allele frequencies of benign cases and negative controls (OR=1 06, [95% CI=0 49-2 29], p=0 87)

3.4.4. Comparison between Malignant Group and Controls

When statistically analyzed through logistic regression analysis, no significant difference was perceived between the genotype frequencies of malignant samples and negative controls under the DM (OR=1.20, [95% CI=0 39-3 63], p=0 74) (Table 3 14) Significant difference between the genotype frequencies of malignant cases and controls under the RM could not be analyzed as no malignant sample with genotype homozygous polymorphic was amplified

Similarly, univariate logistic regression analysis also did not reveal any significant difference between the allele frequencies of malignant samples and negative controls (OR=1, [95% CI=0 37-2 67], p=1 00)

3.4.5. Comparison between Benign and Malignant Groups

The data of the benign patients was also compared with malignant patients statistically (Table 3 14) No significant difference was detected between the genotype frequencies of benign patients and malignant patients under the DM (OR=1 07, [95% CI=0 36-3 15], p=0 88)

Logistic regression analysis also did not divulge any significant difference between the allele frequencies of benign cases and malignant cases (OR=0 93, [95% CI=0 36-2 44], p=0 89)

Table 3.13 Genotype and allele frequency distribution of *ESR1* gene rs9340799 polymorphism among Total Cases and Controls

Genotype	Controls (N=46)	Total Cases (N=79)	Controls vs Total Cases OR (95%CI) (p-value)
AA	32 (71 739%)	56 (70 9%)	1
AG	12 (26 087%)	22 (27 8%)	DM 1 08(0 47-2 46) (0 85)
GG	01 (2 1739%)	01 (1 3%)	
Allele Frequency	**Controls (N=92))**	**Total Cases (N=158)**	**Controls vs Total Cases OR (95%CI) (p-value)**
A	78 (84 783%)	134 (84 8%)	1
G	14 (15 217%)	24 (15 2%)	0 99(0 48-2 04) (0 99)

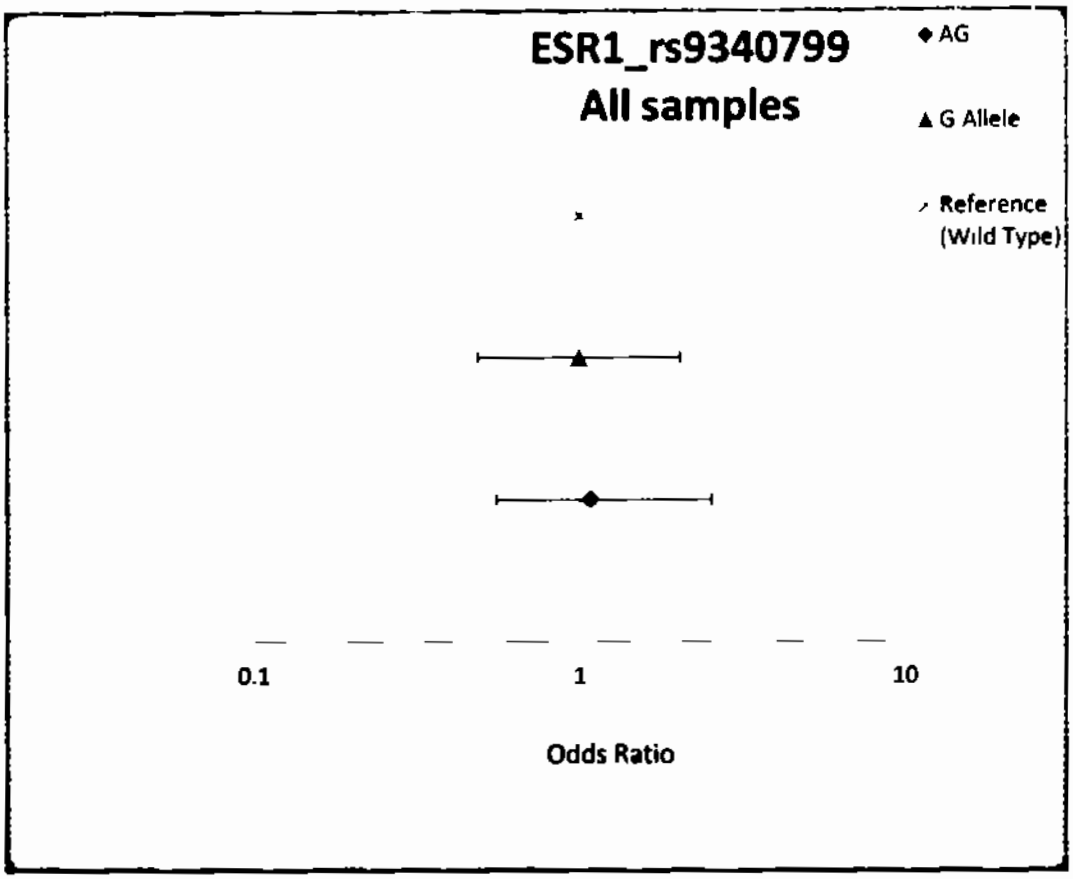


Figure 3.15: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 99 between all cases and controls.

Table 3.14 Genotype and allele frequency distribution of *ESR1* gene rs9340799 polymorphism among Benign cases, Malignant cases and Controls

Genotype	Controls (N=46)	Benign (N=53)	Malignant (N=23)	Controls vs Benign OR (95%CI) (p-value)	Controls vs Malignant OR (95%CI) (p-value)	Benign vs Malignant OR (95%CI) (p-value)
AA	33 (71 739%)	37 (69 8%)	16 (69 6%)	1	1	1
AG	12 (26 087%)	15 (28 3%)	07 (30 4%)	DM 1 11(0 45-2 72) (0 81)	DM.1 20(0 39-3 63) (0 74)	DM 1 07(0 36-3 15) (0 88)
GG	01 (2 1739%)	01 (1 9%)	0 (0 0%)			
Allele Frequency	**Controls (N=92)**	**Benign (N=106)**	**Malignant (N=46)**	**Controls vs Benign OR (95%CI) (p-value)**	**Controls vs Malignant OR (95%CI) (p-value)**	**Benign vs Malignant OR (95%CI) (p-value)**
A	78 (84 783%)	89 (83 96%)	39 (84 8%)	1	1	1
G	14 (15 217%)	17 (16 04%)	07 (15 2%)	1 06(0 49-2 29) (0 87)	1(0 37-2 67) (1 00)	0 93 (0 36-2 44) (0 89)

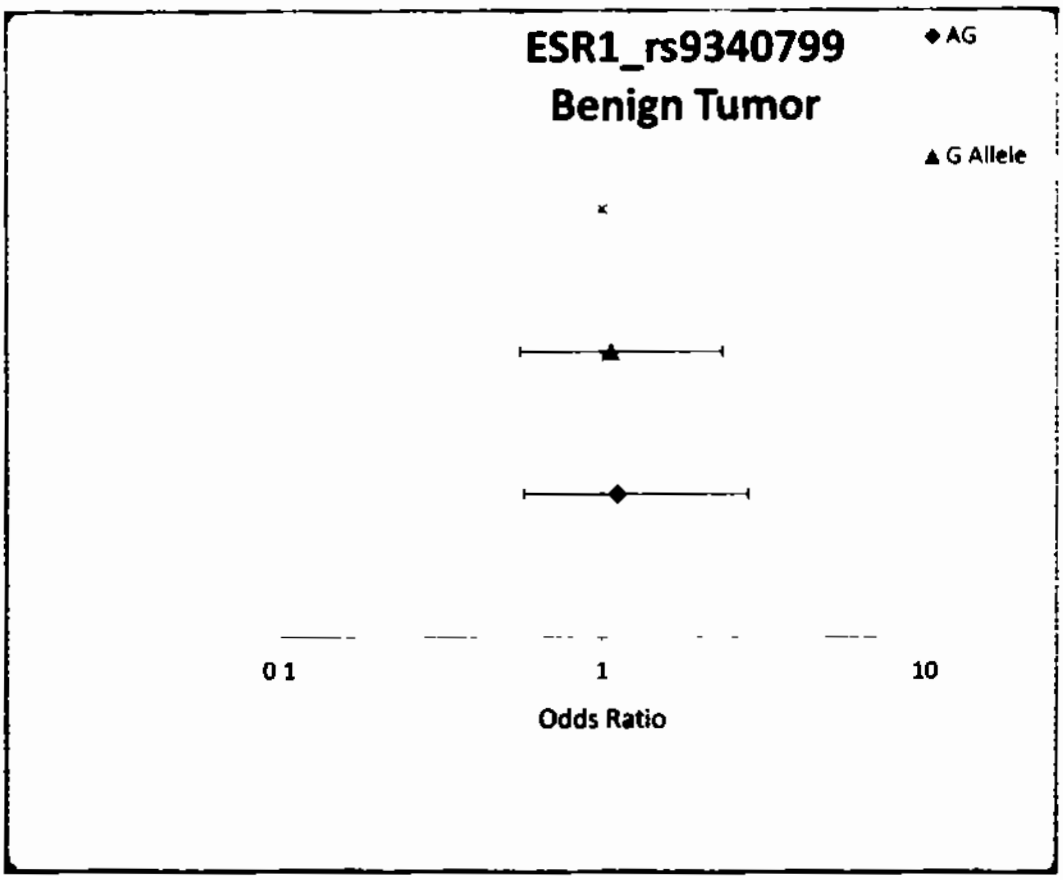


Figure 3.16: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 99 between benign group and control group.

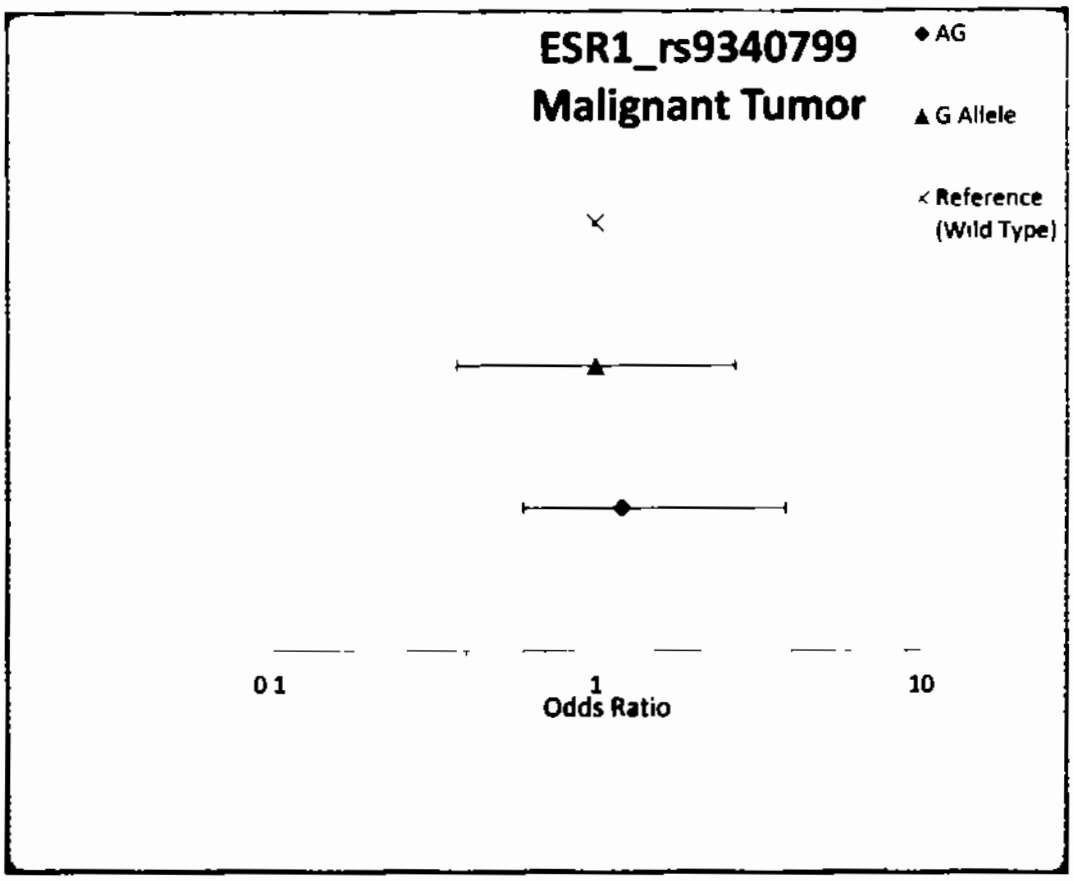


Figure 3.17: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 99 between malignant group and control group.

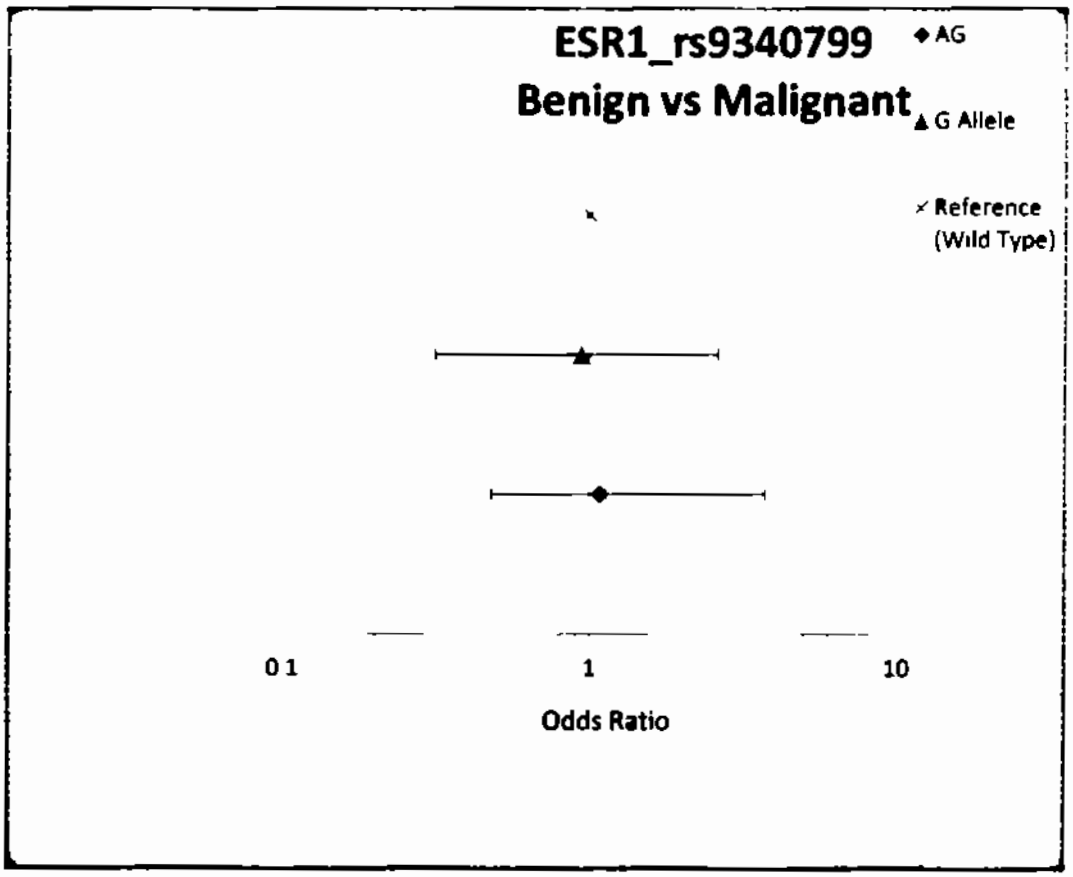


Figure 3.18: Graphical representation of non-significant difference in genotype and allele frequencies of ERA 99 between benign group and malignant group.

CHAPTER 4

DISCUSSION

In the current study 186 patients of ovarian cancer were taken as cases and 177 subjects were taken as negative controls All these cases and negative controls were used while evaluating the patterns and frequency of ovarian lesions in Pakistani population, laterality and grading of malignant ovarian cancer and while assessing the distribution of the disease in different age groups But while investigating the association of rs9340799 and rs2234693 single nucleotide polymorphisms of estrogen receptor alpha with risk of ovarian cancer in our population only 79 cases and 46 controls were amplified using polymerase chain reaction Some of the samples were degraded and some of them were containing the minute quantity of DNA which was insufficient for amplification

In our study the frequency of surface epithelial tumors was the highest among the major histological classes i e 82 3% (153/186) This finding is quite closer to the data reported in other studies from West (Guppy *et al*, 2005) and India (Tyagi *et al*, 1993) i e 90% and 70% respectively but does not correlate to the information reported in a study from Karachi, Pakistan i e 64% (Ahmad *et al*, 2000) This dissimilarity may be because of difference in the sample size or might be environmental and genetic factors are involved The frequency of sex cord stromal tumors in our study was evaluated as 4 3% (8/186) which is very similar to the frequency reported in the study that was carried out in the United Kingdom i e 5% (Morrison, 2005) The frequency of germ cell tumors in present study was 13 4% (25/186) which contradicts to the frequency reported in a study conducted in Pakistan recently i.e 43 31% (Ashraf *et al*, 2012) and the frequency reported in the study carried out in Nepal i e 42 2% (Jha and Karki, 2008) but somehow comparable to the frequency reported in two different studies carried out in Pakistan i e 27 13% (Ahmad *et al*, 2000) and 20 5% (Tanwani, 2005)

Out of 186 ovarian cancer cases, 74 patients were malignant (39 78%), 102 patients were benign (54 84%) and only 10 patients were of borderline tumor (5 38%) This finding is related to the data reported in a study conducted in Pakistan in which the reported frequency of benign tumors was 59 18% (506/855) and the frequency of

malignant tumors was 40 81% (349/855) (Ahmad *et al* , 2000) But our findings were slight varied from another study in Pakistan i e 46 4% (148/319) were benign tumors and 22 2% (71/319) were reported malignant in this study (Tanwani, 2005) Similarly our results were also dissimilar to the study carried out in Nepal According to their study the frequency of benign tumors was 83 9% (135/161) and the frequency of malignant cases was 16 1% (26/161) (Jha and Karki, 2008) This deviation might be because of different sample size or may be genetic and environmental factors are involved

While evaluating the frequency of malignant ovarian lesions the most frequent histologic subtype observed in our population was of 'serous cystadenocarcinoma' i e 41 9% (31/74) followed by 'mucinous cystadenocarcinoma' i e 17 6% (13/74) The most widespread histologic subtype of benign tumor was 'serous cystadenoma' i e 47 06% (48/102) followed by 'mucinous cystadenoma' i e 22 55% (23/102) and 'mature cystic teratoma' (dermoid cyst) i e 18 63% (19/102) These results correlate to the previous epidemiological studies of ovarian cancer in Pakistan Tanwani (2005) conducted a study to evaluate the most frequent histologic subtypes of benign and malignant ovarian cancer and his results were correlated to our study The most frequent benign tumor evaluated in his study was 'serous cystadenoma' (50 7%) followed by 'mucinous cystadenoma' (16 9%) and 'mature cystic teratoma' (18 9%) Similarly the most frequent malignant subtype assessed in his study was serous cystadenocarcinoma' (35 2%) followed by 'mucinous cystadenocarcinoma' (28 2%) Another study in Pakistan reported the same results as reported in our study i e the most common malignant subtype was 'serous cystadenocarcinoma' and the most commonest benign tumor was 'serous cystadenoma' according to their findings (Danish *et al*, 2012) Similarly the evaluation of the most prevalent histologic subtype of malignant ovarian cancer was also done in a study carried out in India and their results reported that the most prevalent type observed was of serous adenocarcinoma' (49 69%) in India and the second most prevalent type was 'endometrioid adenocarcinoma' (19 1%) followed by 'mucinous adenocarcinoma

(10 42%) (Saini *et al* , 2016), unlike in our population as in our study the second most prevalent type was 'mucinous adenocarcinoma'. A study conducted in Egypt also evaluated 'serous cystadenocarcinoma' as the most common histologic subtype of malignant ovarian cancer i e 58% (Mostafa *et al* , 2012) The findings of a study carried out in Nepal on 161 cases of ovarian cancer contradicts to our observations regarding the most frequent benign tumor but were similar regarding the most prevalent malignant ovarian cancer subtype. Their data reported that the most prevalent benign tumor in their population was 'mature cystic teratoma' (48 2%) and the most common malignant tumor was 'serous adenocarcinoma (46 2%) (Jha and Karki, 2008) Similarly another study that was carried out in Lahore, Pakistan reported the same results as presented in our study regarding the most frequent malignant subtype but varied regarding the most common benign ovarian tumor Their data showed that the most frequent benign tumor in our population was 'dermoid cyst' (31/82) followed by 'serous cystadenoma' (20/82) and the most prevalent malignant tumor was 'serous cystadenocarcinoma' (11/45) followed by 'mucinous cystadenocarcinoma' (9/45) (Ashraf *et al* , 2012) Life style factors or genetic factors or the difference in the sample size could be the possible reason for this deviation in the relative frequencies of the histological patterns of ovarian cancer

In our study the benign tumors has observed to be more common than malignant ones in the age groups, <20, 20-34, 35-44, and 45-54 The benign ovarian tumors have found to culminate between the second and the middle of third decade of life while the malignancy has observed to be increased as compared to the benign tumors in the age groups ranging from 55-64 and continue to increase as the age increases This finding is similar to the ovarian cancer statistics in the United States, reported recently in 2016 Their data showed that the number of malignant ovarian cancer patients was highest in the age group of 55-64 i e 24 2% (SEER Cancer Statistics Review, 2016)

Another aspect of the present study was to investigate the association of rs2234693 (*PvuII*) and rs9340799 (*XbaI*) single nucleotide polymorphisms (SNPs) with risk of ovarian cancer in Pakistani population This was the first time that the association of these SNPs with ovarian cancer risk was investigated in any population Previously no such study was available that documented the association of these genetic polymorphisms of estrogen receptor alpha with risk of ovarian cancer

In case of *PvuII* polymorphism, no significant association was observed between this polymorphism and risk of ovarian cancer There was an insignificant difference in heterozygous genotype TC (P=0 83) and homozygous variant genotype CC (P=0 79) between cases and controls Similarly an insignificant change was observed in risk allele 'C' between cases and controls (P=0 79) Further investigations were made and an association of *PvuII* polymorphism was determined separately with benign group and malignant group of patients, also benign group was compared with malignant group but the results were insignificant

Similarly, in case of *XbaI* polymorphism, no significant association was observed The difference in the heterozygous genotype AG distribution between cases and controls and difference in the polymorphic G allele frequency between cases and controls was insignificant (P=0 85 and P=0 99, respectively) The results were insignificant also while comparing the genotype and allele frequencies between benign group and controls, between malignant group and controls and between benign group and malignant group

Although we didn't find association between estrogen receptor alpha gene intron variants rs2234693 and rs9340799 and risk of ovarian cancer in our population but it is obvious from the previous studies that ERα plays a significant role in ovarian carcinogenesis (Willcocks *et al*, 1983, Pujol *et al*, 1998, Li *et al*, 2003, Cunat *et al*, 2004, Sun *et al*, 2005) There is another single nucleotide polymorphism 19kb downstream of *ESR1* gene i e rs2295190 (G>T) that was found to be associated

with risk of invasive ovarian cancer, odds ratio (OR) of 1 24 [95% confidence interval (CI), 1 06-1 44, P=0 006] (Doherty *et al* , 2010)

In our population the heterozygous genotype AG of *Xba*I polymorphism and heterozygous genotype TC of *Pvu*II polymorphism has been reported to be associated with the risk of infertility (Liaqat *et al*., 2015) Similarly these polymorphisms have also been found to be associated with idiopathic premature ovarian failure in Chinese population (Liu *et al*., 2013) but do not found to be associated with the same disease in Serbian women and do not contribute to the genetic basis of the disease (Li *et al* , 2013), suggesting that the association of genetic polymorphisms with a particular disease or with different diseases may vary from population to population

The frequency of homozygous polymorphic genotype GG of rs9340799 SNP has observed to be very less in our population but need further investigations for confirmation and to determine whether the findings are generalizable to other populations

Conclusion

This study was performed to achieve two major goals Although we couldn't find association between ERA 93 and ERA 99 single nucleotide polymorphisms with risk of ovarian cancer in our population, possible reason for which could be small sample size but we successfully evaluate frequency and patterns of ovarian cancer in our population Our results are suggesting that serous epithelial tumors are the most frequent occurring tumors in Pakistani population, therefore, assessing association specifically with them might be significant The reason for the degradation of large number of samples could be the sample storage conditions in the repositories of the histopathology departments of the respective hospitals as FFPE tissue samples need to be stored at relative low temperature but in the repositories they were stored at room temperature, might resulted into an increase number of sample degradation

Future Prospects

- Association of ERA 93 and ERA 99 with each of the particular histologic subtypes of ovarian cancer can be evaluated which might lead to an invention of targeted therapy against any particular subtype
- Expression analysis of ERα can be done in different subtypes of ovarian cancer in order to determine the estrogen receptor alpha positive and negative subtypes

CHAPTER 5

REFERENCES

Ahmad Z, Kayani N, Hasan S H, Muzaffar S., and Gill M S (2000) Histopathological pattern of ovarian neoplasm. *J Pak Med Assoc*, 50, p 416-9

Ashraf A, Shaikh S A, Ishfaq A, Akram A, Kamal F, and Ahmad N (2012) The relative frequency and histopathological pattern of ovarian masses *Biomedica*, 28, p 98-102

Bennett L M, McAllister K A, Malphurs J, Ward T, Collins N K, Seely J C, Gowen L C, Koller B H, Davis B J, and Wiseman R W (2000) Mice heterozygous for a Brca1 or Brca2 mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol *Cancer Res*, 60 p 3461–3469

Boyapati S M., Shu X O, Ruan Z X., Cai Q, Smith J R, Wen W, Gao Y T, and Zheng W (2005) Polymorphisms in ER-alpha gene interact with estrogen receptor status in breast cancer survival. *Clin Cancer Res*, 11, p 1093–1098

Chang S, and Risch H A (1997) Perineal talc exposure and risk of ovarian cancer *Cancer*, 79, p 2396–2401.

Chang H L., Cheng Y J., Su C K., Chen M C, Chang F H, Lin T G, and Yang C C (2012) Association of estrogen receptor α gene *Pvu*II and *Xba*I polymorphisms with non-small cell lung cancer *Oncology Letters*. 3(2). p 462–468

Chen V W, Ruiz B, Killeen J L, Cote T R, Wu X C, Correa C N, and Howe H L (2003) Pathology and classification of ovarian tumors *Cancer*, 97, p 2631-2642

Cook J (2002) Family history of ovarian cancer *Curr Obstet Gynaecol*, 12, p 47-51

Christie M, and Oehler M K (2006). Molecular pathology of epithelial ovarian cancer *J Br Menopause Soc*, 12

Cramer D W, and Xu H (1995) Epidemiologic evidence for uterine growth factors in the pathogenesis of ovarian cancer *Ann Epidemiol*, 5, p 310–314

Cunat S , Hoffmann P , and Pujol P (2004) Estrogens and epithelial ovarian cancer *Gynecol Oncol*, 94, p 25–32.

Danish F , Khanzada M S , Mirza T , Aziz S , Naz E , and Khan M N (2012) Histomorphological spectrum of ovarian tumors with immunohistochemical analysis of poorly or undifferentiated malignancies *Gomal J Med Sci*, 10, p. 209-15

Doherty J A , Rossing M A , Cushing-Haugen K L , Chen C , Van Den Berg D. J , Wu A. H , Pearce C L (2010) *ESR1/SYNE1* polymorphism and invasive epithelial ovarian cancer risk an Ovarian Cancer Association Consortium study *Cancer Epidemiology, Biomarkers & Prevention A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 19*(1), p 245–250

Ferlay J , Soerjomataram I , Dikshit R., Eser S , Mathers C , Rebelo M , Parkin M J , Forman D , and Bray F (2014) Cancer incidence and mortality worldwide sources, methods and major patterns in GLOBOCAN 2012 *Int J Cancer*, 136, p 359-386

Ferlay J , Foucher S E , Tieulent L J , Rosso S , Coebergh W W J , Comber H , Forman D , and Bray F (2013). Cancer incidence and mortality patterns in Europe Estimates for 40 countries in 2012 *EJC*, 49, p 1374-1403

Gemignani M L , Schlaerth A C , Bogomolniy F , Barakat R R , Lin O , Soslow R , Venkatraman E , and Boyd J (2003) Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma *Gynecol Oncol*, 90, p 378-381

Goff B A , Mandel L S , Drescher C W , Urban N , Gough S , and Schurman K M (2007) Development of an ovarian cancer symptom index possibilities for earlier detection *Cancer*, 109(2), p 221-7

Gu Z , Wang G , and Chen W (2014) Estrogen receptor alpha gene polymorphisms and risk of prostate cancer a meta-analysis involving 18 studies *Tumor Biol*, 35, p 5921-5930

Guppy A E., Nathan P D , and Rust G J (2005) Epithelial Ovarian Cancer a review of current management. *Clin oncol (R coll Radiol)*, 17, p 399-411

Hanif M , Zaidi P , Kamal S , and Hameed A (2009) Institution-based cancer incidence in a local population in Pakistan Nine year data analysis *Asian Pacific J Cancer Prev*, 10, p 227-230

den Heijer T , Schuit S C , Pols H A , van Meurs J B , Hofman A , Koudstaal P J , van Duijn C M , Uitterlinden A.G , and Breteler M M (2004) Variations in estrogen receptor alpha gene and risk of dementia, and brain volumes on MRI. *Mol Psychiatry*, 9, p 1129–1135

Hilton J L , Geisler J P , Rathe J A , Hattermann-Zogg M A , DeYoung B , and Buller R E (2002). Inactivation of BRCA1 and BRCA2 in ovarian cancer *J Natl Cancer Inst*, 94, p 1396–1406

Howlader N , Noone A M , Krapcho M., Miller D , Bishop K , Altekruse S F , Kosary C L , Yu M., Ruhl J , Tatalovich Z , Mariotto A , Lewis D R , Chen H S , Feuer E J , and Cronin K A (eds) SEER Cancer Statistics Review, 1975-2013, National Cancer Institute Bethesda, MD, http //seer cancer gov/csr/1975_2013/, based on November 2015 SEER data submission, posted to the SEER web site, April 2016

Hu Y , Ghosh S , Amleh A , Yue W , Lu Y , Katz A , and Li R (2005) Modulation of aromatase expression by BRCA1· a possible link to tissue-specific tumor suppression *Oncogene*, 24, p 8343–8348

Jha R , and Karki S (2008) Histological pattern of ovarian tumors and their age distribution *Nepal Med Coll J*, 10, p 81-85

Jones L L (2004) Ovarian tumors an overview *Atlas Genet Cytogenet Oncol Haematol*, 8(2), p 110-114.

Lane P H (2008) Estrogen receptors in the kidney lessons from genetically altered mice *Gend Med*, 5, S11–S18

Lazennec G (2006) Estrogen receptor beta, a possible tumor suppressor involved in ovarian carcinogenesis *Cancer Letters*, 231(2), p 151–157

Lee H R , Kim T H , and Choi K C. (2012) Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse *Laboratory Animal Research*, 28(2), p 71–76

Leung P C. K , and Choi J H. (2007) Endocrine signaling in ovarian surface epithelium and cancer *Hum Reprod Update*, 13, p 143–162

Li A J , Baldwin R L , and Karlan B Y (2003) Estrogen and progesterone receptor subtype expression in normal and malignant ovarian epithelial cell cultures *Am J Obstet Gynecol*, 189, p 22–27

Li J , Vujovic S , Dalgleish R , Thomson J , Dragojevic-Dikic S , and Al-Azzawi F (2013) Lack of association between ESR1 gene polymorphisms and premature ovarian failure in Serbian women *Climacteric*, 17, p 247-251

Liaqat S , Hasnain S., Muzammil S , and Hayat S (2015) Polymorphism analysis in estrogen receptors alpha and beta genes and their association with infertile population in Pakistan *EXCLI Journal*, 14, p 1085–1094

Lindgren P R , Cajander S , Backstrom T , Gustafsson J A., Makela S , and Olofsson J.I (2004). Estrogen and progesterone receptors in ovarian epithelial tumors *Mol Cell Endocrinol*, 221, p 97–104

Liu L , Tan R , Cui Y , Liu J and Wu J (2013) Estrogen receptor α gene (ESR1) polymorphisms associated with idiopathic premature ovarian failure in Chinese women *Gynecol Endocrinol*, 29, p 182-185

Massart F (2005) Human races and pharmacogenomics of effective bone treatments *Gynecol Endocrinol*, 20, p 36–44.

Mirzapour H , Shafighian Z , and Shahidi Z (2014) Association of estrogen receptor 1 rs9340799 polymorphism with implantation failure in Iranian infertile women *Ann Mil Health Sci Res*, 12(3), p 116-120.

Moore M A , Ariyaratne Y , Badar F., Bhurgri Y , Datta K , Mathew A , Gangadharan P , Nandakumar A , Pradhananga K K , Talukder M H , Yeole B B , and Sobue T (2009). Cancer epidemiology in South Asia - past, present and future *Asian Pacific J Cancer Prev*, 10, p 49-67

Morrison J Advances in the understanding and treatment of ovarian cancer J Br Menopause Soc, 2005, 11 66-71

Mostafa M F , Etreby N E , and Awad N (2012) Retrospective analysis evaluating ovarian cancer cases presented at the clinical oncology department, Alexandria University *Alexandria Journal of Medicine*, 48, p 353-360

Murthy N S , Shalini S , Suman G , Pruthvish S , and Mathew A (2009) Changing trends in incidence of ovarian cancer - the Indian scenario *Asian Pac J Cancer Prev*, 10 (6), p 1025-30

Obata K , Morland S.J , Watson R H , Hitchcock A , Chenevix-Trench G , Thomas E.J , and Campbell I G. (1998) Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors *Cancer Res*, 58, p 2095-2097

Palacios J , and Gamallo C (1998) Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas *Cancer Res*, 58, p 1344-1347

Prat J , Ribé A , and Gallardo A (2005) Hereditary ovarian cancer *Hum Pathol*, 36, p 861–870

Pujol P , Rey J M , Nirde P , Roger P , Gastaldi M , Laffargue F , Rochefort H , and Maudelonde T. (1998) Differential expression of estrogen receptor-alpha and beta messenger RNAs as a potential marker of ovarian carcinogenesis *Cancer Res*, 58, p. 5367–5373

Ratanaphan, A (2012) A DNA repair BRCA1 estrogen receptor and targeted therapy in breast cancer *Int J Mol Sci*, 13(11), p 14898–14916

Ricciardelli C , and Oehler M K (2009) Diverse molecular pathways in ovarian cancer and their clinical significance *Maturitas*, 62, p 270-275

Roa B , Boyd A., Volcik K , and Richards C (1996) Ashkenazi Jewish population frequencies for common mutations in BRCA1 and BRCA2 *Nat Genet*, 14, p 185–187

Roy R , Chun J , and Powell S N. (2012) BRCA1 and BRCA2 different roles in a common pathway of genome protection *Nature Reviews Cancer*, 12, p 68-78

Ryerson A B , Eheman C , Burton J , McCall N , Blackman D , Subramanian S , and Richardson L C. (2007) Symptoms, diagnoses, and time to key diagnostic procedures among older U S women with ovarian cancer *Obstet Gynecol*, 109, p 1053–61

Saini S K , Srivastava S , Singh Y , Dixit A K , and Prasad S N (2016) Epidemiology of epithelial ovarian cancer, a single institution-based study in India *Clin Cancer Investig J*, 5, p 20-4

Salehi F , Dunfield L., Phillips K.P , Krewski D , and Vanderhyden B C (2008) Risk factors for ovarian cancer an overview with emphasis on hormonal factors *J Toxicol Environ Health B Crit Rev*, 11, p 301-321

Segnitz B , and Gehring U (1995) Subunit structure of the nonactivated human estrogen receptor *Proc Natl Acad Sci USA*, 92, p 2179–2183

Senguven B , Baris E , Oygur T , and Berktas M (2014) Comparison of methods for the extraction of DNA from formalin-fixed, paraffin-embedded archival tissues *Int J Med Sci*, 11(5), p 494-499

Singer G , Oldt R 3rd , Cohen Y , Wang B G , Sidransky D , Kurman R J , and Shih IeM. (2003) Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma *J Natl Cancer Inst*, 95, p 484-486

Singer G , Stöhr R., Cope L , Dehari R , Hartmann A , Cao D F , Wang T L , Kurman R.J , and Shih IeM (2005) Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis a mutational analysis with immunohistochemical correlation *Am J Surg Pathol*, 29, p 218-224

Sun P M , Wei L H , Sehouli J., Denkert C , Zhao D , Gao M , Sun X L , Litchtenegger W (2005) Role of estrogen receptor-related receptors alpha, beta and gamma in ovarian cancer cells *Zhonghua Fu Chan Ke Za Zhi*, 40, p 544-8

Tanwani A.k (2005) Prevalence and Patterns of ovarian lesions *Ann Pak Inst Med Sci*, 1, p 211-214

Tyagi S P , Maheswari V , Tyagi N , Saxena K , Sharma R , and Hameed I (1993) Solid tumors of the overy *J Indian med assoc*, 91, p. 227-30

Wedrén S , Lovmar L , Humphreys K , Magnusson C , Melhus H , Syvanen A C , Kindmark A , Landegren U , Fermér M L , Stiger F , Persson I Baron J A , and Weiderpass E (2008) Estrogen receptor alpha gene polymorphism and endometrial cancer risk--a case-control study, *BMC Cancer*, 8, p 322

Weiser M.J , Foradori C D , and Handa R J (2008) Estrogen receptor beta in the brain from form to function *Brain Res Rev*, 57(2) p 309–320

Welcsh P L , and King M R (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer *Hum Mol Genet*, 10 (7), p 705-713

Willcocks D , Toppila M., Hudson C.N , Tyler J P , Baird P J , and Eastman C J (1983) Estrogen and progesterone receptors in human ovarian tumors *Gynecol Oncol*, 16, p 246-53

Willner J , Wurz K , Allison K H , Galic V , Garcia R L , Goff B A , and Swisher E M (2007) Alternate molecular genetic pathways in ovarian carcinomas of common histological types *Hum Pathol*, 38, p 607-613

Young H A , Mills P K , Riordan D G , and Cress R D (2005) Triazine herbicides and epithelial ovarian cancer risk in central California *J Occup Environ Med*, 47, p 1148–1156

Zhong Q , Chen C F , Li S , Chen Y , Wang C C , Xiao J Chen P L , Sharp Z D and Lee W H (1999) Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response *Science*, 285, p 747–750

Book References:

Tavassoli F A , and Devilee P (Eds) (2003) World Health Organization Classification of Tumors Pathology and Genetics of Tumors of the Breast and Female Genital Organs Lyon, France IARC Press

Scully R E , and Sobin L H (1999) World Health Organization (International Histological Classification of Tumors) Histological typing of ovarian tumors, Volume 9 (2nd ed) New York, USA Springer Berlin

Reports Cited:

American Cancer Society (2016) Cancer Facts and Figures 2016 Atlanta, GA Retrieved from http //www cancer org/acs/groups/content/@research/documents/document/acspc-047079 pdf

Punjab cancer registry report (2014) Retrieved from http //www punjabcancerregistry org pk/reports/PCR_2014 pdf

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