Role of XRCC1 DNA repair gene polymorphisms (Arg^{399}Gln and Arg^{194}Trp) on outcome of Platinum based chemotherapy for Non-small cell lung cancer in North Indian Population

Dissertation

Submitted in partial fulfillment of the requirement for the award of degree of

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Submitted

Under supervision of

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CERTIFICATE

This is to certify that dissertation entitled, ***"Role of XRCC1 DNA repair gene polymorphisms ($Arg^{399}Gln$ and $Arg^{194}Trp$) on outcome of Platinum based chemotherapy for Lung Cancer in North Indian Population"*** submitted by Ms. Poonam Naik in partial fulfillment of the requirements for the award of M. Sc in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance. To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

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DECLARATION

I, the undersigned, hereby declare that the research work presented in the M. Sc dissertation entitled ***"Role of XRCC1 DNA repair gene polymorphisms ($Arg^{399}Gln$ and $Arg^{194}Trp$) on outcome of Platinum based chemotherapy for Lung Cancer in North Indian Population"*** has been carried out by me under the supervision and guidance of Dr. Siddharth Sharma, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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CHAPTER1

INTRODUCTION

Vulnerability of cells to physical and chemical agents including ionizing radiation and other toxic chemicals results in DNA damage. To offset the consequences, DNA repair pathway as a whole, safeguard the cell‟s vital genetic information from the impairment by DNA damaging agents, which can modulate the function of encoded proteins. Cells have evolved a network of DNA repair mechanisms to remove different types of DNA damage. There are five different types of DNA repair mechanism. Base excision repair (BER) is one of the major defense mechanism and described as a highly coordinated pathway of consecutive enzymatic reactions, which owes the removal of DNA lesions by many proteins (Dexheimer, 2013).

XRCC1 (X-ray cross complementing group 1) gene is one important component of BER. The *XRCC1* gene products play pivotal role in facilitating the repair of single strand breaks in mammalian cells formed by various exogenous and endogenous DNA damaging agents, (Caldecott, 2003) by acting as a scaffold and interacting with other DNA repair proteins through its different domains. Genetic polymorphisms that has been identified in *XRCC1* gene are at exon 6, codon 194 (C >T base substitution at position 26304, yielding an amino acid change, Arg to Trp) and at exon 10, codon 399 (G>A base substitution at position 28152, Arg to Gln), (Beabes *et al.,* 2001).

Polymorphism in DNA repair genes may be associated with individuals‟ difference in DNA repair efficiency and may prone an individual to cancer risk. Because reduced DNA repair capacity may lead to genetic instability and the damage left unrepaired may cause cancer.

Cancer is characterized by uncontrolled proliferation, progression, invasion and metastasis. When a cell breaks constraint of normal cell‟s division process and begins to follow its own directory for proliferation; it leads to cancer. These abnormal cells form a tumor which may nest in the tissue from which they originated or may get down, permeating nearby tissues via bloodstream and lymph vessels. It becomes life threatening when it starts disrupting the vital organ‟s functioning.

According to WHO Lung cancer is a major cause of cancer mortality worldwide accounting 1.59 million deaths (2012).

Lung cancer is the uncontrolled growth of abnormal cells that start off in one or both lungs; usually in the cells that line the air passages. Lung cancer is mainly of two types, non small cell lung cancer and small cell lung cancer. Non small cell lung cancer (NSCLC) accounts 80-85% while small cell lung cancer (SCLC) accounts for the remaining 15-20%.

Smoking is by far the biggest cause of lung cancer. 90% of lung cancer causes is due to smoking and a small proportion caused by exposure to second hand smoke in non smokers (passive smoking). Cigarette smoke contains a myriad of genotoxic agents and carcinogens such as nitrosamine 4-(methylnitroamine)-1-(3-pyridyl)-1-butanone (NNK) which is cancer causing agent (Natukula *et al.,* 2013) which can covalently bind to DNA and form adducts.

Chemotherapy has been the backbone of treatment for advanced Non-Small Cell Lung Cancer. Of the various types of chemotherapy regimens, Platinum drugs demonstrate activity in a wide range of tumors including ovarian, cervical, testicular, head and neck, and non–small-cell lung cancer and have been shown to improve overall survival. However, response rates to platinum-based regimens are less than 30% in NSCLC patients, compared with greater than 70% in ovarian, testicular, and head and neck cancer patients. The drugs paired with platinum include microtubule targeted agents and DNA damaging agents (Walsh *et al.,* 2008). Platinum compounds exert their effects through formation of DNA adducts which result in bulky distortion of DNA, destabilization of the double helix, inhibition of DNA replication and cytotoxicity of the drug and hence clinical outcome seems to be correlated with the level of platinum-DNA adducts in the circulation (Wei Q *et al.,* 2002). Therefore, an alternative hypothesis regarding DNA repair capacity and NSCLC outcome is that suboptimal DNA repair within the tumor actually may lead to the decreased removal of platinum-DNA adducts and therefore increased clinical response. According to this line of reasoning, deficit DNA repair capacity may predict better response to platinum chemotherapy and may be a prognostic factor for improved survival in advanced NSCLC (Matakidou *et al.,* 2007).

To understand this paradox in this thesis I have studied how polymorphisms in *XRCC1* DNA repair gene effects outcome of platinum based chemotherapy for lung carcinoma.

CHAPTER 2

REVIEW OF LITREATURE

- Cancer
- DNA repair
- DNA repair and Cancer
- XRCC1
- Polymorphism
- Chemotherapy

2.1 CANCER:

Cancer results from a series of molecular events that fundamentally alters the normal properties of cell. Cancer cells rapidly reproduce despite restriction space, nutrients, or signals sent from body for apoptosis producing population of cells called as tumor and tumor produce the symptoms which individual experience as cancer. DNA is in every cell and directs all its actions. In a normal cell, when DNA is damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn‟t die like it should. Instead, the cell goes on making new cells that the body doesn‟t need. These new cells all have the same damaged DNA as the first abnormal cell does (Varmus *et al.,* 1993).

2.1.1 LUNG CANCER:

Lung cancer, also known as bronchogenic carcinomas has become leading cause of cancer death worldwide. Based on current and projected smoking pattern, it is anticipated that lung cancer will be the leading cause of cancer death in world (American cancer society 2012).

2.1.2 ETIOLOGY:

Tobacco smoke:

Smoking is the leading cause of lung cancer, accounting for about 85% of lung cancer. Cigarettes contain multiple carcinogens (more than 60) that have been shown to induce cancer. The more we smoke, the more likely we are to get lung cancer but the length of time you have been a smoker is even more important than how many cigarettes you smoke a day (Arnold, 2004).

- Polycyclic aromatic hydrocarbons (PAH) such as benzo[α]pyrene produce mutations in the *p53* gene. G to T transversion within the p53 gene is a molecular signature of lung tumours caused by tobacco mutagens.
- N-nitroso compounds are a major group of chemicals found in tobacco smoke, several of which are potent animal carcinogens.
- Nicotine: causes addiction to cigarette smoking and is also a promoter for carcinogenesis.

Passive smoking (breathing in other people's cigarette smoke) increases the risk of lung cancer. The risk of lung cancer for passive smokers goes up the more cigarettes smoke they are exposed to. A non-smoker who lives with a smoker has about a 20% to 30% greater risk of developing lung cancer. Workers who have been exposed to tobacco smoke in the workplace are also more likely to get lung cancer. Secondhand smoke is thought to cause more than 3,000 deaths from lung cancer each year (Steliga *et al.,* 2011).

Genetics:

There is a growing realization that genetic constitution of an individual has influence on getting cancer that may predispose an individual to cancer or act as protective factor. This genetic susceptibility may result from inherited polymorphism in genes controlling carcinogen metabolism and repair of DNA damage .Mutation/polymorphisms in DNA repair genes are associated with repair efficiency of DNA damage and deficit in this repair mechanism may prone an individual to cancer risk (Chaudhry *et al.,* 2011)

Other risk factors:

Some other things increase lung cancer risk. But they increase the risk by only a small amount and far less than smoking. They are

- Exposure to radon gas
- Exposure to certain chemicals
- Air pollution
- Previous lung disease
- A family history of lung cancer
- Past cancer treatment
- Previous smoking related cancers
- Lowered immunity

2.1.3 CLASSIFICATION OF INVASIVE LUNG CANCER:

There are two major types of lung cancer, Non-small cell lung cancer and Small cell lung cancer both of which are further sub divided:

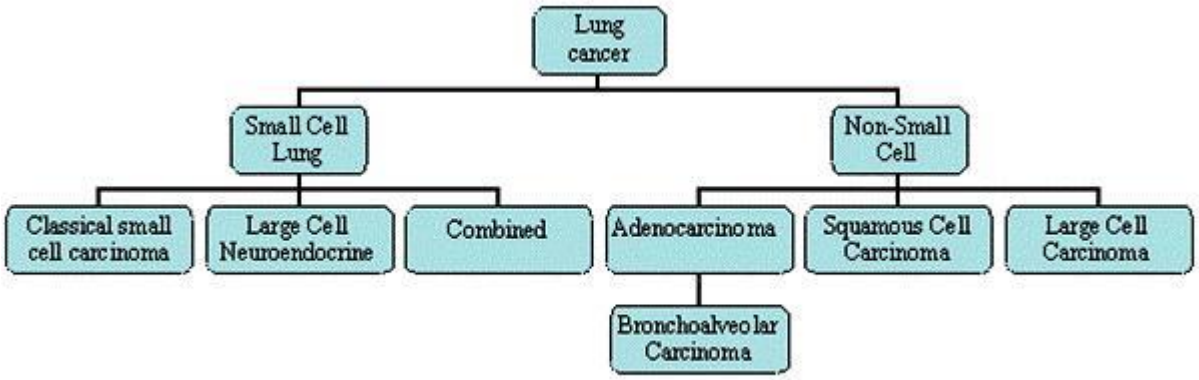


Figure: 2.1 Classification of invasive lung cancer (Ingledew *et al.,* 2013)

Small Cell Lung Cancer:

Small cell lung cancer (SCLC) accounts for10-15% of lung cancers. SCLC is strongly associated to cigarette smoking with only 1% of these tumors occurring in non-smokers. It‟s very rare for someone who has never smoked to develop it. Small cell lung cancer is called this because under the microscope the cancer cells look small and are mostly filled with the nucleus. It is also called oat cell cancer. SCLC grows more rapidly and metastasizes rapidly to many sites (brain, liver, bone) within the body and are most often discovered after they have spread extensively, leading to the worst prognosis. It is more responsive to chemotherapy and radiation. SCLC often spreads quite early on and so oncologist may recommend chemotherapy treatment rather than surgery. SCLC is a neuroendocrine carcinoma that exhibits aggressive behavior, frequent association with distinct paraneoplastic syndromes: commonly secrete ADH (SIADH) or ACTH (ectopic Cushing syndrome), including hypercalcemia (elevated calcium level in the blood), Eaton-lambert syndrome (muscle weakness of limbs), syndrome of inappropriate diuretic hormone and many others (Steliga *et al.,* 2011). It generally arises in central part of lungs *i.e.* from pulmonary

neuroendocrine cells, which are responsible for making neurotransmitters, growth factors, and vasoactive substances.

Non-small cell lung cancer:

Non-small cell lung cancer (NSCLC) is the most common lung cancer, accounting for about 85-90% of all cases. There are 3 main subtypes of NSCLC. The cells in these subtypes differ in size, shape, and chemical make-up when looked at under a microscope. These are grouped together because the way of response to treatment and prognosis (outlook) are often very similar. Occasionally it is not possible to work out which type of non small cell lung cancer the patient has. It may not be possible to tell if only a few cells are taken during a biopsy. It can also be difficult if the cells are undeveloped. Undeveloped cancer cells are known as undifferentiated cells. So oncologist will say that patient has undifferentiated non small cell lung cancer (Herbst *et al.,* 2008). This will not usually make any difference to patient‟s treatment, because most non small cell lung cancers are treated in the same way.

NSCLC has three main types designated by the type of cells found in the tumor. They are:

- Squamous cell carcinoma
- Adenocarcinoma
- Large cell carcinoma

Squamous cell (epidermoid) carcinoma:

Squamous cell carcinoma (SQCC) accounts about 25 to 30% of all lung cancers are; also known as epidermoid carcinomas. This cancer start in early versions of squamous cells, which are flat cells that line the inside of the airways in the lungs (Eldridge, 2014). They are strongly associated with smoking and tend to be found in the middle of the lungs, mostly intrathoracic spread rather than distant metastasis; therefore, best prognosis. SQCC tend to create obstruction and cause distal atelectasis (blockage of an airway).

Adenocarcinoma:

About 40% of lung cancers are adenocarcinomas (ADCC). They originate in early versions of the cells that would normally secrete substances such as mucus. This type of lung cancer occurs mainly in current or former smokers, but it is also the most common type of lung cancer seen in non-smokers. It is more common in women than in men, and more likely to occur in younger people than other types of lung cancer. Adenocarcinoma also develops from small airway epithelial and type II alveolar cells but it develops from a particular type of cells that produces mucus (phlegm) and tends to form glands and secrete mucin (Miller, 2005). It is often found in the outer areas of the lungs. It tends to grow slower than other types of lung cancer, and is more likely to be found before it has spread outside of the lung. People with a type of adenocarcinoma tend to have a better prognosis than those with other types of lung cancer. There are different subtypes of adenocarcinoma of the lung. Bronchiolo alveolar carcinoma (BAC) is one of these subtypes that frequently develops at multiple sites in the lungs and spreads along the preexisting alveolar walls. It may also look like pneumonia on a chest X-ray. It is increasing in frequency and is very common in non-smoking women and in the Asian population (Travis *et al.*, 2011).

Large cell (undifferentiated) carcinoma:

Large cell carcinomas, sometimes referred as undifferentiated carcinomas, are the least common type of NSCLC, accounting for 10-15% of all lung cancers. It can appear in any part of the lung. It tends to grow and spread quickly, which can make it harder to treat. A subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer that is very similar to small cell lung cancer (Brambilla, 2005). Large cell lung cancer is called this because the cells look large and rounded under a microscope. This type of cancer has a high tendency to spread to the lymph nodes and distant sites (Beasley *et al.*, 2005). Large cell carcinomas behave similar to adenocarcinomas but the lesions formed tend to be somewhat larger.

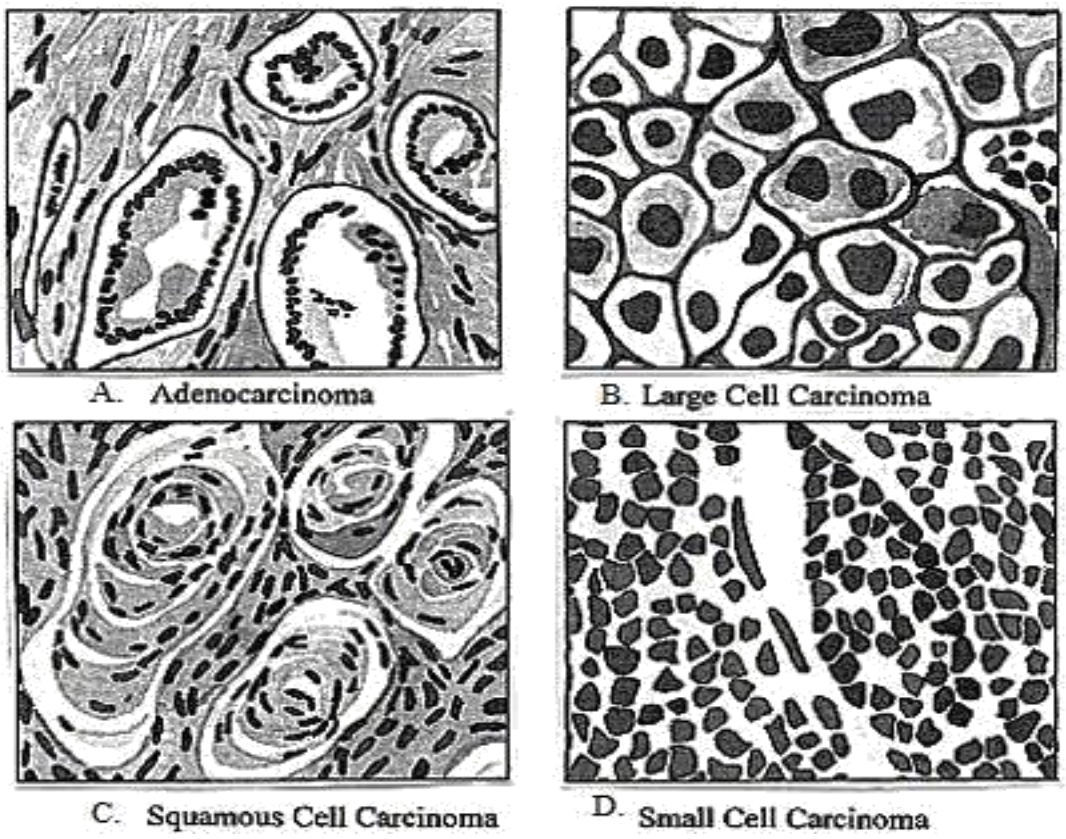


Fig 2.2: Different lung carcinomas. A. Adenocarcinoma; B. Large cell carcinoma; C. Squamous cell carcinoma; D. Small cell lung carcinoma (Source-Anfuso 2011).

2.2 DNA REPAIR:

Fifty years after discovery of the structure of DNA (Watson and Crick, 1953), DNA repair has become one of the most interesting topics in modern biology. Because DNA is the repository of genetic information in each living cell, its integrity and stability are essential to life. DNA however, is not inert; rather, it is a chemical entity subject to assault from the environment. Every day there is approximately 10^4 spontaneous DNA injuries inside every normal body cell. Luckily most of us filled with an insurance policy against such damages in form of our DNA repair mechanism in order to respond to alterations. Generally, these responses take one of two forms. Cells can either repair the damage and restore the genome to its normal physical and functional state, or they can tolerate lesions in a way that reduces their lethal effects (Friedberg *et al.,* 1995**).** DNA repair processes exist in both prokaryotic and eukaryotic organisms, and many of the proteins involved have been highly conserved throughout evolution. In fact, cells have evolved a number of mechanisms to detect and repair the various types of damage that can occur

to DNA, no matter whether this damage is caused by the environment or by errors in replication (Clancy 2008). In human > 70 genes are involved in the five major DNA repair pathways (Park *et al.,* 2002). The sequencing of the human genome (Lander *et al.,* and Venter *et al.,* 2001) yielded a first overview of the huge number of proteins involved in the protection of the genome DNA-repair genes can be sub-grouped into genes associated with signaling and regulation of DNA repair on the one hand and on the other into genes associated with distinct repair mechanisms.

2.3 DNA REPAIR AND CANCER:

DNA repair probably represent the most important cancer avoidance mechanism. Defects in these repair pathways in man are associated with a series of cancer-prone genetic disorders attests to the complexity of DNA repair and its relationship to carcinogenesis. Cells with defective DNA-repair mechanism generally show increased sensitivity to genotoxic agents and increased mutation rate. Individuals with inherited disorders of DNA repair display increased sensitivity, increased level of chromosomal aberration and mutation in somatic cells and predisposition to cancer (Karran *et al.,* 2003 and Lees *et al.,* 2003) e.g. a defect in XP (Xeroderma pigmentosum) results in individual who are very sensitive to UV light and have 1000 fold increase in the incidence of all skin types of skin cancer.

2.3.1 TYPES:

To compensate for the many types of DNA damage that occur, organisms have evolved a complex and intricate series of interrelated repair pathways by which all types of different subset of DNA lesions can be repaired and processed (Dexheimer 2013). At a minimum, most would agree that mammalian cells utilize five major DNA repair mechanisms**):** Base excision repair, direct repair, nucleotide excision repair, double-strand break repair, and repair of inter strand cross-links (Sancar *et al.,* 2004).

Table 2.1: Essential genes of the five major DNA repair mechanisms

Pathways		Genes involved
Base excision repair (BER)		DNA glycosylase, APE1, XRCC1, PNK, dRpase, DNA polymerase ß, FEN1, DNA polymerase δ or Ɛ ,PCNA, PARP
Nucleotide excision repair (NER)		XPC-Rad23B-CEN2, UV-DDB (DDB1-XPE), CSA,CSB, TFIIH, XPB, XPD, XPA, RPA, XPG, ERCC1-XPF, DNA polymerase δ or Ɛ
Direct repair		DNA photolyases, Methylguanine DNA methyltransferase
Cross link repair		Rad51, Rad52,XRCC2,XRCC3,RPA
Double strand break repair(DSB)	**Homologous recombination (HR)**	Mre11-Rad50-Nbs1, CtIP, RPA, Rad51, Rad52, BRCA1, BRCA2, Exo1, BLM-TopIIIα, GEN1-Yen1, Slx1-Slx4, Mus81/Eme1
	Non-homologous end-joining (NHEJ)	Ku70-Ku80, DNA-PKc, XRCC4-DNA ligase IV, XLF

BASE EXCISION REPAIR:

The term base excision repair (BER) was coined to emphasize that this DNA repair mechanism is characterized by the excision of nucleic acid base residues in the free form (Friedberg *et al*,. 1995).

Initial step of BER is characterized by release of the target base to form an abasic (AP) site in the DNA by DNA glycosylase (Figure 2.3). AP sites can also be a direct damage product (Memisoglu *etal.,* 2000). There are DNA glycosylases that recognize **(**Sancar *et al.,* 2004) oxidized/reduced bases, alkylated (usually methylated) bases, deaminated bases (*e.g.* uracil, xanthine), or base mismatches. Some DNA glycosylases are simple glycosylases, catalyzing only the hydrolytic removal of the base so as to form an apurinic/apyrimidinic (AP) site, whereas

others cleave off the base by a lyase mechanism and catalyze a subsequent AP lyase reaction. Generally, lyase reactions are associated with glycosylases that remove oxidized bases, but not with those that remove normal or alkylated bases. After the lyase reaction, an AP endonuclease removes the 3′-sugar residue incising 5′-to the abasic sugar to form a gap which is filled by DNA polymerase, and the resulting nick is ligated. In cases where the glycosylase lacks lyase activity, the 5′ incision is first made by APE1 in mammalian cells, and the abasic sugar can be removed by the dRPase (deoxyribophosphodiesterase) activity of DNA polymerase ß (Polß) (Matsumoto *et al.,* 1995), which concurrently fills in the 1-nucleotide gap. The 1-nucleotide replacement pathway is called the short-patch base excision repair. In mammalian cells, DNA Polß, APE1, and DNA ligase III-*XRCC1* are utilized for short-patch base excision repair. Short-patch BER represents approximately 80–90 % of all BER. Long-patch BER is normally only initiated as a result of 5′-blocking lesions that are refractory to DNA polymerase β lyase activity. Long-patch BER demands several proteins. APE1 makes the 5′ incision to the AP site, and DNA polymerase ß, δ, or Ɛ accompanied by PCNA elongate the 3′-OH into the repair gap and displace the 5′-lesion as part of a DNA fragment (Frosina *et al.,* 1996) or „flap‟ oligonucleotide(2–10 nucleotides). The flap structure is then removed by FEN1 and DNA ligase I sequentially seals the nick that has been relocated downstream of the original nucleotide damage site (Lindahl *et al.,* 1969; Robins *et al.* 1994).

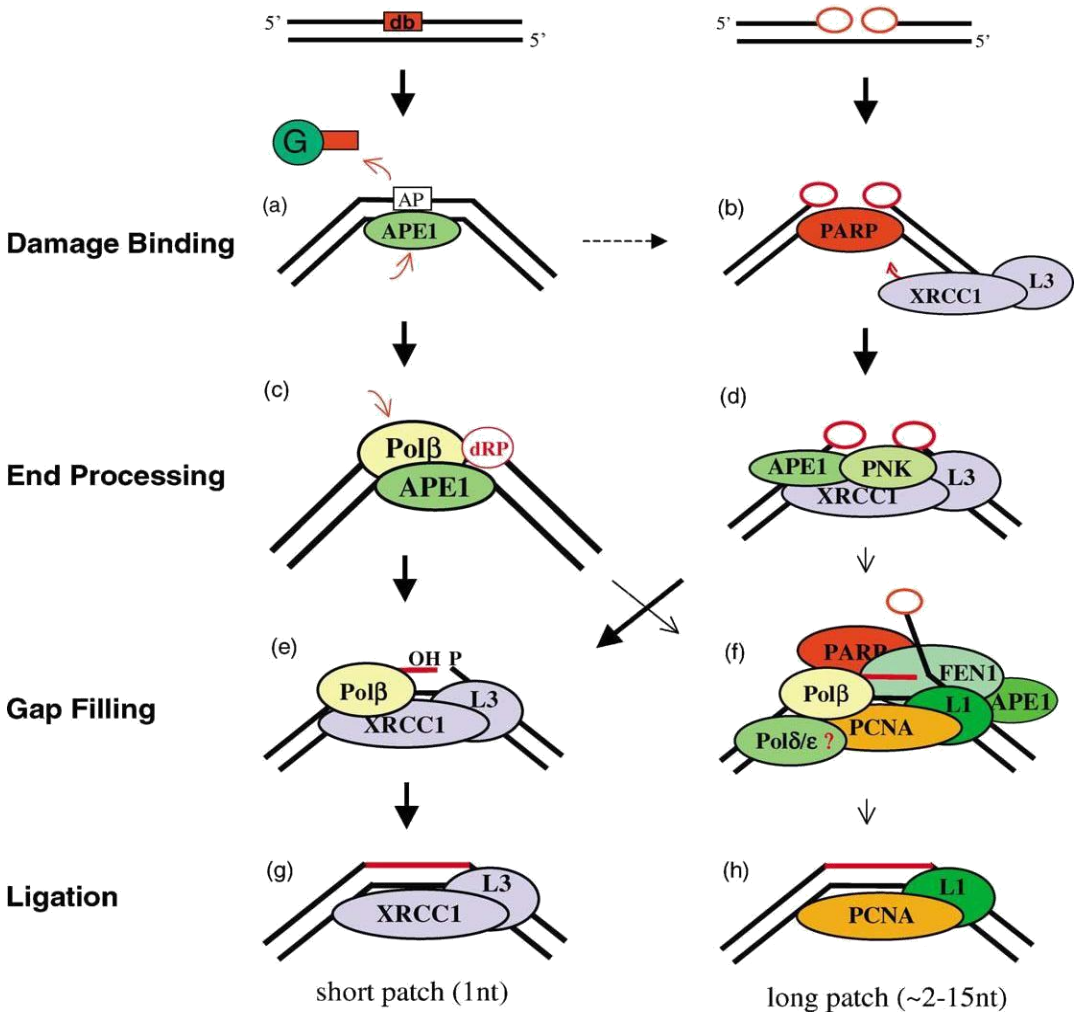


Fig 2.3: Model for mammalian Single Strand Break Repair (Caldecott 2003).
NOTE: DNA base ("db DNA glycosylase ("G"), abasic site ("AP") by APE1
5′-deoxyribose phosphate terminus ("dRP")
The major pathways are indicated by large arrows and the minor pathways by small arrows.

DIRECT REPAIR:

There are two direct repair mechanisms in the majority of organisms: the photoreversal of UV-induced pyrimidine dimers by DNA photolyase and the removal of the O^6-methyl group from O^6-methylguanine (O^6MeGua) in DNA by methylguanine DNA methyltransferase (Sancer *et al.*, 2004). Initially DNA photolyase binds to pyrimidine dimers in a light independent reaction (Seltow *et al.*, 1965; Wulff and Rupert 1962). Upon subsequent exposure to light of wavelength greater than 300nm, the enzyme cleaves the dimer and dissociates it from the substrate, leaving the original primary structure of the DNA (Yasbin 2002). Methylguanine DNA

methyltransferase, is presumed to recognize damage by three-dimensional diffusion, and after forming a low stability complex with the DNA backbone at the damage site, it is thought to flip-out the O^6MeGua base into the active site cavity, where in the methyl group is transferred to an active site cysteine (Lindahl *et al.,* 1988). The protein then dissociates from the repaired DNA, but the C-S bond of methylcysteine is stable, and therefore, after one catalytic event the enzyme becomes inactivated and is accordingly referred to as a suicide enzyme (Sancer *et al.,* 2004).

NUCLEOTIDE EXCISION REPAIR (NER)

NER is characterized by the excision of damaged bases in oligonucleotide fragments. Unlike BER, NER is believed to require no more than 5 proteins to complete the entire process. There is good evidence that in eukaryotes the events that precede repair synthesis and DNA ligation during NER require the participation of between 15 and 20 gene products (Freidberg *et al.,* 1995).

The general properties of NER include a five step mechanism

- Recognition of bulky lesion in the DNA.
- Hydrolyzing a phosphodiester bond in deoxyribose backbone on the 5‟side of lesion.
- Excising the lesion (along with a limited number of nucleotide on its 3‟side).
- Filling in the resulted gap using the information from the complementary strand.
- Closing the nicked DNA to generate intact strand (Schendel 1981).

NER consists of two distinct pathways termed global genomic repair (GGR) and transcription-coupled repair (TCR). GGR is thought to be largely transcription-independent and removes lesions from the non-transcribed domains of the genome and the non-transcribed strand of transcribed regions (Christmann *et al.,* 2003). TCR removes different RNA-polymerase-blocking lesions from the transcribed strand of active genes (Bohr *et al.,* 1985; Mellon *et al.,* 1987).

DOUBLE-STRAND BREAK REPAIR

Double-strand breaks (DSB) are produced by ionizing radiation and chemicals that generate reactive oxygen species and also a normal result of V(D)J recombination and immunoglobulin class switching processes. But occur unnaturally during replication as a consequence of

replication fork arrest and collapse. Double-strand breaks are repaired either by homologous recombination (HR) or nonhomologous end-joining (NHEJ) mechanisms (Petrini 1999; Khanna *et al.,* 2001).

HOMOLOGOUS RECOMBINATION (HR)

This process has three steps:

- strand invasion
- branch migration
- Holliday junction formation

The Holliday junction is then resolved into two duplexes by the structure-specific endonucleases. Strand invasion and branch migration is initiated by Rad51 in eukaryotes (Sung P 1994), or RecA in prokaryotes (Kowalczykowski 2000).

NONHOMOLOGOUS END-JOINING (NHEJ)

In this form of repair in eukaryotes, the Ku heterodimer binds to the two ends of a double-strand break and recruits DNA-PKcs (Ramsden *etal.,* 1998) and the ligase4-XRCC4 heterodimer, which then ligates the two duplex termini regardless of whether the two ends come from the same chromosome. Genetic data indicate that HR is important for the recovery of collapsed replication forks. In contrast, NHEJ is essential for V(D)J recombination and is thought to be the major pathway for repair of double-strand breaks induced by ionizing radiation and radiomimetic agents.

CROSS-LINK REPAIR

Interstrand DNA cross-links (ICLs) are formed by natural products of metabolism and by chemotherapeutic reagents (Muniandy *et al.,* 2010). ICLs are highly toxic DNA lesions that prevent transcription and replication by inhibiting DNA strand separation (Deans *et al.,* 2011)**.**

This has been taken as evidence that the structure-specific XPF•ERCC1 nuclease plays a special role in cross-link repair aside from its function in nucleotide excision repair. When cross-linked DNA is used as a substrate for the human excision nuclease in vitro, identified two activities involving incisions on one strand on either side of the ICL (unhooking) producing a gapped

intermediate with the incised oligonucleotide (22–28nt) attached to the intact strand. The gap is filled by recombinational repair or lesion bypass synthesis. The remaining monoadduct is then removed by nucleotide excision repair (Muniandy *et al.,* 2010).

2.4 ***XRCC1*:**

Human X-ray repair cross complimenting group1(*XRCC1)* was cloned more than 10 years ago, yet experimental analysis of the *XRCC1* gene product is still unveiling new insights into the DNA damage response. The most striking feature of *XRCC1* to have emerged during the past 10 years is its ability to interact with other DNA repair proteins. Indeed, although lacking any known enzymatic activity itself, *XRCC1* interacts with enzymatic components of each stage of DNA strand break repair (Keith W. Caldecott 2003).

XRCC1 the major DNA repair gene in the base excision repair (BER) pathway, acts as scaffold of different activities involved in repair by interacting with components at site of damage. Human *XRCC1* is located on chromosome 19q13.2-13.3 (Thomposon *et al.,* 1989; Mohrenweiser and Jones 1998) spans a genomic distance 33 kb, contains 17 exons, and transcripts a protein of 633 amino acids (69.5 kDa) (Natukula *et al.,* 2013).

2.4.1 INTERACTING PARTNERS OF XRCC1

Although *XRCC1* has no known enzymatic activity, it can interact with several important repair proteins through its different domains, such as DNA ligase III at its breast cancer susceptibility gene C terminus II (BRCT-II) domain, DNA polymerase ß at its NH2 terminus, poly (ADP-ribose) polymerase (PARP) 1 and 2 at its BRCT-I domain, human AP endonuclease**,** polynucleotide kinase (PNK), human 8-oxoguanine DNA glycosylase (OGG1) and proliferating cell nuclear antigen (PCNA) at the central section of *XRCC1* protein (Heng Xu *et al.,* 2007).

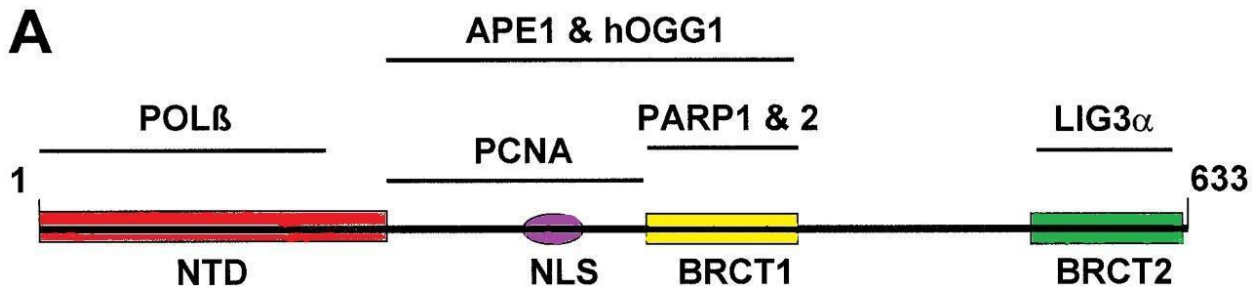


Fig 2.4: Protein–protein interactions mediated by human *XRCC1*. NTD: N-terminal domain; NLS: nuclear localization signal (Fan *et al.,* 2004) BRCT1: breast cancer Terminus domain1; BRCT2: breast cancer Terminus domain 2.

Poly ADP-ribose polymerase (PARP) 1 and 2:

One of the immediate eukaryotic cellular responses to DNA breakage is the covalent post-translational modification of nuclear proteins with poly (ADP-ribose) from NAD+ as precursor, mostly catalysed by poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is an abundant nuclear protein that functions as a molecular nick sensor, and is important for genetic stability and for cellular resistance to ionizing radiation and alkylating agents (Burkle 2001, Murica *et al.,* 1994). PARP-1 appears to strongly influence chromatin architecture and gene expression (Tulin *et al.,* 2002). In addition, PARP-1 is also implicated in the induction of cell death (Chiarugi *et al.,* 2001). PARP-2 is required for efficient base excision DNA repair in association with PARP-1 and *XRCC1* (Schreiber *et al.,* 2002). PARP-1 appears to function as a homodimer, and possibly as a heterodimer with PARP-2. The ability of PARP-1 to bind rapidly to DNA strand breaks is conferred by two unusual amino-terminal zinc fingers.

PAR glycohydrolase, degrades PAR rapidly within minutes. The rapid dissociation of PARP-1 from breaks is important as it allows other DNA repair proteins subsequent access to the break. Binding of PARP-1 to DNA breaks may also serve a signaling role to initiate DNA repair reactions by recruiting other proteins (Masson *et al.,* 1998).

AP endonucleases-1(APE1):

APE1 is the major AP endonuclease activity in mammalian cells and cleaves the sugar phosphate backbone immediately 5‟ of abasic sites (Mol *et al.,* 2000). Abasic sites arise by spontaneous base loss or as intermediates during the BER of modified bases. In addition, modified abasic sites can arise from oxidative damage to deoxyribose. APE1 is thus the major source of indirect Single Strand Breaks arising during BER. At abasic sites recruitment of APE1 may displace the

DNA glycosylase from the abasic site created by DNA glycosylase during BER and then both recruit DNA polymerase-ß (Polß) and cleave the abasic site (Okano *et al.,* 2003)

The ordered sequence of step-wise interactions of different proteins during base excision repair has prompted a model in which an enzyme involved in one step of the reaction receives its DNA substrate as an enzyme-product complex from the previous step (Wilson *et al.,* 2000). In this way, repair intermediates can be handed from one enzyme to the next in a molecular relay that is driven by sequential protein–protein and protein–DNA interactions .The termini of strand breaks are often of nonconventional chemistry and require enzymatic processing to convert them to the 3‟-hydroxyl and 5‟-phosphate moieties necessary for gap filling and DNA ligation. In addition to its ability to cleave abasic sites, APE1 is also able to remove, even though to a lesser extent, 3‟-phosphoglycolate and 3‟-phosphate from direct breaks and 3-unsaturated aldehydes that result from cleavage of abasic sites by an AP lyase activity (Winters *et al.,* 1994). In long patch base excision repair APE1 may help recruit and/or stimulate PCNA, FEN1, and DNA ligase I activities. However, *XRCC1* also weakly stimulated the 3′-end processing activity of APE1.

Polynucleotide kinase (PNK):

Human polynucleotide kinase (PNK), a homologue of T4 PNK, is a key component of both the base excision repair (BER) and nonhomologous end-joining (NHEJ) DNA repair pathways. PNK acts as a 5'-kinase/3'-phosphatase to create 5'-phosphate/3'-hydroxyl termini, which are a necessary prerequisite for ligation during repair. PNK possesses both 5‟-DNA kinase activity and 3‟-phosphatase activity (Jilani *et al.,* 2002). PNK prefers gapped or nicked DNA as a substrate and process it (Busheri *et al.,* 1998). 3‟-Phosphate termini are present at many direct Single Strand Breaks arising from sugar damage by oxidizing agents and free radicals. *XRCC1* stimulates both the 5′-kinase and 3′-phosphatase activities of PNK. In addition to stimulating PNK activity, it has been proposed that the interaction with *XRCC1* serves to recruit PNK to chromosomal Single Strand Breaks.

DNA polymerase-ß (pol ß):

Pol ß is a small, single-subunit, DNA polymerase that is associated primarily with filling small gaps during DNA base excision repair (Wilson 1998). In addition, Pol ß is also implicated in gap filling at direct Single strand breaks. However, there is extensive redundancy in the gap filling

role fulfilled by Pol ß. Pol ß with Pol δ/ε seemingly able to compensate for the absence of this polymerase (Fortini *et al.,* 1998). Pol-ß appear to be the primary polymerase employed for both processes though the PCNA-dependent polymerases Pol δ/ε can also conduct gap filling and may be involved in long patch repair under some circumstances. In 1996, Polß was identified as a partner of *XRCC1*. This interaction occurs within the palm–thumb domains of the DNA polymerase active site and an amino terminal domain (NTD) of *XRCC1* (Fig.2.4). The *XRCC1*–

Pol ß complex might surround a DNA molecule at the site of a Single strand breaks, perhaps protecting the undamaged strand from nucleolytic attack, which might otherwise create a Double strand break (Marientchev *et al.,* 2000). This interaction could also help recruit *XRCC1* to a Pol ß bound Single strand break or conversely Pol ß to an *XRCC1*-bound Single strand breaks depending on which pathway is in use.

DNA ligase III:

Although involved in the last step of repair, DNA ligase III was the first *XRCC1* partner to be discovered (Caldecott *et al.,* 1994). The interaction between Lig3 α and *XRCC1* is mediated by their C-termini, both of which encode a BRCT (breast cancer terminal) domain (Nash *et al.,*

1997; Mackey *et al.,* 1997) (Fig. 2.4). *XRCC1* is required for the stability of ligase III; in addition to that, an important function of the interaction with *XRCC1* is to target the DNA ligase to sites of strand breakage. Deletion or mutation of the BRCT domain in *XRCC1* that binds Lig3 α prevents the ability of *XRCC1* to facilitate rapid rejoining of cellular Single strand breaks.

Proliferating cell nuclear antigen (PCNA):

DNA polymerase ß, δ, or Ɛ accompanied by PCNA elongate the 3‟OH into the repair gap and displace the 5‟-lesion as part of a DNA fragment or „flap‟ oligonucleotide. PCNA is an auxiliary factor for the DNA polymerase ε and δ, and found to be important for DNA replication and repair. PCNA was initially thought to be a moving platform for DNA polymerase δ or ε but recent studies indicate that PCNA is involved in a variety of important cellular processes including cell cycle control, DNA replication and excision repair (Jonsson *et al.,* 1997; Prosperi 1997).

2.5 POLYMORPHISM:

Genetic polymorphism is common throughout the genome. The most common type of polymorphism, single nucleotide polymorphisms (SNPs) can occur as frequently as 1 out of every 300 base pair and there are probably more than 10 million SNPs in human population (The international HapMap consortium 2006). Polymorphism can occur both in exon and intron of the genes, those which occur in exon have impact on structure and function of the protein and sometimes lead to amino acid exchange in conservative domains.

In *XRCC*1 more than 300 validated single nucleotide polymorphisms (SNPs) has been reported in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), three of which are common and leads to amino acids substitution(Liu *et al.*, 2013).

Table 2.2 SNP Characteristics:

Gene	rs no.	SNP	Codon	Exon	Change in amino acid
XRCC1	rs1799782	C/T	194	6	Arg>Trp
	rs25489	G/A	280	9	Arg>His
	rs25487	G/A	399	10	Arg>Gln

2.5.1 *XRCC1 Arg^{399}Gln* and *Arg^{194}trp*:

Codon 399 resides in functionally important PARP binding domain (Seedhouse *et al.,* 2013). PARP is a zinc-finger containing enzyme that detects DNA strand breaks (Shall and de Murica 2000).

XRCC1 gene codon 194 was at a conserved residue in humans (Lamerdin *et al.,* 1995), suggesting the functional significance of this site. Codon 194 resides in a linker region connecting the domains that interacts with PARP and DNA polymerase ß (Seedhouse *et al.,* 2002). Theoretically, these polymorphisms could affect the response to cancer therapy through the removal of DNA adducts, hence influence the overall survival of patients (Zhigang *et al.,* 2012).

Several epidemiological studies have explored the relationship between XRCC1 polymorphisms and the development of different type of cancers. These studies have reached inconsistent conclusions on whether any *XRCC1* genetic variant could serve as biomarker of lung cancer, with some studies showing association (Haijun *et al.*, 2011).

The tables (2.3 and 2.4) in the next page show the role of the SNPs in *XRCC1* gene and its association with different cancer

Table 2.3: Association of *XRCC1* polymorphism with different cancer in worldwide population:

SNPs	Cancer	Study	Year	Country	Result	OR	*p*-value
Arg^{194}*Trp*	Colorectal	Sherif Z Adbel-Rahman *et al.,*	2000	Egypt	Increased risk	2.56	0.08
Arg^{399}*Gln* (Heterozygous+Homozygous variant)					Increased risk	3.98	<0.001
Arg^{194}*Trp*	Systemic lupus erythematous	Saeedeh Salami *et al.,*	2014	Iran	No association		
Arg^{399}*Gln*					protective	0.42	0.01
Arg^{399}*Gln* (Homozygous variant)	Breast	Karolina przbylowska-sygut *et al.,*	2013	Poland	Higher disease free survival		
Arg^{399}*Gln* (Heterozygous)	Acute myeloblastic leukemia	Clarie *et al.,*	2002	UK	Protective survival in controls	0.44	0.03
Arg^{399}*Gln*	Brain tumor risk	Sahika L. Cengiz *et al.,*	2008	Turkey	No risk		0.13
Arg^{399}*Gln* (Homozygous variant)	Bladder	Karl T.Kelsey *et al.,*	2004	New Hampshire	Reduced risk		0.6
Arg^{399}*Gln*	Bladder	Sei Chung Sak *et al.,*	2007	UK	No association		
Arg^{194}*Trp*					No association		
Arg^{194}*Trp* (Homozygous variant)	Differenciated thyroid carcinoma(DTC)	Feng Yu Chiang *et al.,*	2008	Taiwan	Increased risk	1.85	0.018
Arg^{399}*Gln*					No significance		
Arg^{399}*Gln*	Gastric	Wen Qiao *et al.,*		China	Risk	2.0	0.006

Table 2.4: Association of XRCC1 polymorphism with different cancer in Indian population**:**

SNPs	Cancer	Study	Year	Result	OR	*p*-value
Arg^{399}*Gln* (Homozygous variant)	Lung	Leela Kumari Sreeja *et al.,*	2007	Higher	2.1	0.007
Arg^{399}*Gln*	Lung	Kirmani Natukula *et al.,*	2013	Risk in males with smoking habbits		<0.05
Arg^{399}Gln	Breast	Priya Chacko *et al.,*	2005	Higer risk	2.14	0.003
Arg^{194}*Trp* (Homozygous variant + Heterozygote)					1.98	0.017
Arg^{399}*Gln+ Arg*^{194}*Trp*				Predisposition		
Arg^{399}*Gln*	Colorectal	Nighat Parveen Khan *et al.,*	2013	Protective role		
Arg^{399}*Gln*	Gallbladder	Jingwen Wang *et al.,*	2010	Increase risk	1.65	
Arg^{399}*Gln* (Homozygous variant)	Gallbladder	G.Choudhuri *et al.,*	2009	Low risk	0.62	0.039
Arg^{194}*Trp*				No association		
Arg^{194}*Trp*	Hepatitis related hepatocellular carcinoma	Manjula Kiran	2009	Risk	2.27	<0.001
Arg^{399}*Gln*	Prostrate	Nega Berhane *et al.,*	2011	Increased risk	2.06	0.033
Arg^{399}*Gln* (Homozygous variant)	Sporadic breast cancer	Volga S.Syamala *et al.,*	2008	Not significant		

2.6 CHEMOTHERAPY:

Chemotherapy (chemo) has been the backbone of treatment for broad range of malignant disease. Chemotherapy is treatment with anti-cancer drugs injected into a vein or taken by mouth. These drugs enter the bloodstream and go throughout the body, making this treatment useful for cancer anywhere in the body (American Cancer Society 2014).

Of the various types of chemotherapy regimens available, platinum-based drugs such as cisplatin or carboplatin are used to treat many types of cancer. In case of NSCLC they have been shown to improve overall survival (Xinchen *et al.,* 2009). However, it is difficult to identify platinum resistance in clinical treatment. Genetic factors are thought to represent important determinants of drug efficacy.

Platinum based drugs work by binding to DNA and forming DNA adducts leading to intrastrand or interstrand cross-links which disrupt the structure of the DNA molecule, leading to steric changes in the helix (van de vaart *et al.,* 2000). Alteration in the structure of the DNA molecule leads to cellular DNA damage recognition and repair which can result in the continued viability of the cell resulting in platinum resistance. It appears that tumor cells can have intrinsic differences in DNA repair mechanisms when compared with their normal counterparts (Martin *et al.,* 2008).

2.6.1 MECHANISM OF ACTION DNA LESIONS:

Upon entering a cell, all platinating agents become aquated, losing chloride or oxalate ions, and gaining two water molecules. This positively charged molecule is then able to interact with nucleophilic molecules within the cell, including DNA, RNA, and proteins. It is generally agreed that DNA is the preferential and cytotoxic target for platinating agents (Cara *et al.,* 2006). When binding to DNA, platinating agents favor the N7 atoms of the imidazole rings of guanosine and adenosine. Three different types of lesions can form on purine bases of DNA:

- monoadducts
- intrastrand crosslinks
- interstrand crosslinks

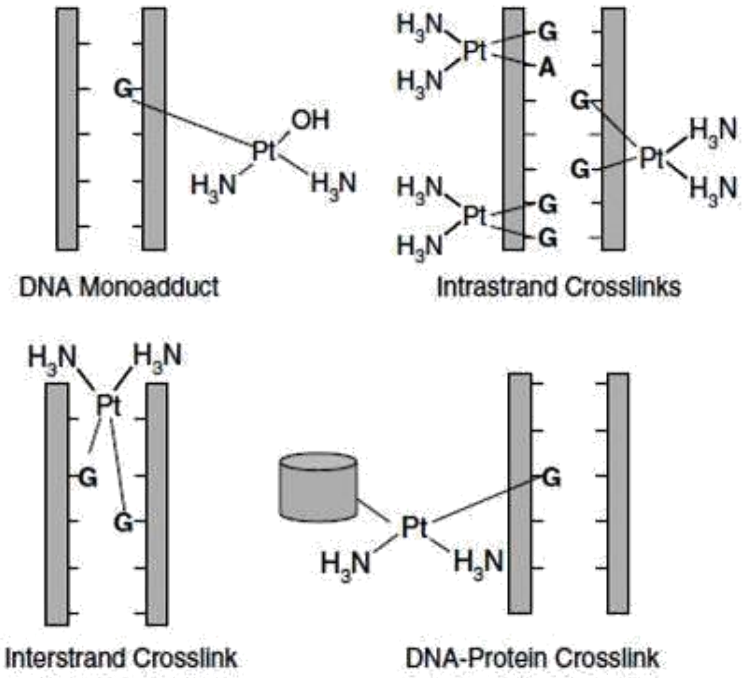


Fig. 2.5: Platinating agent adducts on DNA (Cara *et al.,* 2006**).**

2.6.2 <u>ROLE OF DNA REPAIR IN OUTCOME OF CHEMOTHERAPY:</u>

Defects in DNA repair mechanism have at least two important implications in cancer biology. First these defects cause genetic instability, which promotes malignant transformation of cells. Second, they lead to cellular hypersensitivity to DNA-damaging agents, which can contribute to efficient chemo/radiotherapy (Toshiyasu 2012). Inhibition of DNA repair pathway can sensitize cancer cells to chemotherapy. Deficit DNA repair capacity is may be due to genetic variability of the individual. The genetic polymorphism in DNA repair gene may result in subtle structural alteration of repair enzymes and hence decrease removal of adducts or DNA damages caused by environmental exposure. During the chemotherapy session, decrease removal of adducts formed by the plating agents leads to distortion in DNA helix structure inhibiting the DNA replication and high Cytotoxicity levels of drugs.

As *XRCC1* is a major component of BER pathway, polymorphism in this gene may be associated with deficit DNA repair capacity hence may show good response to chemotherapy.

Different studies conducted so far in various populations to examine the role of polymorphisms of *XRCC1* gene towards overall survival of patients. The table **2.5** in the next page summaries meta-analysis conducted by Zhigang *et al.,* 2013.

Table 2.5: *XRCC1 Arg^{194}Trp* and *Arg^{399}Gln* polymorphisms and prognosis.

SNPs	Study	Year	Country	Outcome
Arg^{399}Gln	Qian *et al.,*	2010	China	TR
Arg^{399}Gln	Cheng *et al.,*	2011	China	TR
Arg^{399}Gln	Giachino *et al.,*	2007	Italy	TR/OS
Arg^{399}Gln	Butkiewicz *et al.,*	2010	Poland	OS
Arg^{399}Gln	de las Penas R. *et al.,*	2006	Spain	OS
Arg^{399}Gln	Yin *et al.,*	2009	China	OS
Arg^{399}Gln	Sreeja *et al.,*	2008	India	OS
Arg^{399}Gln	Gurubhagavatula *et al.,*	2004	USA	OS
Arg^{399}Gln	Kalikaki *et al.,*	2009	Greece	OS
Arg^{399}Gln	Liu *et al.,*	2008	China	OS/PFS
Arg^{399}Gln	Han *et al.,*	2011	Korea	OS/PFS
Arg^{399}Gln	Yao *et al.,*	2009	China	OS
Arg^{399}Gln	Ding *et al.,*	2010	China	TR
Arg^{194}Trp	Yuan *et al.,*	2006	China	TR
Arg^{194}Trp	Jin *et al.,*	2006	China	TR
Arg^{194}Trp and Arg^{399}Gln	Gao *et al.,*	2006	China	TR
Arg^{194}Trp and Arg^{399}Gln	Shi *et al.,*	2006	China	TR
Arg^{194}Trp and Arg^{399}Gln	Sun *et al.,*	2009	China	TR

Arg^{194}*Trp and* *Arg*^{399}*Gln*	Song *et al.,*	2007	China	TR
Arg^{194}*Trp and* *Arg*^{399}*Gln*	Wang *et al.,*	2004	China	TR
Arg^{194}*Trp and* *Arg*^{399}*Gln*	Hong *et al.,*	2009	China	TR

NOTE: TR: objective response, OS: overall survival, PFS: progression-free survival

XRCC1 Arg^{194}*Trp* and *Arg*^{399}*Gln* polymorphisms were the commonest one among more than 60 validated SNPs in *XRCC1* gene and showed no major variations by ethnicity (Hung *et al.,* 2005). *XRCC1* SNPs have been reported to be associated with an altered DNA repair activity (Zienolddiny *et al*., 2006; Shen *et al.,* 2005). Previous reports have also suggested that *XRCC1* polymorphisms might be risk factors for the development of lung cancer and promising predictive or prognostic makers for lung cancer patients (Yin *et al.,* 2009; Shreeja *et al.,* 2008; Gurubhagavatula *et al.,* 2004). Therefore, functional SNPs in *XRCC1* gene may relate with platinum sensitivity and have prognostic values among lung cancer patients.

CHAPTER 3

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

The present piece of work is an attempt to study the following aspects that might be associated towards the associative risk for lung cancer.

- To find a correlation of *XRCC1Arg*^{399}*Gln* and *Arg*^{194}*Trp* polymorphisms towards the risk of developing lung cancer and also the clinico-pathological features associated with it

- To carry out the overall survival analysis of lung cancer patients undergoing platinum based chemotherapy in association with *XRCC1Arg*^{399}*Gln* and *Arg*^{194}*Trp* polymorphisms.

CHAPTER 4

METHODOLOGY

- Study subjects
- Isolation of DNA from peripheral blood
- DNA quantification
- Resolution of DNA fragments on agarose gels
- Polymerase chain reaction (PCR) amplification of *XRCC1Arg*^{399}*Gln* and *Arg*^{194}*Trp*
- Restriction digestion of *XRCC1Arg*^{399}*Gln* and *Arg*^{194}*Trp*
- DNA agarose gel electrophoresis
- Statistical Analysis

4.1 STUDY POPULATION AND SAMPLE COLLECTION:

Approximately 200 Lung cancer patients were enrolled from the Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India for this study. The current study had been reviewed and ethically approved by the Institute ethics committee of PGIMER. Written Informed consent was obtained from all participants or from patient‟s representatives if direct consent could not be obtained. In brief, eligible cases included all patients who were newly diagnosed with primary lung cancer. All the recruited patients were histopathologically diagnosed as having NSCLC and SCLC. There were no age, gender, smoking, histological, or TNM stage restrictions, but patients with a prior history of cancer were excluded from this study. Controls were randomly selected from a pool of healthy volunteers that visited the general health check-up center of PGIMER. Each control was pair-matched by sex, age (±10 years) and smoking parameters to a patient with lung cancer. These characteristics allowed us to obtain control population without any possible risk bias for lung cancer. Information concerning age, sex, native place, smoking status and past history was obtained for each case and control by a trained interviewer. Smokers reported tobacco habits such as smoking of cigarette and/or beedi (a native cigarette like stick of coarse tobacco hand-rolled in a dry tembuhurni leaf). As an indication of cumulative smoking exposure, pack-years were calculated by the following formula: [(cigarettes or beedis per day / 20) X years smoked]. While medical information of cases, including Histology, TNM classification, clinical staging, primary tumor size, involvement of lymph node and metastasis were obtained from medical records of the hospital. Approximately 3ml of venous blood was collected from each participant.

4.1.1 CLINICAL EVALUATION:

Of the 200 lung cancer patients, we identified a subset of 105 patients whose successful follow up was conducted. One of our major priorities was to have complete data regarding diagnosis date, corresponding genotype of the patient and dates on which the patient died. As a result our study was limited to 90 patients who had complete outpatient records and XRCC1 genotype. All patients received chemotherapy as the first line treatment. All the patients were followed every two months until death or end of the study period. The overall survival was the end point. Survival time was calculated from the date of diagnosis to the date of last follow-up and death

from any causes. All patients were followed from august 2012 to mid June of 2014. All patients were followed up by telephone.

4.2 ISOLATION OF DNA FROM PERIPHERAL BLOOD

REQUIREMENTS

- Washing buffer
- Lysis buffer
- Phenol:Chloroform:Isoamylalcohol (25:24:1)
- Chloroform:Isoamylalcohol (24:1)
- Isopropanol
- TE buffer

PROCEDURE

Preparation of Buffers

Washing buffer, Lysis buffer and TE buffer were prepared as shown in tables below.

Table no 4.1 Preparation of Washing buffer :

STOCK CONCENTRATION	WORKING CONCENTRATION
1M sucrose	320 mM sucrose
100% Triton X-100	1% Triton X-100
100mM Magnesium Chloride	5mM Magnesium Chloride
100mM Tris-HCl pH (8.0)	10mM Tris-HCl pH (8.0)

Table 4.2 Preparation of Lysis Buffer:

STOCK CONCENTRATION	WORKING CONCENTRATION
1M Tris HCl pH (8)	400mM Tris HCl pH (8)
10% SDS	1% SDS
0.5M EDTA	60mM EDTA
5M NaCl	150mM NaCl
10mg/ml Proteinase-K	100 µg/ml Proteinase–K

Isolation of DNA

Took 3ml of blood and added 3ml of washing buffer (0.96ml 1M Sucrose, 0.03 ml Triton X-100, 0.15ml MgCl2, 0.03 ml 100mM Tris HCl and 1.83ml of water) and mix it thoroughly. Centrifuged it at 3500 rpm for 5 minutes.

- Discarded the supernatant and added 3ml of washing buffer to the pellet and resuspended the pellet in the buffer and centrifuged again (repeat this step thrice).

- Dissolved the pellet in 3ml of Lysis buffer (1 M Tris HCl 1.2ml,10% SDS 0.3ml ,0.5 M EDTA 0.37ml, 5M NaCl 0.09ml,10mg/ml Proteinase-K 0.03ml and water 1.01ml) and incubated at 44 °C overnight.

- Added an equal volume of Phenol: Chloroform: Isoamyl alcohol (PCI) 25:24:1(1.5ml Phenol, 1.44ml Chloroform and 0.06ml Isoamyl alcohol) and mixed slowly.

- Centrifuged at 8000 rpm for 10 minutes at 4°C. Took the upper aqueous layer and again add PCI mix and centrifuged.

- Took the aqueous layer and added equal volume of Chloroform: Isoamyl alcohol (24:1)(2.87ml Choloroform and 0.13ml Isoamyl alcohol).

- Centrifuged it at 6500 rpm for 5 minutes and took the aqueous layer.

- To the aqueous layer added equal volume of chilled isopropanol or 2.5 times volume of absolute ethanol and mixed it gently.
- Freeze it at -20°C for 1-2 hours.
- Centrifuged it at 12,000 rpm for 10 min at 4°C. Supernatant Discarded and the pellet of DNA washed with chilled 70% ethanol twice at 10,000 rpm for 5 minutes.
- Decanted ethanol and air dry the pellet.
- Dissolved the pellet in 50µl-200µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White, 2003).

4.3 DNA QUANTIFICATION:

The NanoDrop Spectrophotometer (Thermoscientific) is designed for measuring nucleic acid concentrations in sample volumes of one microliter. The key to this advanced spectrophotometer is its unique sample retention technology that overcomes the need for cuvettes and capillaries when taking measurements. This is accomplished by placing the sample directly on top of the detection surface and using the surface tension to create a column between the ends of optical fibers. Thus the measurement optical path is formed.

Procedure

- Pipetted 1µl of deionised water onto the lower optical surface to clean it.
- Opened the nanodrop software and selected nucleic acid module.
- Performed a blank measurement by loading 1µl of TE and selecting "blank" from the screen.
- Measured the nucleic acid sample by loading 1µl of DNA sample and selecting "measure"
- Concentration and purity of DNA samples were calculated automatically.

DNA concentration otherwise can be calculated as:

DNA concentration (µg/ml) = O.D at 260nm X 50 X Dilution factor

Where 50µg/ml of DNA is equal to 1 O.D

Purity of DNA = O.D at 260nm/O.D at 280nm

NOTE: A ratio of ~1.8 indicates pure for DNA; a ratio of ~2.0 is generally accepted as pure for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants.

4.4 PCR –RFLP(Polymerase chain reaction-restriction fragment length polymorphism):

PCR is rather inexpensive and easy to use. The genes of interest are amplified with a thermocycler until the number is large enough to be visualized on an agarose or acrlamide gel, stained with ethidium bromide. Restriction enzymes that cleave the DNA specially for different alleles are used and the each allele of each sample can be observed as a specific band pattern on the gel. Together these two techniques form the basis of PCR-RFLP analysis. This analysis was performed to determine the genotype of the polymorphisms of the *XRCC1* gene. The 615bp and 375bp fragments encompassing the $Arg^{399}Gln$ and $Arg^{194}Trp$ polymorphisms respectively of *XRCC1* gene was amplified using specific primers. The reaction mixture was placed in 200µl PCR tubes and the amplification was carried out in T100TM Thermal Cycler,Bio-Rad

Table 4.3 Primers used for *XRCC1* gene

SNPs	Primers sequences	Amplification fragment(bp)	Region	Annealing temperature ºC
rs25487	5‟- TTGTGCTTTCTCTGTGTCCA3‟ (Forward) 5‟-TCCTCCAGCCTTTTCTGATA3‟ (Reverse)	615	Exon10	62
rs1799782	5‟-CCCTTTGGCTTGAGTTTTGT-3‟ (Forward) 5‟-TCAGACCCAGGAATCTGAGC-3‟ (Reverse)	375	Exon6	52

Requirements for PCR

- 10X PCR buffer
- BSA
- Forward Primer
- Reverse Primer
- dNTP,,s
- Taq DNA polymerase
- Water
- DNA sample

Table 4.4 :PCR was performed in 25µl of reaction mixture containing the following

Reagents	Stock concentration	Working concentration	Quantity used
PCR grade water	------	------	11.25 µl
BSA	100X	10X	2.5 µl
Buffer	10X	1X	2.5µl
Mg conc.	15mM	1.5mM total	0.75 µl
Forward Primer	10µM	0.5µM	1.25µl
Reverse Primer	10µM	0.5µM	1.25 µl
dNTP's	10mM each	0.2mM each	0.5 µl
Taq polymerase	2.0U/µl	0.8U	1 µl
DNA Template	100ng/µl	400ng	3 µl

Table 4.5: The thermal cycling parameters were set as follows:

Steps	Temperature(°c)	Time
Initial Denaturation	95°C	5min
Denaturation	94°C	30sec
Annealing	62°C for *XRCC1 Arg^{399}Gln*	45sec
	52°C for *XRCC1 Arg^{194}Trp*	45sec
Polymerization	72°C	30sec
Final Extension	72°C	5min

The reaction was carried out for 30 cycles.

The qualitative estimation of amplified DNA was done by agarose gel electrophoresis. 100bp ladder (G-Biosciences) was loaded in one of the wells for reference to fragment size of amplified DNA. Gel imaging was performed in UV light.

RESOLUTION OF DNA FRAGMENTS ON AGAROSE GELS

Requirements

- Electrophoresis buffer (TAE or TBE)
- Ethidium bromide solution
- Electrophoresis-grade agarose
- 6X loading dye
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs (slot formers)
- DC power supply

Procedure

Table 4.6: Preparing 5X TBE (1000ml)

Reagents	Quantity
Tris base	54 g
Boric Acid	27.5g
EDTA (0.5M)	20ml
Make up final volume with water	

Table 4.7: Preparing 6X Loading Dye (20ml)

Reagents	Quantity
0.25% Bromophenol blue	0.05gm
0.25% Xylene Cyanol	0.05gm
40% Sucrose	8gm
Make up final volume with TE buffer	

Preparing the gel

- Prepared an adequate volume of electrophoresis buffer.

- Added the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. For example for genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared while for the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.

- Melted agarose was cooled to 55°C in a water bath before pouring onto the gel platform to prevent warping of the gel apparatus.

- Before pouring ethidium bromide solution was added to the electrophoresis buffer to a final concentration of 0.3μg/ml to facilitate visualization of DNA when seen under UV transilluminator.

- Poured the melted agarose onto gel casting apparatus between 0.5 and 1 cm thick and inserted the gel comb, making sure that no bubbles were trapped underneath the combs and all bubbles on the surface of the agarose were removed before the setting of the gel.

Loading and running the gel

- After the gel got hardened, gel comb was withdrawn taking care not to tear the sample wells.

- Placed the gel casting platform containing the set gel in the electrophoresis tank. Added sufficient electrophoresis buffer to cover the gel until the tops of the wells are submerged. Made sure no air pockets were trapped within the wells.

- DNA samples were prepared by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water in case of genomic DNA or by mixing 5µl DNA with 2µl of 6X loading dye in case of PCR product.

- Samples were typically loaded into the wells with micropipette. Care was taken to prevent mixing of the samples between wells.

- Appropriate DNA molecular weight marker was also loaded in case of PCR products.

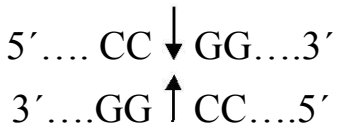
- Connected the electrodes to a power pack, turned on the power, and allowed the electrophoresis run at 60 V until the marker dyes migrated the desired distance.

- Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs

- The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

After confirmation of successful amplification, the PCR products of both polymorphisms were then digested overnight by respective restriction enzymes. The enzymes used by us were:

*Msp*I is a restriction enzyme isolated from *Moraxella* species. It has following restriction site.



*Pvu*II is a restriction enzyme isolated from *Vulgaris* species. It has following restriction site

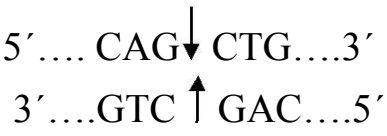


Table 4.8 : SNP and RFLP analysis for *XRCC1* polymorphisms:

SNPs	Restriction enzyme	Genotype(bp)
rs25487	*Msp*I	GG:376,239 GA:615,376,239 AA:615
rs1799782	*Pvu*II	CC:375 CT:375,254,121 TT:254,121

Note: SNP(Single nucleotide polymorphism)

PROCEDURE

Digestion of *XRCC1Arg^{399}Gln*

The total reaction mixture of 20µl consisted of 2.2 µl 10X NEB (New England Biolabs, Inc.,Beverly,MA) buffer, 0.35µl (3U) of 20U/µl *MspI* enzyme (NEB), 10µl of PCR amplified product and 7.4µl water. The buffer used for the process was provided with the enzyme by NEB for increased activity of enzyme. Since the G to A transition in exon 10 of *XRCC1* abolishes the recognition site for *MspI* enzyme, the Gln/Gln genotype yields an undigested band of 615bp, Arg/Arg results in two fragments 376bp and 239bp and Arg/Gln genotype with three fragments of 615bp, 376bp, 239bp. All the samples were incubated at 37°C overnight. The enzyme reaction was stopped by keeping the samples at -20°C and the digested products were then electrophoresed on 1.7% agarose gel in 0.5X TBE, containing 0.5µg/ml EtBr and visualized under UV light. 100bp ladder (G-Biosciences) was used for reference to fragment size. Also, gel imaging was performed.(Vettriselvi *et al.,2007)*

Digestion of *XRCC1Arg^{194}Trp*

The total reaction mixture of 20µl consisted of 2.2 µl 10X NEB 4 buffer, 0.2µl (2U) of 10U/µl *PvuII* enzyme (NEB), 10µl of PCR amplified product, 0.2 µl BSA and 7.4µl water. The buffer used for the process was provided with the enzyme by NEB for increased activity of enzyme. The C to T transition in exon 6 of *XRCC1* gene creates a recognition site for *PvuII* enzyme. The Arg/Arg genotype was characterized by an undigested band 375bp, Trp/Trp genotype results in 254bp and 121bp fragments and Arg/Trp genotype had three bands 375bp, 254bp, and 121bp. All the samples were incubated at 37°C overnight. The enzyme reaction was stopped by keeping the samples at -20°C and the digested products were then electrophoresed on 2% agarose gel in 0.5X TBE, containing 0.5µg/ml EtBr and visualized under UV light. 100bp ladder (G-Biosciences) was used for reference to fragment size. Also, gel imaging was performed. (Vettriselvi *et al.,2007)*

4.5 STATISTICAL ANANLYSIS

Adjusted odds ratios (ORs) with adjustment for possible confounders (age and pack-years of smoking as continuous variables; and gender as a nominal variable) together with their corresponding 95% confidence intervals (CIs) were used to assess the strength association between the *XRCC1* polymorphisms and the risk of lung cancer using a logistic regression model. The genotypes were categorized into three groups (major allele homozygous, heterozygous, homozygous variant) keeping the major homozygous allele of both polymorphisms of *XRCC1* gene were used as the reference in calculating the ORs and 95%CI. All *p* values were two sided, and a *p* value of <0.05 was considered statistically significant. In some cases, we combined the heterozygous genotype with homozygous rare genotype to see the combined effect for particular cases. All the statistical analyses were performed with Medcalc version 13.3.0.0 (Medcalc Software, Ostend, Belgium).To compare the distributions of demographic variables and selected risk factors between patients and controls, Chi-square tests (χ^2 test) were used. The Hardy-Weinberg equilibrium (HWE) (p2+2pq+q2=1), where p is the frequency of the variant allele (q = 1 – p) was tested by a goodness-of-fit χ 2 test to compare the observed genotype frequencies with expected genotype frequencies in cancer-free controls with SNP Analyzer™

2.0(ISTECH).The Kaplan-Meier method was adopted to estimate survival curves and the log-rank test was used to compare patients‟ survival time between genotype groups.

Primary death from lung cancer was defined as the failure event, and the time of survival as the time between diagnosis and death. If a patient died of other causes other than lung cancer, he was censored at the date of death. All surviving patients were censored at the last date of follow-up. Statistical significance was defined as two-sided *p* value of less than 0.05.

CHAPTER 5
RESULTS AND DISCUSSIONS

- Genotyping
- Epidemiology
- Lung cancer risk with *XRCC1Arg^{399}Gln* genotype
- Lung cancer risk with *XRCC1Arg^{194}Trp* genotype
- Distribution of *Arg^{399}Gln* genotype of *XRCC1* gene among patients with different type of Lung cancer
- Distribution of *Arg^{194}Trp* genotype of *XRCC1* gene among patients with different type of Lung cancer
- Effects of smoking status on the association between *Arg^{399}Gln* and Lung cancer
- Effects of smoking status on the association between and *Arg^{194}Trp* Lung cancer
- Effect of *XRCC1Arg^{399}Gln and Arg^{194}Trp* polymorphism on overall survival of Lung cancer patients
- Combined Effect of *XRCC1Arg^{399}Gln and Arg^{194}Trp* polymorphism on overall survival of Lung cancer patients

5.1 DNA ISOLATION:

DNA was isolated from blood (as discussed in section 3.2) using a simple and efficient procedure and the samples were run on 0.7% gel. This total DNA from blood samples were used as template in PCR.

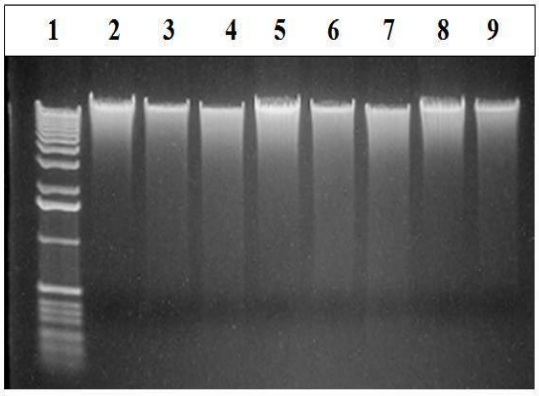


Figure 5.1: Genomic DNA isolated from peripheral blood

5.2 PCR AMPLIFICATION of *XRCC1 $Arg^{399}Gln$ and $Arg^{194}Trp$:*

In order to amplify *XRCC1 $Arg^{399}Gln$ and $Arg^{194}Trp$* gene suitable sets of primers were used. The temperature cycling parameters as employed for *$Arg^{399}Gln$ and $Arg^{194}Trp$* during PCR are given in Table 3.3. The PCR amplified products were separated on 1.7% agarose gel containing ethidium bromide. The DNA bands were clearly visible and distinct which indicated that the primer combinations worked well for both the polymorphism. Figure 4.2 and 4.3 shows the PCR amplified DNA products obtained using set of primer pair specific for *XRCC1 $Arg^{399}Gln$ and $Arg^{194}Trp$* respectively.

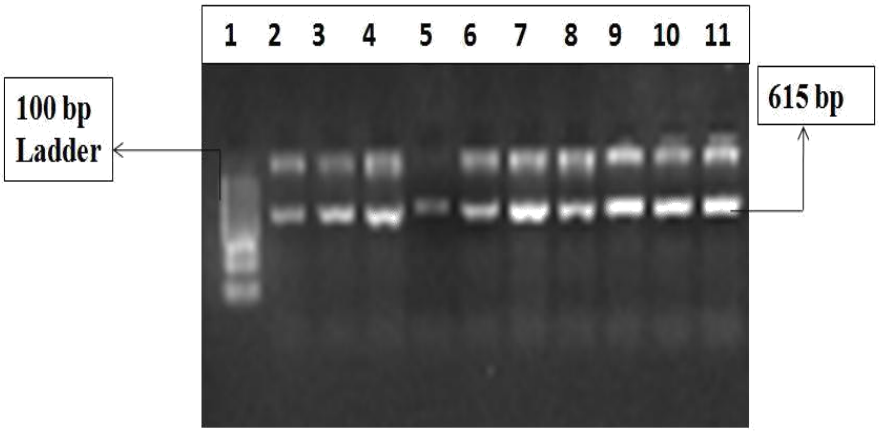


Fig. 5.2: Agarose gel electrophoresis picture for PCR amplified product for *Arg*^{399}*Gln.*
Lane1 – 100bp Ladder; Lane2-Lane11 – DNA products amplified for *Arg*^{399}*Gln* (615bp)

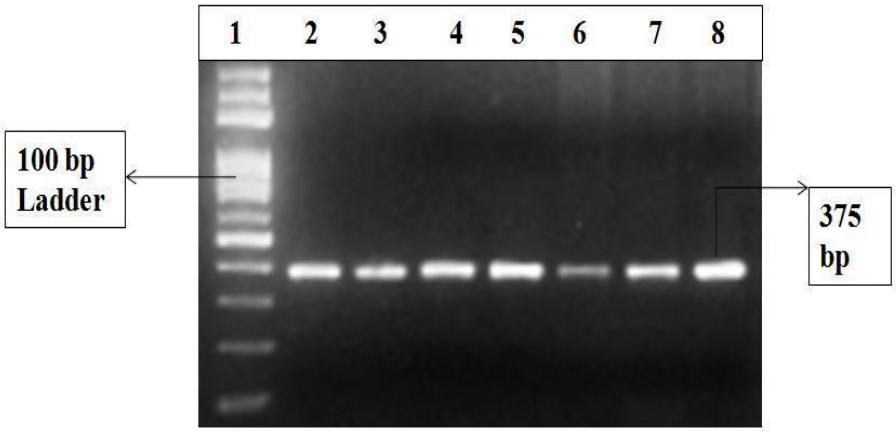


Fig. 5.3: Agarose gel electrophoresis picture for PCR amplified product for *Arg*^{194}*Trp.*
Lane1 – 100bp Ladder; Lane2-Lane8 – DNA products amplified for *Arg*^{194}*Trp* (375bp)

5.3 RESTRICTION DIGESTION OF *XRCC1Arg*^{399}*Gln*

PCR product of *Arg*^{399}*Gln* was digested with the restriction enzyme *MspI.* Then the digested samples were allowed to run on 1.7 % polyacrylamide gel containing ethidium bromide. The genotypic analysis of samples was done on the basis of fragments seen in the gel picture: 2 bands

(376bp and 239bp) – Arg/Arg (Homozygous wild) genotype; 3 bands (615bp, 376bp and 239bp) – Arg/Gln (Heterozygous) genotype; 1 band (615bp) – Gln/Gln (Homozygous variant) genotype.

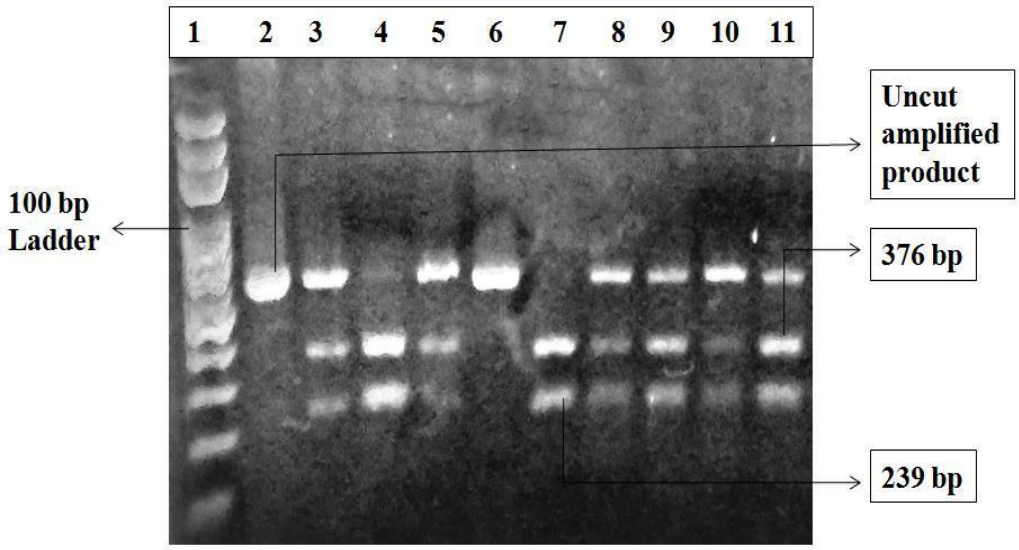


Fig.5.4: Agarose gel electrophoresis picture for restriction enzyme (*Msp1*) digested products, showing Arg/Arg (homozygous wild), Arg/Gln (heterozygous genotype), and Gln/Gln homozygous variant genotype.

Lane 1- 100bp ladder**;** Lane 2- Uncut amplified product (615bp fragment); Lane 3-5 and 8-11 Arg/Gln (Heterozygote) genotype; Lane7- Arg/Arg (Homozygous wild) genotype; Lane 6 Gln/Gln (Homozygous variant) genotype.

5.4 RESTRICTION DIGESTION OF *XRCC1* *$Arg^{194}Trp$*

PCR product of *$Arg^{194}Trp$* was digested with the restriction enzyme *PvuII.* Then the digested samples were allowed to run on 2 % polyacrylamide gel containing ethidium bromide. The genotypic analysis of samples was done on the basis of fragments seen in the gel picture: 2 bands (254bp and 121bp) – Trp/Trp (Homozygous variant genotype); 3 bands (375bp, 254bp and 121bp) – Arg/Trp (Heterozygous genotype); 1 band (375bp) – Arg/Arg (homozygous variant) genotype.

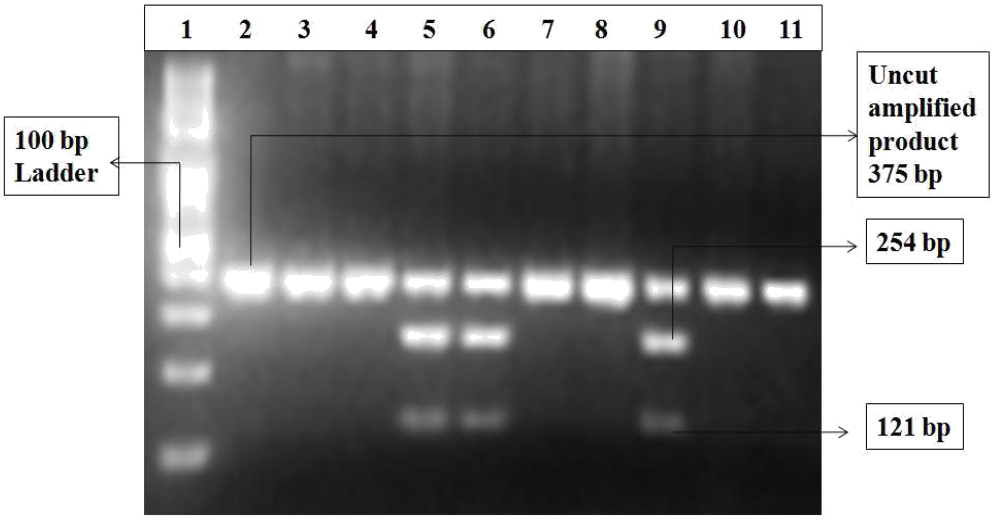


Fig.5.5: Agarose gel electrophoresis picture for restriction enzyme (*PvuII*) digested products, showing homozygous wild Arg/Arg and heterozygous Arg/Trp genotype.

Lane 1- 100bp ladder**;** Lane 2- Uncut amplified product (375bp fragment); Lane 3,4,7,810,11 Arg/Arg (Homozygous wild) genotype and Lane5,6,9- Arg/Trp (Heterozygous) genotype.

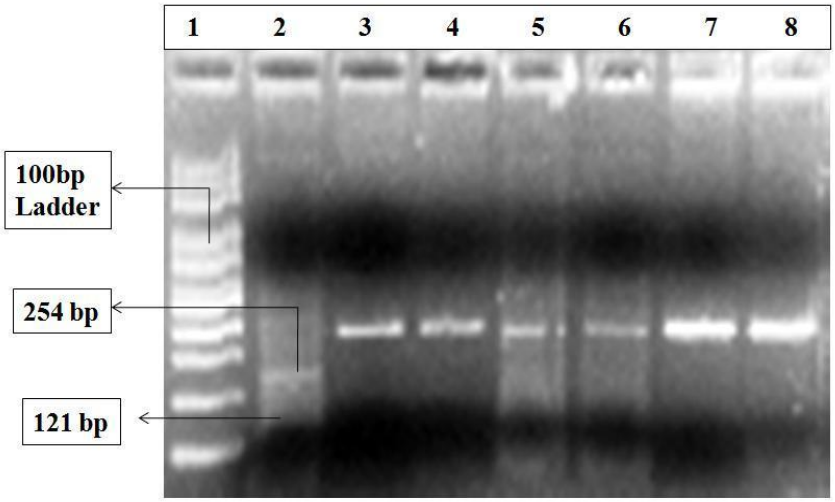


Fig.5.6: Agarose gel electrophoresis picture for restriction enzyme (*PvuII*) digested products, showing homozygous wild Arg/Arg and homozygous variant Trp/Trp genotype.

Lane 1- 100bp ladder**;** Lane 2- Homozygous variant(254bp and121bp fragments); Lane 3-8 Arg/Arg (Homozygous wild) genotype.

5.5 EPIDEMIOLOGY:

Characteristics of Subject

Table 5.1a: summarizes the distribution of demographic characteristics for this population which includes age, gender, smoking status, pack years, histological subtypes, TNM staging and other clinical parameters. The demographic characteristics of the population recruited for the study of *Arg*^{399}*Gln* polymorphism gave following: the mean age was 57.98 ± 9.90 (range 31- 86) for the cases and 52.66 ±10.12 (range 30-80) in controls, respectively. The cases of *Arg*^{399}*gln* included 173 (86%) males and 28(14%) females; controls of *Arg*^{399}*Gln* included 192 (96.5%) males and 7 (3.5%) females. There was significant difference in distribution of males and females between cases and controls and hence suggesting inadequate matching (p=0.0005). In the present study, 81.1% of the cases were smokers and 18.9% were non-smokers, where as in the control group 76.4% were smokers and 23.6% were non-smokers, with no association with risk of lung cancer as p value came out to be 0.3031. Furthermore, among the smokers numbers of pack years were significantly higher in cases as compared to controls (33.5±31.1 vs. 25.8±21.0, p=0.0111). Regarding the histology, squamous cell carcinoma, adenocarcinoma, small cell lung carcinoma represented 90 (44.8%), 55 (27.4%), 49 (24.4%) of the total 201 Lung cancer patients, respectively and 7(3.4%) were having unknown cellular types. Lung cancer patients had following stages. Stage I and II comprised of 2 (0.9%) and 11 (5%) patients respectively. whereas in stage III and IV, each had 90 (44.8%) and 83 (41.2%) patients respectively with 15(7.5%) unclassified. When the whole study group was considered, there was no difference in allele frequencies among cases and controls. The distribution of the studied genotype in the cases and control group was in the Hardy–Weinberg equilibrium ($\chi^2 < 3.8$). The cases were also stratified according to Tumor size maximum number of cases were found in T4 stage (39.4%), followed by T3 (18.9%), T2 (9.9%) and T1 (3.9%), respectively rest cases couldn‟t be classified as data was unavailable for them. Cases were stratified according to lymph node involvement and metastasis which were accounted as follows: (16%) N0, (8.4%) N1, (29.3%) N2, (18%) N3, (2%) N4 and rest 26.3% cases were unclassified also, 33.9% cases showed metastasis.

Table 5.1a: Distribution of demographic variables for patients and controls showing *Arg*^{399}*Gln* polymorphism.

SD = Standard Deviation
$p < 0.05$ was considered statistically significant

Variable	Patients with *Arg*^{399}*Gln* polymorphism, (N= 201) N (%)	Controls, (N = 199) N (%)	*p* – value
Age (years)			
Mean ± SD	57.98 ± 9.90	52.66 ±10.12	<0.0001
Range	(31 – 86)	(30 – 80)	
Gender			
Male	173 (86)	192 (96.5)	0.0005
Female	28 (14)	7 (3.5)	
Smoking Status			
Smokers	163 (81.1)	152 (76.4)	0.3031
Non – Smokers	38 (18.9)	47 (23.6)	
Pack Years			
Mean ± SD	33.5±31.1	25.8±21.0	0.0111
Histological Types			
SQCC	90 (44.8)		
ADCC	55 (27.4)		
SCLC	49 (24.4)		
Other carcinoma	7(3.4)		
TNM Staging			
I	2 (1)		
II	11 (5.5)		
III	90 (44.8)		
IV	83 (41.2)		
Unclassified	15 (7.5)		
Tumor Size			
T1	8 (3.9)		
T2	20 (9.9)		
T3	38 (18.9)		
T4	79 (39.4)		
Unknown	56 (27.9)		
Lymph Node Involvement	32 (16)		
N0	17 (8.4)		
N1	59 (29.3)		
N2	36 (18)		
N3	4 (2)		
N4	53 (26.3)		
Unknown			
Metastasis			
M0	84 (41.8)		
M1	67 (33.3)		
Unknown	50 (24.9)		

p-values were derived from pearson Chi – square test except age; Student t-test was used for age. All *p*- values are two – sided

Characteristics of Subjects

Table 5.1b: summarizes the distribution of demographic characteristics for this population which includes age, gender, smoking status, pack years, histological subtypes, TNM staging and other clinical parameters. In study of $Arg^{194}trp$ polymorphism the recruited population gave the following demographic results: the mean age was 57.48 ± 10.15(range 31-86) for the cases and 52.98 ± 10.29 (range 30- 83) in controls, respectively. The cases of Arg194Trp included males 189 (86.3%) as well as 30(13.7%) females; controls of Arg194Trp included 199 (97.5%) males and 5 (2.5%) females. There was significant difference in distribution of males and females between controls and cases suggesting adequate matching (p=0.0001). In the present study, 79.4% of the cases were smokers and 20.6% were non-smokers, where as in the control group 77.4% were smokers and 22.6% were non-smokers, with no association with risk of lung cancer as p value came out to be 0.3031. Furthermore, among the smokers numbers of pack years were significantly lower in cases as compared to controls (33.8±30.9 vs. 26.6±21.3, p=0.0152). Regarding the histology, squamous cell carcinoma, adenocarcinoma, small cell lung carcinoma represented 96 (44%), 61 (27.8%), 55 (25.1%) of the total 219 Lung cancer patients, respectively. Also 7 (3.1%) were having unknown cellular types. Lung cancer patients had following stages. Stage I and II comprised of 2(1%) and 11(5.5%) patients respectively, whereas in stage III and IV each had 98 (44.8%) and 91 (41.5%) patients respectively with 17 (7.8%) unclassified. When the whole study group was considered, there was no difference in allele frequencies among cases and controls. The distribution of the studied genotype in the cases and control group was in the Hardy–Weinberg equilibrium ($\chi^2 < 3.8$). On stratification according to Tumor size(T), maximum number of cases were found in T4 stage (39.2%), T3 stage had 18.8% representation,T2 stage showed 11.5% representation while T1 stage had minimum representation of 4.5% and rest were unclassified. Cases were also stratified according to lymph node involvement and metastasis which were accounted as follows: 16% N0, 8.2%N1, 30.1%N2, 19.1%N3, 2%N4 and rest 24.6% unclassified also, 34.3% patients had metastasis.

Table 5.1b: Distribution of demographic variables for patients and controls showing *Arg*^{194}*Trp* polymorphism

Variable	Patients with *Arg*^{194}*Trp* polymorphism, (N= 219) N (%)	Controls, (N = 204) N (%)	*p* – value
Age (years)			
Mean ± SD	57.48 ± 10.15	52.98 ± 10.29	<0.0001
Range	(31 – 86)	(30 – 83)	
Gender			
Male	189 (86.3)	199 (97.5)	0.0001
Female	30 (13.7)	5 (2.5)	
Smoking Status			
Smokers	174 (79.4)	158 (77.4)	0.7024
Non – Smokers	45 (20.6)	46 (22.6)	
Pack Years			
Mean ± SD	33.8±30.9	26.6±21.3	0.0152
Histological Types			
SQCC	96 (44)		
ADCC	61 (27.8)		
SCLC	55 (25.1)		
Other carcinoma	7 (3.1)		
TNM Staging			
I	2 (0.9)		
II	11 (5)		
III	98 (44.8)		
IV	91 (41.5)		
Unclassified	17 (7.8)		
Tumor Size			
T1	10 (4.5)		
T2	25 (11.5)		
T3	41 (18.8)		
T4	86 (39.2)		
Unknown	57 (26)		
Lymph Node Involvement			
N0	35 (16)		
N1	18 (8.2)		
N2	66 (30.1)		
N3	42 (19.1)		
N4	4 (2)		
Unknown	54 (24.6)		
Metastasis			
M0	92 (42)		
M1	75 (34.3)		
Unknown	52 (23.7)		

SD = Standard Deviation

$p < 0.05$ was considered statistically significant

p-values were derived from pearson Chi – square test except age; Student t-test was used for age. All *p*- values are two – sided

5.6. LUNG CANCER RISK WITH *XRCC1Arg^{399}Gln* GENOTYPE

Table 5.2a: Frequency distribution of *XRCC1Arg^{399}Gln* genotype and associated risk

Arg^{399}gln rs25487 (G/A)	Patients with lung cancer, (N = 201) n (%)	Controls, (N =199) N (%)	Adjusted OR (95% CI)	*p*- value
GG	57 (28.3)	55 (27.7)	1.00 (Reference)	
GA	104 (51.7)	110 (55.3)	0.82 (0.50 - 1.33)	0.4389
AA	40 (20)	34 (17)	1.08 (0.58 – 2.00)	**0.052**
(GA + AA) vs. GG	144 (71.6)	144 (72.3)	0.878 (0.55- 1.39)	0.579
G	218 (54.2)	220 (55.2)	------	------
A	184 (45.8)	178 (44.8)	------	------
Minor allele frequency	0.45	0.44	------	------

OR odds ratio and (95% CI) confidence interval were calculated by logistic regression and adjusted for age, gender and smoking

Table 5.2a: shows the frequency distribution and the association risk of the *XRCC1Arg^{399}Gln* genotypes towards lung cancer. The distribution of *XRCC1Arg^{399}Gln* genotype in the study population as depicted had 28.3% GG (homozygous wild type), 51.7% GA (heterozygous) and 20% AA (homozygous variant) in cases whereas 27.7% GG, 55.3% GA, and 17% AA genotype in controls. No significant higher odds ratios were observed for *XRCC1* 399genotypes where as AA genotype showed no association with lung cancer (OR=1.08, 95%CI=0.58 – 2.00, *p*= 0.05).

We also looked for the combined effect of these polymorphisms in lung cancer susceptibility. Compared to the *XRCC1* 399 (GA+ AA) vs. GG genotype which being the reference genotype (OR = 0.878, 95% CI= 0.55- 1.39, *p*=0.579).

Pachouri *et al.,* 2007 in a case-control study examined the association of polymorphism in *XRCC1* gene and lung cancer risk and found the polymorphism at codon 399 was protective in lung cancer. A recent study by Karkucak *et al.,* 2012 reported no association of *XRCC1Arg^{399}Gln* polymorphism with lung cancer. Divine *et al.,* 2001 also reported 399Gln allele is more frequent in lung cancer. Kiran *et al.,* 2010 conducted study on south Indian population which has shown that Gln/Gln and Arg/Gln genotypes are associated with high risk of lung cancer.Recent study conducted in Northeastern Chinese lung cancer population by Guo, 2013 reported that heterozygous genotype (Arg/Gln) had much higher risk than other genotypes.

Chen *et al.,* 2002, and Zhang *et al.,* 2005 found no association of lung cancer with *XRCC1* 399 codon in Chinese population.The results are complex and contradictory with each others. Many studies proved that individuals with the 399Gln allele were not able to repair DNA damage and therefore had significant risk of lung carcinogenesis. But the functional consequences still remain unknown. But this study conducted in North Indian population showed no significant association of $Arg^{399}Gln$ polymorphism with lung cancer.

5.7 LUNG CANCER RISK WITH $XRCC1Arg^{194}Trp$ GENOTYPE

Table 5.2b: Frequency distribution of $XRCC1Arg^{194}Trp$ genotype and associated risk

$Arg^{194}trp$ rs1799782 (C/T)	Patients with lung cancer, (N = 219) N (%)	Controls, N = 204 N (%)	Adjusted OR (95% CI)	*p* value
CC	169 (77.2)	172(84.7)	1.00 (Reference)	
CT	49 (22.75)	31 (15.25)	1.38 (0.82 – 2.32)	0.2252
TT	1 (0.05)	1 (0.05)	-------	------
(CT + TT) vs. CC (dominant model)	50 (22.8)	32 (15.6)	1.39 (0.83 – 2.33)	0.2017
C	338 (87.1)	374(91.6)	------	------
T	50 (12.9)	34(8.4)	------	------
Minor allele frequency	0.116	0.081	------	------

OR odds ratio and (95% CI) confidence interval were calculated by logistic regression and adjusted for age, gender and smoking

Table 5.2b shows the frequency distribution and the association risk of the *XRCC1* $Arg^{194}Trp$ genotypes towards lung cancer. The distribution of $XRCC1Arg^{194}Trp$ genotype in the study population as depicted had 77.2%CC (homozygous wild type), 22.75% CT (heterozygous), and 0.05% TT (homozygous variant) in cases. For the case of controls 84.7% had homozygous wild genotype (CC), 15.25% had heterozygous genotype (CT) and homozygous variant genotype (TT) was similar to that of lung cancer i.e. 0.05%. Since the frequency of homozygous variant (TT) genotype was less, we combined the heterozygous and homozygous variant genotype as one genotype compared it with the reference genotype(CC). The combined genotype had a slight risk towards lung cancer, however this associated risk was non-significant (OR = 1.39, 95% CI = 0.83 – 2.33, *P* = 0.2017) had insignificant high odds ratio. Hence Arg194Trp polymorphism with at least one T allele may act as risk factor but there was no significant result observed. A study

by Zhang J *et al.,* 2014 showed no association of *XRCC1Arg*^{194}*Trp* with lung cancer risk. Study in Chinese Han population by Tao Wu *et al.*, showed no obvious association between *Arg*^{194}*Trp* polymorphism and lung cancer risk under the dominant model. Hauang *et al.,* 2011 found *Arg*^{194}*Trp* polymorphism as cancer susceptibility factor among Chinese Mainland population. In Tawanese population there is no association between *Arg*^{194}*Trp* and lung cancer risk found by Wei-Chung Hseih *et al.,* 2008

5.8 DISTRIBUTION OF *Arg*^{399}*Gln* GENOTYPE OF *XRCC1* GENE AMONG PATIENTS WITH DIFFERENT TYPES OF LUNG CANCER

Table 5.3a: Frequency distribution of *XRCC1 Arg*^{399}*Gln* genotype among different histological types of lung cancer

Genotype Arg^{399}gln rs25487 (G/A)	Patients with Lung cancer (N = 201) N (%)	Controls, (N = 199) N (%)	AOR (95% CI)	*P* – value
SQCC	90(44.8)			
GG	23(25.5)	55 (27.7)	1.00 (Reference)	
GA	52(57.8)	110 (55.3)	1.16 (0.63-2.15)	0.6203
AA	15(16.7)	34 (17)	1.12 (0.50-2.53)	0.7733
GA + AA	67(33.3)	144 (72.3)	1.16 (0.65-2.10)	0.6013
ADCC	55(27.4)			
GG	19(34.5)	55 (27.7)	1.00 (Reference)	
GA	23(41.9)	110 (55.3)	0.59 (0.28-1.24)	1.665
AA	13(23.6)	34 (17)	1.03 (0.43-2.49)	0.931
GA + AA	36(65.4)	144 (72.3)	0.71 (0.36-1.39)	0.323
SCLC	49(24.4)			
GG	13(26.5)	55 (27.7)	1.00 (Reference)	
GA	26(53.0)	110 (55.3)	0.96 (0.43-2.14)	0.9294
AA	10(20.5)	34 (17)	1.35 (0.49-3.65)	0.5536
GA+AA	36(73.4)	144 (72.3)	1.06 (0.50-2.24)	0.8758
UNKNOWN	7(3.4)			

OR odds ratio and (95% CI) confidence interval were calculated by logistic regression and adjusted for age, gender and smoking

We further analyzed the risk of the *Arg*^{399}*Gln* polymorphism after stratification according to histology and the three genotypes (homozygous wild, heterozygous, homozygous variant) were compared in cases and controls. Regarding the distribution of *XRCC1 Arg*^{399}*Gln* genotypes in the study population the total no. of SQCC, ADCC, SCLC cases studied were 44, 27.8, and 25.1%

respectively whereas 3.1% cases were unknown. In SQCC 25.5% GG (homozygous wild), 57.8% GA (heterozygous) and had 16.7%AA (homozygous variant) when compared controls had 27.7% GG (homozygous wild), 55.3% had GA (heterozygous) and 17% had AA (homozygous variant). The combined effect of these genotypes on histology was studied and no significant association was observed (OR=1.16, 95% CI =0.65-2.10, *p*=0.6013). In ADCC 34.5% had GG (homozygous wild), 41.9% had GA (heterozygous) and 23.6% had AA (homozygous variant). When compared to 27.7% GG (homozygous wild), 55.3% GA (heterozygous), and 17AA (homozygous variant) in controls no risk was observed within genotype. In SCLC 26.5% had GG (homozygous wild), 53% had GA (heterozygous) and 20.5% had AA (homozygous variant) genotype. When compared to 27.7% GG (homozygous wild), 55.3% GA (heterozygous), 17% AA (homozygous variant) in controls. No significant association was observed within heterozygous genotype (GA) (OR =0.96, 95%CI=0.43-2.14, *p*=0.9294). However a slight risk was observed within homozygous variant (AA) genotype which is however was non-significant (OR=1.35, 95%CI=0.49-3.65, *p*=0.5536)

Jae Young Park *et al.,* 2002 found codon 399Gln allele of *XRCC1* gene was associated with increased risk of squamous cell carcinoma of lung. Divine *et al.,* 2001 who reported an association between the 399AA variant genotype and elevated risk for lung Adenocarcinoma. Park *et al.,* 2002 found 399AA genotype is associated with increased risk of squamous cell carcinoma in Koreans. R.Misra *et al.,* 2003 in their study on male smoker population of Finland found no such direct association between any type of tumor subtype and genotype of *XRCC1*

.DNA repair gene *XRCC1* 399 is a risk for adenocarcinoma of lung cancer. In the study done here we had results somewhat supporting the results discussed by R.Misra *et al.,* for the case of SQCC and SCLC type of cells, in his study on male smokers. O.R. for homozygous variants and the combination of carriers and the homozygous variants were same (O.R 1.16/O.R.1) indicating whereas it was bit different for carries alone (O.R.1.12/O.R.1.35) though which was again not statistically significant. Whereas for ADCC type of cells, the carrier genotype (O.R=0.59) along with combination of carrier and homozygous variants (O.R=0.7) were associated with decreased risk of lung cancer though not statistically significant.

5.9 DISTRIBUTION OF *Arg^{194}Trp* GENOTYPE OF *XRCC1* GENE AMONG PATIENTS WITH DIFFERENT HISTOLOGICAL TYPES OF LUNG CANCER

5.3b: Frequency distribution *XRCC1Arg^{194}Trp* genotype among different histological types of lung cancers.

Genotype *Arg^{194}Trp* rs1799782 (C/T)	Patients with Lung cancer, (N = 219) N (%)	Controls, (N = 204) N (%)	AOR (95% CI)	*P* - value
SQCC	96 (44)			
CC	72 (75)	172 (84.7)	1.00 (Reference)	
CT	23 (24)	31(15.25)	1.50 (0.80-2.84)	0.2031
TT	1(1.0)	1(0.05)	------	------
CT + TT	24 (25)	32 (15.6)	1.54 (0.80-2.84)	0.1696
ADCC	61(27.8)	------	------	------
CC	48(78.7)	172 (84.7)		
CT	13(21.3)	31 (15.25)	1.19 (0.52-2.70)	0.6757
TT	0	1 (0.05)	------	------
CT + TT	13(21.3)	32 (15.6)	------	------
SCLC	55(25.1)			
CC	43(78.1)	172 (84.7)	1.00 (Reference)	
CT	12(21.9)	31 (15.25)	1.10 (0.49-2.48)	0.8101
TT	0	1 (0.05)	------	------
CT +TT	12(21.9)	32 (15.6)	------	------
UNKNOWN	7(3.1)			

OR odds ratio and (95% CI) confidence interval were calculated by logistic regression and adjusted for age, gender and smoking

We further analyzed the risk of the *Arg^{194}Trp* polymorphism after stratification according to histology and the three genotypes (homozygous wild, heterozygous, homozygous variant) were compared in cases and controls. The population recruited for the study of histology in association with lung cancer for *XRCC1Arg^{194}Trp* polymorphism had 44% SQCC, 27.8% had ADCC, and 25.1% had SCLC whereas 3.1% had unknown cases. The cases having SQCC histology had 75% CC (homozygous wild), 24% had CT (heterozygous), and 1% had TT (homozygous variant). When compared to their normal counterparts which had 84.7 %(homozygous wild), 15.25 % had (heterozygous) and 0.05 % had (homozygous variant). A slight risk was found in case of CT (heterozygous) which however was non-significant (OR=1.50, 95%CI=0.80-2.84, *p*=0.2031).When the genotypes were combined slight risk was found (OR=1.54, 95%CI=0.80-

2.84, p=0.1696) which was non-significant. Sturgis *et al.*, 1999 reported that $Arg^{194}Trp$ polymorphism was associated with a reduction in risk of squamous cell carcinoma.

5.10 EFFECTS OF SMOKING STATUS ON THE ASSOCIATION BETWEEN $Arg^{399}Gln$ GENOTYPE AND LUNG CANCER RISK

Table5.4a: Frequency distribution of *XRCC1*$Arg^{399}Gln$ genotype among smokers and non-smokers

XRCC1 $Arg^{399}Gln$	Genotype	Cases(N)	Controls(N)	OR(95%CI)	*p*-value
Smokers	G/A	90	85	1.08(0.69-1.8)	0.75
	A/A	29	22	1.39(0.67-2.69)	0.84
	G/A+A/A	119	107	1.13(0.69-1.8)	0.5
	GG	44	45	Reference	------
Non-smokers	G/A	14	25	0.51(0.19-1.36)	0.11
	A/A	11	12	0.7(0.22-2.25)	0.59
	G/A+A/A	25	37	0.51(0.19-1.36)	0.18
	GG	13	10	Reference	------

OR odds ratio and (95% CI) confidence interval were calculated by logistic regression and adjusted for age, gender and smoking

Table 5.4a shows the effect of smoking on lung cancer risk in relation with the genotype of *XRCC1*$Arg^{399}Gln$. Smokers and Non Smokers of cases and controls were further stratified according to genotype. As observed, 81% of the cases studied were smokers while 19% were non-smokers. Of which 21.9, 44.8, and 14.5% smokers were homozygous wild, heterozygous and homozygous variant respectively and 6.4, 7, and 5.4% non-smokers were homozygous wild, heterozygous and homozygous variant respectively .On the other hand 76.4% of controls were smokers and 23.6% were non-smokers. In control smokers 22.7% (homozygous wild), 42.8 % (heterozygous), 11% (homozygous variant) were accounted. Whereas in control non-smokers 5% (homozygous wild), 12.5% (heterozygous), 6% (homozygous variant) were reported.When the smokers present in cases were compared according to its genotype a slight risk was found in case

of homozygous variant (OR=1.39, 95%CI 0.67-2.69, *p*=0.84) which however was statistically insignificant. In case of non-smokers comparatively protective effect was found.

Wei Zhou *et al.,* 2003 found that cumulative smoking plays an important role in altering the direction and magnitude of the association between the *XRCC1* and lung cancer. In a study in Finland conducted by Rita misra *et al.,* (2003) reported that *XRCC1* codon 399 genotype and lung cancer was modified by the amount of smoking. Hidmani *et al.,* 2004 found that

XRCC1Arg^{399}*Gln* polymorphism might the risk of lung cancer attributable to cigarette smoking exposure.

5.11 EFFECTS OF SMOKING STATUS ON THE ASSOCIATION BETWEEN *Arg*^{194}*Trp* GENOTYPE AND LUNG CANCER RISK

Table 5.4b: Frequency distribution of *XRCC1Arg*^{194}*Trp* genotype among smokers and non-smokers

XRCC1 Arg*^{194}*Gln	**Genotype**	**Cases(N) N=219(%)**	**Controls(N) N=219(%)**	**OR(95%CI)**	***p*-value**
Smokers	C/T+T/T	43(19.5)	28 (13.7)	1.5 (0.89-2.6)	0.12
	CC	131 (60)	130 (63.8)	Reference	------
Non-smokers	C/T	7(3.2)	4 (2)	1.93 (0.19-1.36)	0.32
	CC	38(17.3)	42 (20.5)	Reference	------

Table 5.4b shows the effect of smoking on lung cancer risk in relation with the genotype of *XRCC1Arg*^{194}*Trp*. As observed, 79.5% of the cases studied were smokers while 20.5% were non-smokers. On the other hand 77.4% of controls were smokers and 22.6% were non-smokers. Smokers and Non Smokers with lung cancer were further stratified according to genotype. In case

of smokers when heterozygous and homozygous variant were combined which accounts 19.5% a slight risk was found (OR=1.5, 95%CI 0.89-2.6, *p*= 0.12). Again in case of non-smokers risk was found for heterozygous genotype (OR=1.93, 95%CI 0.19-1.36, *p*=0.32).

5.12: EFFECT OF *XRCC1 ARG^{399}GLN* AND *ARG^{194}TRP* POLYMORPHISM ON OVERALL SURVIVAL OF LUNG CANCER PATIENTS

Table 5.5 : Association of *XRCC1 Arg^{399}Gln* and *Arg^{194}Trp* genotype with overall survival of lung cancer patients.

Parameter	Genotype	Events/ censored	Median survival time (months)	Log rank *p* Value
Arg^{399}Gln	GG	11/15	12.8	0.9
	GA	17/26	12.9	
	AA	5/13	11.8	
Arg^{194}Trp	CC	25/47	12.8	0.5
	CT	5/13	13.9	
Arg^{399}Gln+ Arg^{194}Trp	GG+CC	9/9	12.5	0.8
	GA+CT	7/21	11.8	

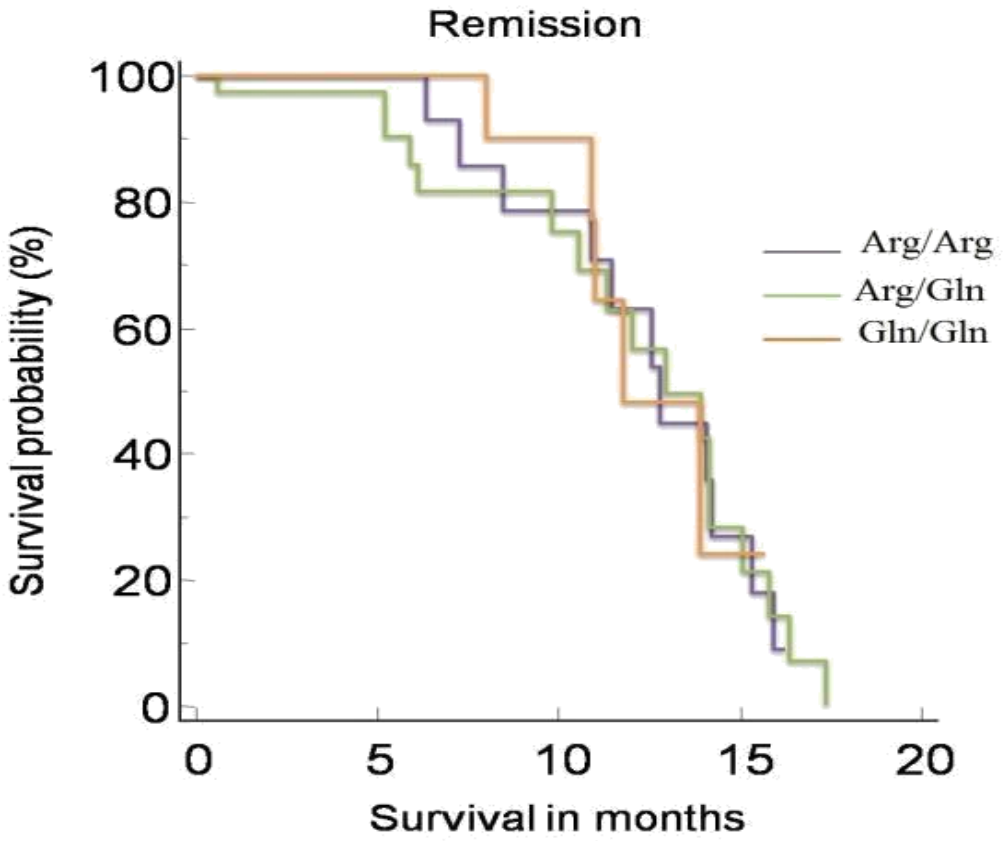


Fig.5.7 :Kaplan-Meier plot of *Arg*^{399}*Gln* polymorphism and log-rank test(*p*=0.9) for overall survival .The log-rank test was based on full data.Note: Arg/Arg=Homozygous wild genotype, Ar/Gln=Hetero

Data of 90 patients was available for the survival analysis. The above Kaplan-Meier survival curve is for *XRCC1Arg*^{399}*Gln* in which X-axis represents survival time in months and Y-axis represents survival probability. According to the graph heterozygous genotype(Arg/Gln) had maximum medial survial time in months *i.e.* 12.9 (95% CI =10.533-15.067). As shown in table( ) the median survial time for homozygous wild genotype(Arg/Arg) was 12.8 months (95%CI =10.9 -15.367) and 11.8 months (95%CI = 10.933–13.900) for homozygous variant (Gln/Gln)(log rank *p* value 0.9). And hence patients with heterozygous genotype have good response towards chemotherapy.

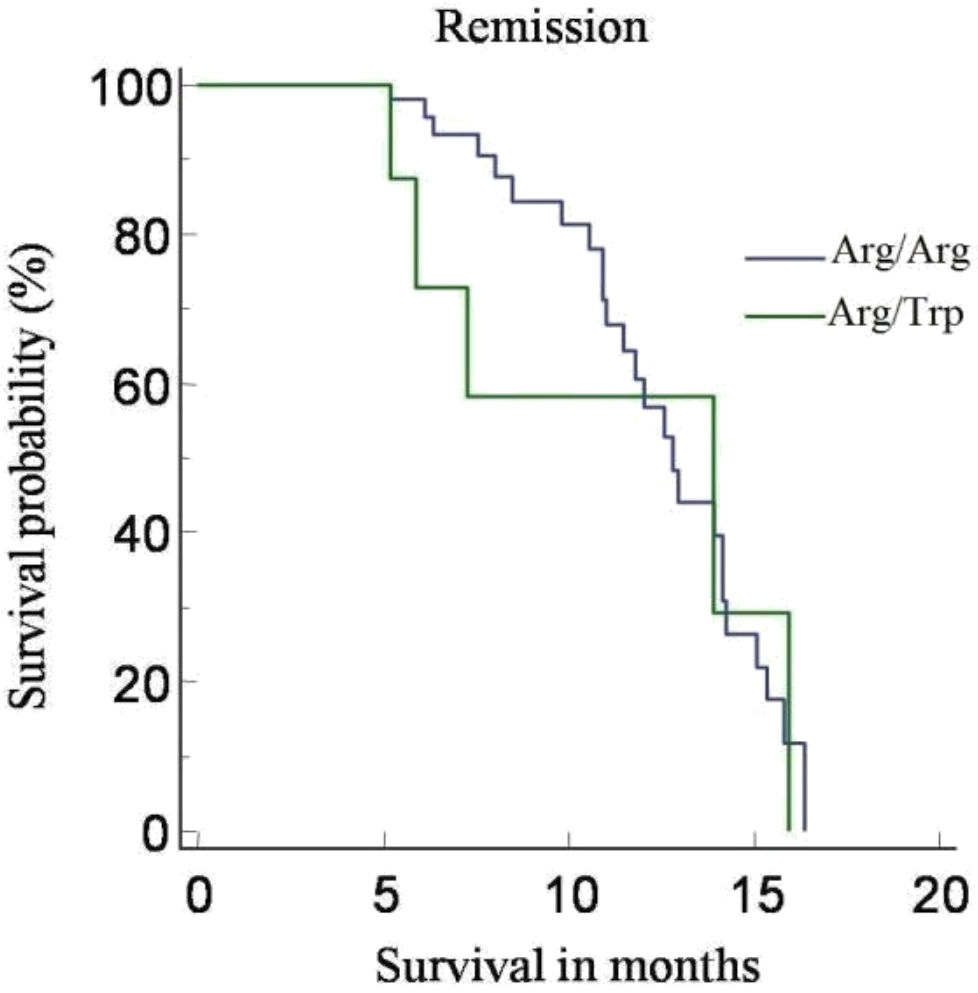


Fig5.8:Kaplan-Meier plot of *Arg*^{194}*Trp* polymorphism and log-rank test(p=0.5) for overall survival .The log-rank test was based on full data.

The above figure represents the Kaplan-Meier survival curve for the gene *XRCC1Arg*^{194}*Trp* where X-axis represents survival time in months and Y-axis represents survival probability.The survivalbility of heterozygous genotype(Arg/Trp) had the maxium median survial time of 13.9 (95% CI =5.9-15.933) months whereas homozygous wild genotype(Arg/Arg) had 12.8 months (95% CI =11.467-14.133) of median survival time (log rank p value =0.5). Hence patients with heterozygous genotype have increased response towards chemotherapy.

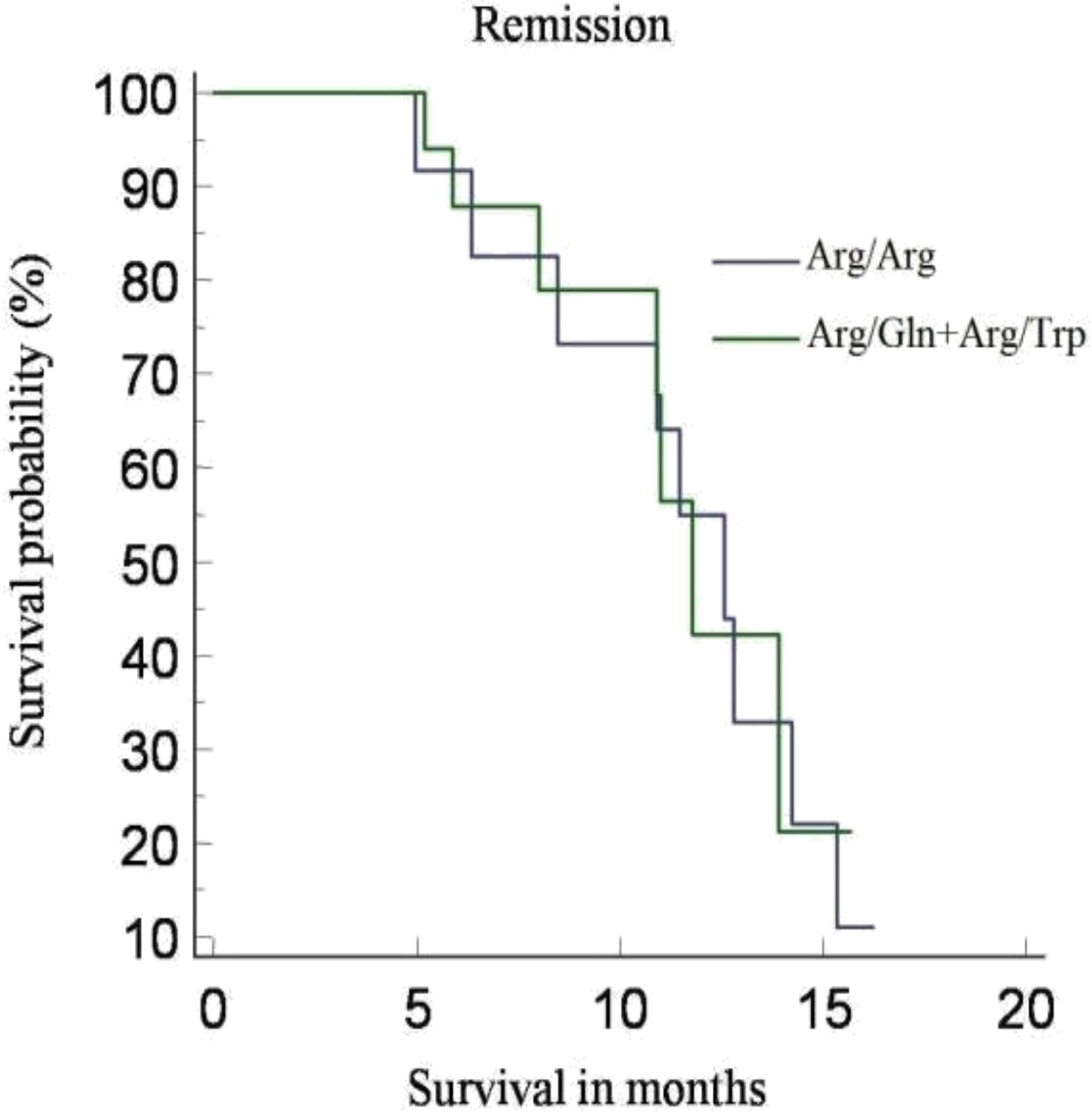


Fig 5.9: Kaplan-Meier plot of combination of *Arg*^{399}*Gln and Arg* ^{194}*Trp* polymorphisms and log-rank test(p=0.8) for overall survival .The log-rank test was based on full data .

The above figure is Kaplan-Meier survival curve for the combination of the two polymorphisms of *XRCC1Arg*^{399}*Gln* and *XRCC1 Arg*^{194}*Trp* where X-axis represents survival time in months and Y-axis represents survival probability.The combination was of common heterozygous and homozygous wild genotype of both polymorphism as there was no homozygous variant in *XRCC1 Arg*^{194}*Trp* polymorphism and hence homozygous variant of *Arg*^{399}*Gln* was converted to heterozygous genotype. Maximum median survial time was observed for homozygous wild

genotype(Arg/Arg) *i.e.*12.5 months (95% CI=8.5-14.2). Heterozygous genotype (Arg/Gln+Arg/Trp) had 11.8 months (95% CI=10.9 to 13.9) of median survival time(log rank *p* value=0.8).

A study by Hseih *et al.,* (2008) in Taiwanese population showed that patient with $Arg^{194}Trp$ homozygous variant genotype had lower survival rate and 1.84 fold risk. Sun *et al.,* (2008) found no considerable association between *XRCC1399* polymorphism and treatment response to platinum based chemotherapy in Chinese population. Gurubhagavatula *et al.,* (2004) reported that individuals with *XRCC1399,* the variant genotype (Gln/Gln) were associated with shorter overall survival, which is same as our result. Daniel *et al.,* (2007) reported that the $Arg^{399}Gln$ homozygous variant genotype was associated with higher median survival time which is contradictory to our result. In a meta-analysis Junjie Wu *et al.,*(2012) found 194Trp allele may be indicative of better response rates to platinum based treatment than 194Arg allele and 399Gln allele is associated with poorer response rate to platinum-based treatment than 399Arg allele. Horang AM *et al.,* (2011) also found association of $Arg^{399}Gln$ with clinical outcome of chemotherapy. Cui Z *et al.,* (2012) reported that both $Arg^{399}Gln$ and $Arg^{194}Trp$ were significantly associated with response to treatment of lung cancer patients.

CONCLUSION

The present case/control study pertains to patients visiting the Post Graduate Institute of Medical Education and Research, which is a referral center for patients from states like Haryana, Himachal Pradesh, Punjab, Uttar Pradesh, Jammu & Kashmir and Chandigarh. The following points are evident from the present study

- The two polymorphisms of the gene *XRCC1*, *Arg*^{399}*Gln* and *Arg*^{194}*Trp* did not account as a risk factor for lung cancer and even not found to be protective also.
- No significant association was found for any of the three types of histology with genotype.
- There was no significant association of *Arg*^{399}*Gln* and *Arg*^{194}*Trp* genotypes in smokers.
- In case of *XRCC1 Arg*^{399}*Gln* and *Arg*^{194}*Trp* polymorphism, the heterozygous genotype showed a maximum median survival time and hence indicating a good survival response.
- When a combined survival analysis was carried out for both the polymorphic sites (*Arg*^{399}*Gln* and *Arg*^{194}*Trp)* the individuals carrying homozygous wild genotype showed maximum median survival time indicating good prognosis.

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