A Study on Association of XRCC3 gene Polymorphism in Gastric cancer risk.

Dissertation Submitted for the Award of the Degree of Master of Philosophy in Biochemistry

By

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UNDER THE JOINT SUPERVISION OF

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(NAAC Accredited Grade "A")

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CERTIFICATE

Certified that the work embodied in the dissertation entitled ***"A Study on Association of XRCC3 Gene Polymorphism in Gastric cancer risk"*** is the bonafide work of**Mr. Haamid Bashir** and has been carried out under the joint supervision of**Prof. SabhiyaMajid** (Head, Department of Biochemistry, Govt. Medical College Srinagar, Research Centre-University of Kashmir), **Dr. Rabia Hamid** (Department of Biochemistry, University of Kashmir) in the Department of Biochemistry, Govt. Medical College Srinagar and Department of Biochemistry, University of Kashmir. The work is suitable for the award of M.Phil degree in Biochemistry.

It is further certified that no work under this heading has previously been submitted to the University of Kashmir for the award of any degree or diploma to the best of our belief.

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DECLERATION

I, **Haamid Bashir,** declare that the work embodied in this dissertation entitled **"A Study on Association of XRCC3 Gene Polymorphism in Gastric cancer risk"** has been carried out by me in the Department of Biochemistry, Government Medical College, Srinagar (Research Centre university of Kashmir), and Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

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Date:

DEDICATED TO MY PARENTS

Who cherished my dreams through sacrifice

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μg	Microgram
μl	Microlitre
μM	Micromolar
bp	Base pair
BPB	Bromophenol blue
cm	Centimetre
DDW	Double distilled water
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribosenucleotide –triphosphate
EDTA	Ethylene diaminetetraacetic acid
GC	Gastric Cancer
EtBr	Ethidium bromide
Fig.	Figure
gm	Grams
GMC	Govt. Medical College
h	Hours
Kb	Kilobase pair
Kd	Kilo Dalton
M	Molar
mg	Miligram
min	Minutes

ml Mililitre

mM mili molar

mRNA messenger ribonucleic acid

PCR Polymerase Chain Reaction

RFLP Restriction Fragment Length Polymorphism

NaCl Sodium chloride

ng Nanogram

O.D Optical density

O.R Odds ratio

^{o}C Degree Celsius

PAGE Polyacrylamide gel Electrophoresis

pmol Pico mole

rpm Revolutions per minute

RT Room temperature

S.M.H.S. Shri Maharaja Hari Singh

SDS Sodium dodecyl sulphate

sec Second

SES Socio economic status

SNP Single nucleotide polymorphism

STE Sodium chloride-Tris-Ethylenediaminetetraacetic acid

TAE Trisacetic EDTA

Taq *Thermus aquaticus* DNA polymerase

Tm Melting temperature

Tris Tris(hydroxymethylaminomethane)

UV Ultraviolet

XRCC3 X-ray cross complementing protein-3

NlaIII Nisseria lacteamcia

H.O.D Head of Departement

χ^2 Chi-square

Gastric Cancer (GC) is among the common and fatal cancer in the world. DNA repair plays a critical role in protecting the genome of the cell from insults of cancer-causing agents. Inherited polymorphisms of DNA repair genes may contribute to variations in DNA Repair capacity (DRC) and genetic susceptibility to different cancers. Mammalian cells are constantly exposed to a wide variety of genotoxic agents from both endogenous and exogenous sources. In human beings, 70 genes are involved in the five major DNA repair pathways: direct repair, NER, BER, mismatch repair and double-strand break repair. The X-ray repair complementing defective repair in Chinese hamster cells 3 (XRCC3) gene is a member of the RAD51 gene family. It encodes an important protein that functions in the homologous recombination repair of DNA double-strand break. The Kashmir valley has an elevated incidence of GC and its etiology is not understood fully yet, though, we are ethnically and demographically different from the other states of the country and world. The aim of this study was to determine whether single nucleotide polymorphism (SNP) of XRCC3 gene (Thr241Met) of exon7, can influence the risk of gastric cancer in Kashmiri population. About 80 histopathologically confirmed GC cases and 70 healthy controls, age ,gender, ethnicity matched for known genotypes of XRCC3 exon7 were analyzed. Patients medical history and dietary habits were taken for the study as well.

The genotype for this variant was determined using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) in 80 histologically confirmed GC patients and 70 frequency-matched healthy controls in Department of Biochemistry, Government Medical College srinagar. The XRCC3 genotype and allele frequencies were not significantly different between cases and controls (P= 0.92 for genotype; P= 0:72 for allele). The XRCC3 241Met allele frequency (6.6%) was significantly lower in healthy Kashmiri controls than previously reported healthy US Caucasian controls (38.9%). Compared with the XRCC3241Thr/Thr genotype, the variant XRCC3241Thr/Met and Met/Met genotypes were not associated with an increased risk of gastric cancer (adjusted odds ratio (ORa), 1.19; 95% confidence interval (CI), 0.44-3.18). These findings suggest that polymorphisms of XRCC3 Thr241Met may not play a role in the etiology of GC. Further studies with a larger number of subjects and simultaneous measurement of different polymorphisms in DNA repair genes in the same pathway are needed.

1.1 Cancer

Cancer is an English term, dissimilated from Greek word "Karkinos" (Sanskrit Karkata) for crab, the symbol for fourth zodiacal constellation (The CANCER). The word was believed to be first used by Hippocrates, who attributed this affliction to an excess of black bile. Cancer was known in old ages, being described in early writings of Greeks and Romans. Pathological evidences support the bone tumors in dinosaurs and other prehistoric animals but the tumors in Egyptian mummies dating back 5000 years represent the first known human malignant growths. The vast catalog of cancer cell genotypes is a manifestation of following six basic alterations in cell physiology (Hanahan *et al.,* 2000) (a) Self-sufficiency in growth signals (b) Insensitivity to growth-inhibitory (antigrowth) signals (c) Evasion of programmed cell death (apoptosis) (d) Limitless replicative potential (e) Sustained angiogenesis and (f) Tissue invasion and metastasis. In, general malignant cancers cause significant morbidity and will be lethal to the host if left untreated. Exceptions to this appear to be latent, indolent cancers that may remain clinically undetectable (or in situ), allowing the host to have a standard life expectancy.

Cancer is a disease that involves dynamic changes in genomes which result in abnormal gene expression. Individual cancer susceptibility can be the result of several host factors, including differences in metabolism which has been related to the enzymatic polymorphisms involved in activation and detoxification of chemical carcinogens, the presence of millions of such polymorphic gene variants in the human genome (Sachidanandam *et al.,* 2001*,* Subramanian *et al.,* 2001) provide extensive genetic (and eventually phenotypic) variation affecting both normal physiological mechanisms and cancer pathogenesis, loss of functions of DNA repair genes, activation of oncogenes by molecular and chromosomal rearrangements in co-ordination with cellular transcription factors, mutation in tumor suppressor genes or interaction of tumor suppressor gene products with viral oncoproteins, and nutritional status. Besides gene polymorphisms and genetic alterations, epigenetic events also play role in cancer development as epigenetic regulators alter the activities and abilities of a cell without directly affecting and mutating the sequence of the DNA, so perturbation of epigenetic balances may lead to alterations in gene expression, ultimately resulting in cellular transformation and malignant outgrowth. Thus gene function in cancer can be disrupted either through genetic alterations, which directly mutate or delete genes, or epigenetic

alterations, which alter the heritable state of gene expression.

Clinically, cancer appears to be many different diseases with different phenotypic characteristics. As a cancerous growth progresses, genetic drift in the cell population produces cell heterogeneity in such characteristics as cell antigenicity, invasiveness, metastatic potential, rate of cell proliferation, differentiation state, and response to chemotherapeutic agents. At the molecular level, all cancers have several things in common, which suggests that the ultimate biochemical lesions leading to malignant transformation and progression can be produced by a common but not identical pattern of alterations of gene readout.

Epidemiological studies have shown that upto 90% of all cancers are related to environmental factors. Recent estimations suggest that there are over 1.4 million sequence variants known as single nucleotides polymorphisms (SNPs) in the human genome and other polymorphisms like variable number of tandem repeats (VNTR), deletions and gene amplifications in the low penetrance genes which may lead to inter-individual differences in susceptibility and genetic predisposition to cancer. However all individuals exposed to the same type and dose of carcinogen do not develop cancer as there is wide inter-individual variability in genes which eliminate the detoxifying effect of carcinogens and protect the cells from damage. The study of genetic variants in low penetrance group of predisposing polymorphic genes together with environmental influences may lead to identification of susceptible subgroups with increased risk for cancer in exposed individuals perhaps at low doses of carcinogens.

Recent estimates show that there are 10.1 million new cases and more than 6.2 million deaths, and 22.4 million persons living with cancer (WHO. 2006). Global burden of cancer continues to increase and more than 24 million people were living with cancer in the year 2002 (Parkin *et al.,* 2005). Incidence of lung cancer has been found to be the main cancer in world followed by cancer of the breast (Parkin *et al.,* 2005). Although lung cancer is predominant cancer in men it is followed by cancer of esophagus and stomach, cervical being the most common cancer in Indian women (*PBCRs, NCRP Report., 2001*).

1.2 Gastric Cancer

Gastric cancer is one of the most common cancers worldwide with approximately 989,600 new cases and 738,000 deaths per year, accounting for about 8 percent of new

cancers (Jemal *et al .,* 2011). Gastric cancer remains one of the most common cancer in Asia (Inone *et al.,* 2005, Bae *et al.,* 2002). Gastric cancer is the third most common cancer in India and the second leading site of cancer occurrence worldwide (Parkin *et al.,* 2001). Gastric cancer has been described as early as 3000 BC in hieroglyphic inscriptions and papyri manuscripts from ancient Egypt. The first major statistical analysis of cancer incidence and mortality (*using data gathered in Verona, Italy from 1760 to 1839*) showed that gastric cancer was the most common and lethal cancer.

It has remained one of the most important malignant diseases with significant geographical, ethnic, and socioeconomic differences in distribution. gastric cancer, like all cancers results from an abnormality in the body's basic unit of life, the cell. Normally, the body maintains a system of checks and balances on cell growth so that cells divide to produce new cells only when new cells are needed. Disruption of this system of checks and balances on cell growth results in an uncontrolled division and proliferation of cells that eventually forms a mass known as a tumour.

1.2.1 Symptoms

Gastric cancer is often asymptomatic. By the time symptoms occur, the cancer has often reached an advanced stage. Symptoms are indigestion, loss of appetite, abdominal discomfort, abdominal pain in the upper abdomen, bleeding etc

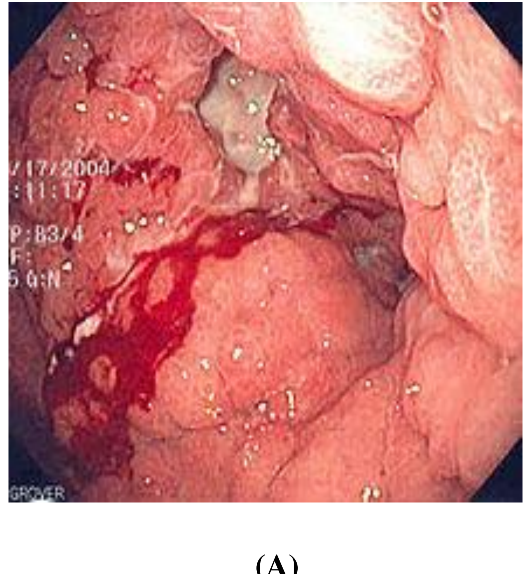
1.2.2 Causes

Multiple factors contribute to the pathogenesis of gastric cancer. Marked differences in gastric cancer incidence among different ethnic groups living in the same geographic area have been observed, pointing to host genetic factors or socio-environmental factors peculiar to a particular racial group (Miller *et al.,* 1996). Environmental factors including, dietary habits, are important in its development (Lee *et al.,* 1995*,* Ahn *et al.,* 1997). Consumption of salted, smoked, pickled and preserved foods rich in salt, nitrite and preformed N-nitroso compounds have been reported to be associated with an increased risk of gastric cancer (Malik *et al*., 2011). Infection by *Helicobacter pylori* is believed to be a cause of most stomach cancers while autoimmune atrophic gastritis, intestinal metaplasia and various genetic factors are associated with increased risk levels. *Helicobacter pylori*, or *H. pylori*, is a spiral-shaped bacterium that grows in the mucus layer that coats the inside of the human stomach. To survive in the harsh, acidic environment of the stomach, *H. pylori* secretes an enzyme called urease, which

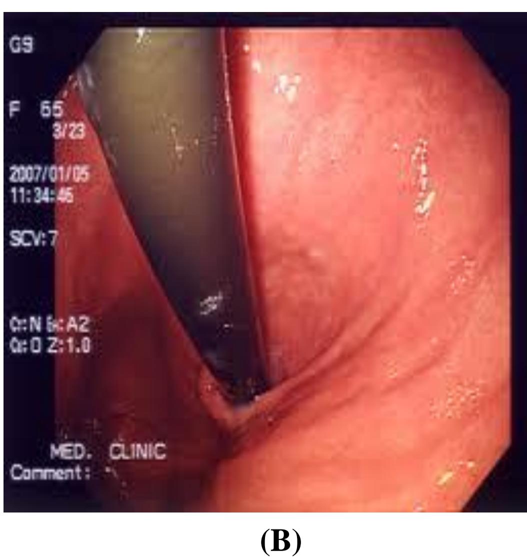
converts the chemical urea to ammonia. The production of ammonia around *H. pylori* neutralizes the acidity of the stomach, making it more hospitable for the bacterium. In addition, the helical shape of *H. pylori* allows it to burrow into the mucus layer, which is less acidic than the inside space, or lumen, of the stomach. *H. pylori* can also attach to the cells that line the inner surface of the stomach.

Although immune cells that normally recognize and attack invading bacteria accumulate near sites of *H. pylori* infection, they are unable to reach the stomach lining. In addition, *H. pylori* has developed ways of interfering with local immune responses, making them ineffective in eliminating the bacteria (Atherton *et al., 2006,* Kusters *et al.,* 2006). *H. pylori* has coexisted with humans for many thousands of years and infection with the bacterium is common. The Center for Disease Control and Prevention (CDC) estimates that approximately two-thirds of the world's population harbors the bacterium, with infection rates much higher in developing countries than in developed nations. A study reported from china supports that gastric cancer is primarily determined by environmental factors and develops in a multistep progression of precancerous lesions (*You et al., 2005*).

Gastric cancer may be seen on gastroscopic exam, endoscopy and computed Tomography (CT-SCAN). The diagnosis is confirmed with a biopsy. Abnormal Tissue seen in gastroscope examination is biopsied by the Surgeon or Gastroenterologist. Treatment and prognosis depend upon the histological type of cancer, the stage (degree of spread), and the patient's performance status. Possible treatments include surgery, chemotherapy, and radiotherapy. With treatment, the five-year survival rate is 14% (Minna *et al.,* 2004).



(A)



(B)

Fig. 1: Picture (**A**) showing stomach cancer where the entire stomach is invaded, leading to leather bottle-like appearance with blood coming out it. Picture **(B)** showing normal view of stomach by endoscopy procedure (*courtesy : wikipedia*)

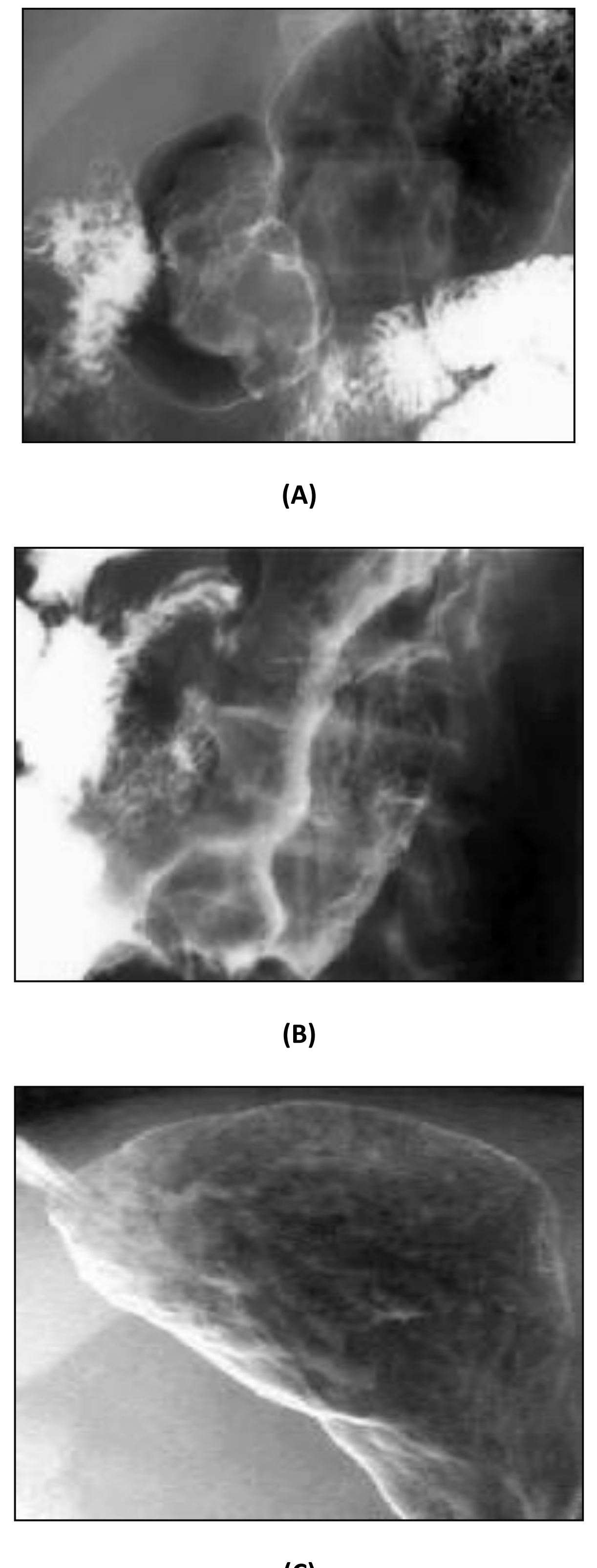
1.3. Gastric carcinoma

Tumors can be benign or malignant when we speak of "cancer," Benign tumors usually can be removed and do not spread to other parts of the body. Malignant tumors, on the other hand, grow aggressively and invade other tissues of the body, allowing entry of tumor cells into the bloodstream or lymphatic system and then to other sites in the body. This process of spread is termed metastasis; the areas of tumor growth at these distant sites are called metastases. Gastric carcinoma is the most common cancer in the world after lung cancer and is a major cause of mortality and morbidity. Though a marked reduction has been observed in the incidence of gastric carcinoma in North America and Western Europe in the last 50 years, 5-year survival rates are less than 20%, as most patients present late and are unsuitable for curative, radical surgery.

Adenocarcinomas account for approximately 95% of all malignant gastric neoplasms. The remaining 5% of tumors are lymphomas, leiomyosarcomas, carcinoids, or sarcomas. Gastric adenocarcinomas are divided into 2 types. Type 1 adenocarcinomas are intestinal tumors and have well-formed glandular structures. This form of gastric carcinoma is more likely to involve the distal stomach and occur in patients with atrophic gastritis (Fig.2). It has a strong environmental association.

1.4. Morphology of the Gastric cancer

According to modern concepts, gastric cancer is sub-divided into three major histological types: intestinal, diffuse and mixed. This classification was proposed by P. Laurent, who grouped cancer by its etiology, morphogenesis, macro-and microcharactersitics, the specific features of this course, and prognosis of the disease. Approximately 90% of gastric cancers are adenocarcinomas. These can be classified according to their differentiation or according to the histomorphological classification of Lauren, which divides the tumours into `intestinal' and `diffuse' (Lauren et al., 1965). Intestinal tumours are usually exophytic, often ulcerating, and are associated with intestinal metaplasia of the stomach. Diffuse tumours are poorly differentiated infiltrating lesions which lead to thickening of the stomach (linitisplastica). Patients with diffuse-type tumours have a worse prognosis than those with an intestinal type. Intestinal cancers appear to be more common in proximal (fundus) localized tumours than in distal lesions, while diffuse, poorly differentiated tumours predominate in younger patients (Lo *et al*., 1996). Interestingly, the diffuse type of gastric carcinoma



(A)

(B)

(C)

Fig. 2: Gastric carcinoma imaging depicts following (A) Polypoid carcinoma of body of stomach, (B) Infilitrating carcinoma in stomach, (C) Atrophic gastritis (courtesy : Wikipedia).

demonstrates a nearly equal sex ratio, compared with a male preponderance in the intestinal form.

1.5. Inherited predisposition to cancer

It is well established that most genetic alterations leading to cancer are somatic. However, a number of cancers (around 5-10%) are due to inherited cancer predisposition syndromes and arise from inherited germline mutations in a cancer susceptibility gene (Garber *et al.,* 2005). Inherited cancer predisposition syndromes are characterized by multiple affected family members usually at an early age of cancer onset, multiple primary cancers and for some rare syndromes, congenital abnormalities (Fearon *et al.,* 1997). To date, over 200 hereditary cancer susceptibility syndromes have been described, the majority of which are inherited in an autosomal dominant manner (Nagy *et al.,* 2004). The lifetime risk of cancer for individuals carrying a mutation in a cancer predisposition gene is high and ranges between 50% and 80% (Ponder et al., 2001). The likelihood of developing cancer depends on the actual gene and the mutant allele as well as on other modifying risk factors, both genetic and non-genetic. Furthermore, it also depends on the complex gene environment interactions which are currently under intense investigation, but at the moment remain poorly understood (Ponder *et al.,* 2001). Recently, a number of genome-wide association studies (*GWAS*) have identified common SNP-based variants conferring low to moderate risk for cancer. These findings have brought us a step closer to a polygenic model for cancer. However, these small effects of multiple genes only explain a small proportion of the observed familial clustering for cancer and an extended analysis with a more complete range of potential susceptibility variants is needed (McCarthy *et al.,* 2008).

1.6. Gastric cancer and XRCC3 repair system

It has been known that genes involved in DNA repair are critical in maintaining the integrity of genetic material as well as protection against mutations that could result in cancer. Exposure to endogenous and exogenous carcinogens and genotoxic compounds can cause damage to DNA. DNA repair process monitors and repairs these DNA alterations using the complex mechanisms. The repair systems include base excision repair (BER), homologous recombination repair (HRR), nucleotide excision repair (NER), mismatch repair, and double strand break repair depending on the type of the damaged DNA (Mohrenweiser *et al.,* 1998). The BER process replaces a single

damaged nucleotide with a normal residue. Maintenance of genomic integrity in mammalian cells depends heavily on the presence of efficient DNA repair systems. Reduction in mammalian DNA repair capacity is associated with increasing birth defects, cancer and reduced lifespan. Although mutations have long been identified as early events in carcinogenesis, defective DNA repair is also a risk factor for many types of cancer. Polymorphisms in several DNA repair genes have been described affecting DNA repair capacity and modulate cancer susceptibility by means of gene-environment interactions (Claire *et al.,* 2002). Genetic polymorphisms of DNA repair genes are thought to result in different phenotypic features compared to the wild type DNA repair gene X-ray repair cross-complementing group-3 (XRCC3) corrects defective DNA strand-break repair and sister chromatid exchange following treatment with ionizing radiation and alkylating agents (Lamerdin *et al.,* 1995). The human gene has 17 exons and spans approximately 17 kb and is assigned to14q32.3 (Robert *et al.,* 1995) Fig. 3.

Homology-directed repair of DNA damage has recently emerged as a major mechanism for the maintenance of genomic integrity in mammalian cells. The highly conserved strand transferase, Rad51, is expected to be critical for this process. XRCC3 possesses a limited sequence similarity to Rad51 and interacts with it. XRCC3 gene participates in HRR of DNA double-strand breaks and cross-links (Tebbs *et al*., 1995, Liu *et al.,* 1998). XRCC3 is required for the assembly and stabilization of Rad51 DNA repair gene family and has a key role in preventing mutations, chromosomal instability and cancer (Khanna *et al.,* 2001). XRCC3-deficient cells do not form Rad51 foci after DNA damage and exhibit genomic in stability and increased sensitivity to DNA-damaging agents (Masson *et al.,*2001*,* Brenemann *et al.,* 2000). Because XRCC3 plays an important role in the DNA repair process (Brenemann *et al.,* 2000*,* Smith *et al.* ,2003). XRCC3 was originally identified as a human gene able to complement the DNA damage sensitivity, chromosomal instability and impaired growth of the mutant hamster cell line irs1SF. More recently, it has been cloned, sequenced and found to bear sequence homology to the highly conserved eukaryotic repair and recombination gene RAD51. The phenotype of irs1SF and the identification of XRCC3 as a member of the RAD51 gene family have suggested a role for XRCC3 in repair of DNA damage by homologous recombination. Homologous recombinational repair (HRR) of a specifically induced chromosomal double-strand break (DSB) was assayed in irs1SF cells with and without transient complementation by human XRCC3. Complementation

Fig. 3: Representative picture showing XRCC3 Gene structure (Source: **NCBI**)

with XRCC3 increased the frequencies of repair by 34- to 260-fold. Studies confirmed XRCC3 in HRR of DNA DSB, and the importance of this repair pathway for the maintenance of chromosomal integrity in mammalian cells (Brenemann *et al.,* 2000, Smith *et al.,* 2003). Growth and development are dependent on the faithful duplication of cells. Duplication requires accurate genome replication, the repair of any DNA damage, and the precise segregation of chromosomes at mitosis; molecular checkpoints ensure the proper progression and fidelity of each stage. Loss of any of these highly conserved functions may result in genetic instability and proneness to cancer (Griffn *et al*., 2000).

The polymorphisms of DNA repair gene XRCC3 have been reported to be associated with the risk of several types of cancer including, colon cancer (Mort *et al.,* 2003, Yeh *et al.,* 2005), squamous cell carcinoma of head and neck (Shen *et al.,* 2002, Rousseau *et al.,* 2011), lung cancer (Butkiewicz *et al.,* 2001, Misra *et al.,* 2003), Skin cancer (Winsey *et al.,* 2000, Duan *et al.,* 2002) and breast cancer (Kuschel *et al.,* 2002, Smith *et al.,* 2003). Few studies have investigated the role of polymorphisms of DNA repair genes in the etiology of gastric cancer, and none has investigated the role of XRCC3 polymorphism in this cancer. No data exists, to our knowledge, on their role in susceptibility to Gastric cancer among Kashmiri population. This study is therefore the first investigative effort in describing the association between XRCC3 exon 7 gene polymorphism and Gastric cancer risk. In this case-control study, the distribution of one genetic variant of XRCC3 gene – codon 241 for any risk of development of gastric cancer in Kashmiri population with gene polymorphism has been looked into.

2.1. DNA damage and its impact on Cancer, aging and longevity

During the lifespanof the cell, DNA is regularly damaged by endogenous and exogenou s carcinogens. In order to protect genome stability, cells have evolved DNA repair mec hanisms. When DNA is exposed to damage, cells activate several pathways suchas cell cycle arrest, transcriptional and post-transcriptional activation of DNA damage respons e genes including DNA repair genes, and in some cases apoptosis.An inability to repair the damage properly due to genetic polymorphisms impairing the DNA repair capacity, can lead to genetic instability and potentially modulate individual's susceptibility to va rious cancers. DNA is the genetic 'instruction manual' found in all our cells. If DNA becomes damaged and is not repaired properly then the cell may get the wrong instruction and start to multiply out of control. This can lead to cancer.

DNA damage, due to environmental factors, and several normal metabolic processes inside the cell, occurs at a rate of 1000 to 1,000,000 molecular lesions per cell per day. While this constitutes only 0.000165% of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as tumor suppressor genes) can impede a cell's ability to carry out its function and appreciably increase the likelihood of tumor formation (Lodish *et al.,* 2004).

The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified. These modifications can in turn disrupt the molecule's regular helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix (Wilson *et al*., 1997). Unlike proteins and RNA, DNA usually lacks tertiary structure and therefore damage or disturbance does not occur at that level. DNA is however, supercoiled and wound around packaging proteins called histones (in eukaryotes) and both super structures are vulnerable to the effects of DNA damage (Watson *et al.,* 2004).

DNA plays a major role in mutagenesis, carcinogenesis and ageing. There are a number of chemical events that lead to DNA damage including hydrolysis, exposure to reactive oxygen species (ROS) and other reactive metabolites (Fig. 4). These events result from metabolic, endogenous processes or are triggered by exposure to exogenous chemicals. It is now becoming apparent that mutations due to DNA damage are caused by endogenous factors that are modulated by exogenous factors (UV, IR radiations etc) and it is probably this combination that plays a major role in many cases of cancer.

Oxidatively modified DNA is present in many tissues including tumor tissue (De Bont *et al,* 2004). Oxidative stress occurs when the production of ROS exceeds the body's natural defense mechanisms, causing damage to macromolecules such as DNA. Any change in the efficacy of DNA repair will alter the steady-state levels of oxidative DNA modifications, which in turn affects the mutation rate and ultimately the cancer incidence. Oxidative base damage and single-strand breaks (SSB) are the most frequent types of DNA damage caused by ROS (Thompson *et al.,* 2000) and if not repaired, can lead to much more serious double-strand breaks that directly contribute to the development of cancer.

The replication of damaged DNA before cell division can lead to the incorporation of wrong bases opposite damaged ones. Daughter cells that inherit these wrong bases carry mutations from which the original DNA sequences is unrecoverable (except in the rare case of a back mutation, for example through gene conversion). Extensive epidemiological and experimental data suggests that ultraviolet radiation is an important environmental carcinogen involved in the initiation and progression of skin cancer (Halpern *et al.,* 1999). UV radiation at short wavelengths induces damage in the form of cyclobutane pyrimidine dimers and pyrimidine (Mohrenweiser *et al.,* 1998*)* photoproducts. At longer wavelengths it causes single-stranded breaks, DNA – protein cross linking and generates free-radicals which cause oxidative damage (Elment *et al.,* 1996).

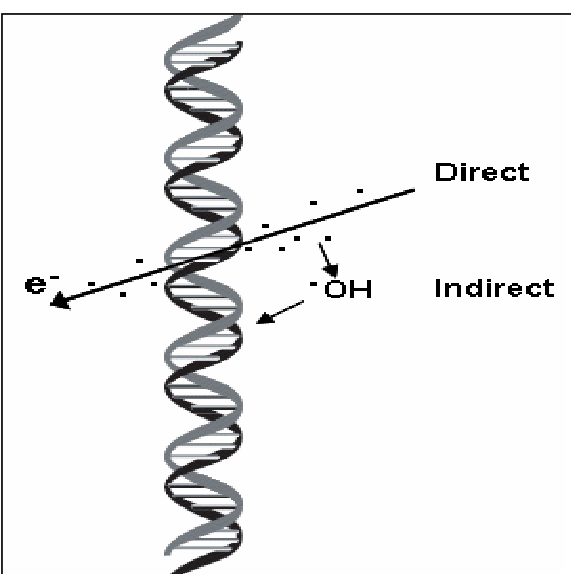


Fig. 4: Mechanisms of direct and indirect actions of ionizing radiations inducing DNA damage (source: Pubmed)

2.2. DNA repair system

DNA repair systems maintain genomic integrity, which is of vital importance for cellular functions. Any kind of failure of these systems has been associated with cancer, birth defects and an accelerated rate of ageing (Ronen *et al.,* 2001). Polymorphisms in DNA repair genes may result in deficient DNA repair, which in turn leads to cumulative genotoxic damage and increased cancer susceptibility (Krajinovic *et al*., 2002*)*.

DNA repair refers to a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome. In human cells, both normal metabolic activities and environmental factors such as UV light and radiation can cause DNA damage, resulting in as many as I million individual molecular lesions per cell per day (Kreiger *et al*., 2004). Many of these lesions cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the effected DNA encodes. Other lesions induce potentially harmful mutations in the cell's genome, which affect the survival of its daughter cells after it undergoes mitosis. As a result, the DNA repair process is constantly active as it responds to damage in the DNA structure. When normal repair processes fail and when cellular apoptosis does not occur, irreparable DNA damage may occur, including double-strand breaks and DNA cross linkages (Bjorksten *et al*., 1971).

The rate of DNA repair is dependent on many factors, including the cell type, the age of the cell and the extra cellular environment. A cell that has accumulated a large amount of DNA damage, or one that no longer effectively repairs damage incurred to its DNA, can enter one of three possible states of either senescence (Leeuwen *et al*., 2002*),* apoptosis or unregulated cell division, which can lead to the formation of a tumor that is cancerous (Braig *et al*., 2006*).* Many genes that were initially shown to influence life span have turned out to be involved in DNA damage repair and protection (Browner *et al.,* 2004*)*. Failure to correct molecular lesions in cells that form gametes can introduce mutations into the genomes of the offspring and thus influence the rate of evolution. Mutations in genes involved in DNA repair are also necessary. About 150 human DNA repair genes have been identified to date (Wood *et al*., 2005*)*, but the real number is probably higher, since less than 50% of known and putative genes have an identified function.

DNA repair systems are responsible for maintaining the integrity of the genome in both somatic and germinal cells by minimizing replication errors, deleterious rearrangements arising via aberrant recombination and by removing DNA damage which might otherwise result in carcinogenesis (Lindahl *et al*., 1997). Thus, DNA repair has a critical role in protecting against mutations that can lead to cancer. Absence or incorrect repair can lead to the development of cancer through activation of oncogenes, inactivation of tumour-suppressor genes or loss of heterozygosity (Bohr *et al*., 1995). Repair of damaged DNA involves many proteins performing functions directly on the damaged DNA, as well as the interaction and interplay with proteins involved in regulation of DNA replication and progression through the cell cycle (Fig. 5) (Lehmann *et al*., 1998). Numerous studies have shown that genes directly involved in DNA repair and maintenance of genome integrity, or genes indirectly involved in the repair of DNA damage through regulation of the cell cycle are critical for protecting against mutations which lead to cancer.

At least four pathways of DNA repair operate on specific types of damaged DNA. Base excision repair (BER) operates on small lesions, while the nucleotide excision repair (NER) pathway repairs bulk lesions. Mismatch repair corrects replication errors. Double-strand DNA break repair (DSBR) actually consists of two pathways, homologous recombination (HR) and nonhomologous end-joining (NHEJ). The NHEJ repair pathway involves direct ligation of the two double strand break ends, while HR is a process by which double-strand DNA breaks are repaired through the alignment of homologous sequences of DNA. It is believed that the predominant pathway used for removal of oxidized and many of the alkylated bases is BER.

2.3. DNA repair capacity and inter-individual differences

Epidemiological studies using functional measurements of DNA repair suggests that DNA repair capability is variable within human population (Setlow *et al*., 1983). As inactivating mutations in DNA repair genes are rare (Yu *et al*., 1999), it has been hypothesized that variation in DNA repair capability in the general population is a product of combinations of multiple alleles that show subtle variations in biological functions (Mohrenweiser *et al*., 1998). The presence of polymorphic alleles in DNA repair genes may alter the repair capacity modifying the biological responses to exogenous and endogenous DNA insults, both at cellular and tissue level and the

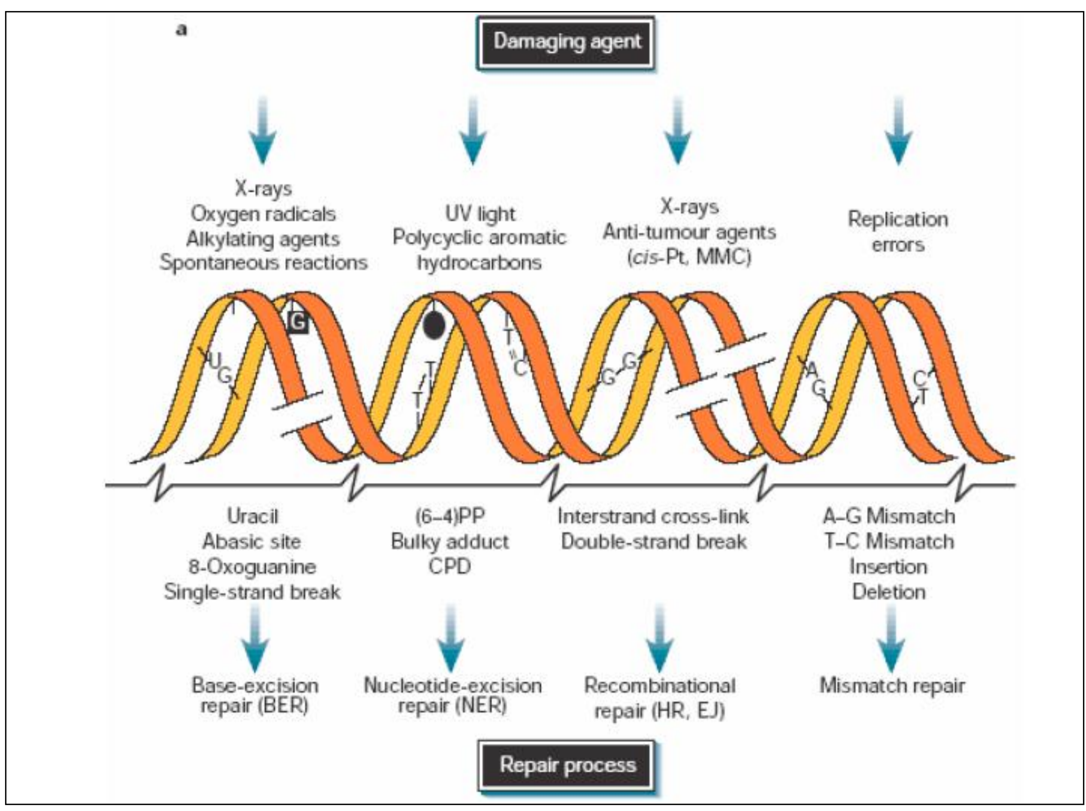


Fig. 5: Four pathways of DNA repair process that operate on specific types of damaged DNA (Source: Science direct).

individual susceptibility in developing different kinds of disease. Inter-individual variation in DNA repair capacity has been shown through the use of lymphocyte assays. Although findings have been difficult to reproduce, individuals with repair capacity of 65-80% of the population mean are more often in the cancer cohorts (Scott *et al*., 1994). The phenotype of reduced repair capacity for one pathway is independent of the phenotype for another pathway; this is consistent with DNA repair being genetically regulated. Measurement of repair capacity in twins (Pero *et al*., 1983) and the elevated frequency of individuals with reduced repair capacity among relatives of cancer patients is further evidence that repair capacity is a genetic trait (Helzouer *et al*., 1995).

A number of polymorphisms in genes that encode DNA repair proteins have been described (Shen *et al*., 1998). These genes namely XRCC1, ERCC1, XPD, XPF and XRCC3, encode enzymes involved in three DNA repair pathways, known to be involved in the correction of UV-induced DNA damage. Many of the variants result in amino acid substitutions and exist at polymorphic allele frequencies (i.e. allele frequencies >0.05). Given the known relationship of DNA repair to cancer, polymorphic, variants in the DNA repair enzymes have the potential to be population risk factors for cancer because of the large number of individuals affected. Polymorphisms in several DNA repair genes have been described affecting DNA repair capacity and modulate cancer susceptibility by means of gene-environment interactions (Claire *et al*., 2002).

2.4 Genetic Polymorphism

The coexistence of multiple alleles at a locus is called genetic polymorphism (Lewin *et al*., 2004). An allele is usually defined as polymorphic if it is present at a frequency of more than 1% in the population. Polymorphism caused by the presence of more than one allele for the same gene which result in more than one phenotype in the organisms. The differences between mutations and polymorp hisms are that polymorphisms generally do not cause any sickness or other problems affecting the reproductive efficiency so that their frequencies in a population are relatively high compared to mutations (more than 1%), whereas a mutation causing a serious disease would affect the reproductive efficiency (Gonzalez *et al.*, 1999). That is the reason why mutations are found at extremely

low frequency (less than 1%) in populations. The other difference between mutation an d polymorphism is that, mutations occur at random sites withinthe DNA, whereas poly morphims are found at specific alleles.

2.4.1 Mechanism of Genetic Polymorphism

Genetic polymorphisms can be categorized into functional and nonfunctional polymorphism depending on their effect on protein profile that the gene is translated to a functional polymorphism is a change in the DNA sequence of a gene that results in different levels of expression of protein or enzyme, or in alteration of the activity, while a nonfunctional polymorphism results in neither of them (Gonzalez *et al*., 1999). The functional polymorphisms can be either in the coding or in the noncoding regions of the gene. Variations in the coding region of gene have the potential to alter enzyme activity or protein function by changing the primary sequence of the protein. The non-coding regions of a gene are comprised of introns –which are spliced of post transcriptionally and regulatory regions, which are not transcribed but regulate the level of expression of the protein. However, if the polymorphism occurs at the splice junction, it can affect the structure of the protein or enzyme. Genetic variability in these noncoding regions is associated with altered levels of protein rather than changes in the protein itself (McKinnon *et al*., 2000).

2.4.2 Types of Genetic Polymorphism

Structurally, polymorphisms could occur as deletions/insertions (InDel), varying number of tandem repeats (VNTR), and single nucleotide substitutions (SNP). Insertion and deletion polymorphism is the insertion or deletion of DNA sequences that are 1 to 1000 nucleotides long. The name microsatellite (VNTR) is usually used when the length of the repeating unit is shorter than 10 bp, and the name minisatellites (VNTR) is used when the length of the repeating unit is between 10 to 100 bp. The high variability of minisatellites makes them useful for genomic mapping. The most widely observed polymorphism in human genome is single nucleotide substitutions (SNP).

2.4.3 Single Nucleotide Polymorphism (SNP)

SNP is the substitution of a single nucleotide with a frequency of more than 1% in at least one population (Evans *et al*., 2001). SNPs are distributed throughout the human

genome at an estimated over all frequency of one in every 1330 bases in the human genome (Lewin *et al*., 2004). In human, more than 130 genes have been identified which codes for proteins of the various DNA repair pathways, and within 80 genes over 400 SNPS characterized (Mohrenweiser *et al*., 2003). Because of the importance of maintaining genomic integrity in order to prevent development of carcinogenesis, DNA repair genes have strong relevance in determining susceptibility to cancer (Cairns *et al*., 1982, Knudson *et al*., 1989, and Shields *et al*., 1981). For that reason, SNPs in DNA repair genes are an important area of investigation for epidemiology of carcinogenesis.

2.5 DNA repair gene XRCC3

XRCC genes, abbreviation of X-ray cross complementing, are components of several different damage recovery pathways and XRCC proteins do not show similarity in biochemical functions.

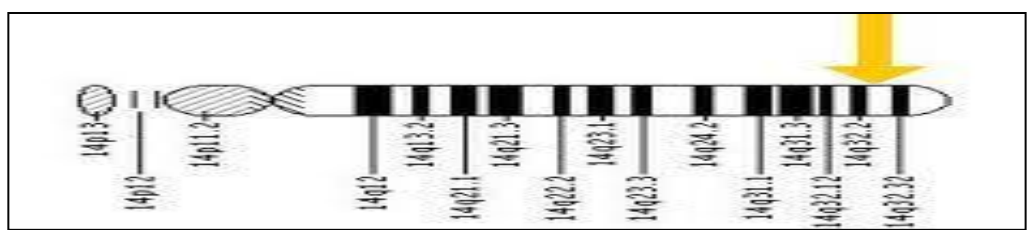


Fig 6: Location of XRCC3 gene on the long arm(q) of chromosome 14.

X-ray cross-complementing group 3 (XRCC3) is a member of homologous DNA repair mechanisms and play a key role in that repair system. XRCC3 contains ATPase motifs composed of the Walker A, and B boxes, and the integrity of the Walker A motif of XRCC3 is required for biological activity and governs Rad51C-XRCC3 complex formation (Yamada *et al*., 2004). Moreover XRCC3 protein interacts with Rad51 protein, enabling Rad51 protein multimers to assemble at the site of damage (Bishop *et al*., 1998) In Homo sapiens, this protein coding gene maps on chromosome 14, at 14q32.3, (Fig. 6) (Robert *et al*., 1995). It covers 17870 bases (NCBI., 2011) on minus strand. The homodimeric protein has a size of 37850 Da with 346 amino acids. Its sub cellular location is the nucleus as it accumulates at sites of DNA damage. This gene is expressed at high level, 3.8 times the average gene. The gene is composed of 17 exons and contains 26 different introns (24 gt-ag, 2 gc-ag)

(Lamerdin *et al*., 1995*)*. Transcription produces 25 different mRNAs, 23 alternatively spliced variants and 2 unspliced form. There are 6 probable alternative promotors (Zhou *et al*., 1998*)*, 6 non overlapping alternative last exons and 9 validated alternative polyadenylation sites. The mRNAs appear to differ by truncation of the 5' end, truncation of the 3' end, presence or absence of 18 cassette exons, overlapping exons with different boundaries, alternative splicing or retention of 2 introns. 3125 bp of this gene are antisense to spliced gene C14orf153 and KLC1, raising the possibility of regulated alternate expression. Four variants were isolated *in vivo*, although it is a predicted target of nonsense mediated mRNA decay (NMD). Twenty-one spliced and 2 unspliced mRNAs putatively encode good proteins, altogether 15 different isoforms (9 complete, 2 COOH complete, 4 partial), some containing domains RAD51 domain (Bork *et al*., 1997*)*, DNA-repair protein XRCC3; N-terminal [Pfam]. The remaining mRNA variant (unspliced) appears not to encode a good protein isoforms are annotated using as Met a Kozak-compatible non-AUG(CTG) start, thereby gaining a minimum of 72 amino acids N-terminal to the first AUG.

Additionally, XRCC3 was originally identified as a human gene able to complement the DNA damage sensitivity, chromosomal instability and impaired growth of the mutant hamster cell line irs1SF. More recently, it has been cloned, sequenced and found to bear sequence homology to the highly conserved eukaryotic repair and recombination gene RAD51. The phenotype of irs1SF and the identification of XRCC3 as a member of the RAD51 gene family have suggested a role for XRCC3 in repair of DNA damage by homologous recombination (Mark *et al*., 2000). Homologous recombinational repair (HRR) of a specifically induced chromosomal double-strand break (DSB) was assayed in irs1SF cells with and without transient complementation by human XRCC3.Complementation with XRCC3 increased the frequencies of repair by 34- to 260-fold. In NHEJ, there is no need to undamaged partner, two DNA ends are degraded limitedly at the termini and the DNA ends are ligated together (Khanna *et al*., 2001). Since there is no template for the repair of damaged DNA, it is often prone to error, and small sequence deletions are usually introduced to the DNA. Schematic representation of double strand break repair is given in (Fig. 7).

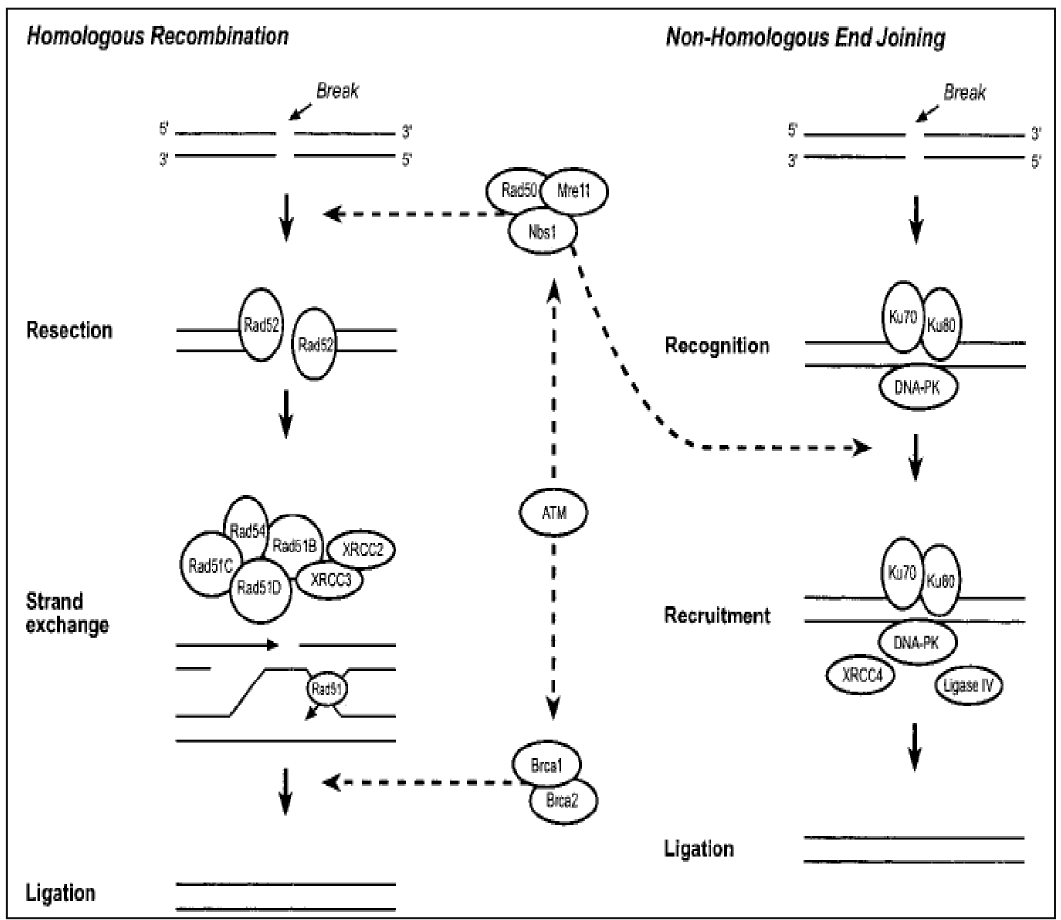


Fig. 7: Schematic representation of Homologous Recombination end joining and Non-homologous recombination end joining (Source: Ellen *et al*., 2002).

A lot of information about XRCC3 function has been derived from mutant mammalian cell lines; to confirm the role of XRCC3 protein on replication similar experiments were conducted using permeable XRCC3 gene-knockout DT40 cells (Takata *et al.,* 2001). As observed in wild-type hamster cells, cisplatin treatment of wild-type DT40 cells resulted in a dose-dependent reduction in replication, whereas cisplatin treatment of XRCC3 DT40 cells did not. Taken together, these data demonstrate that cisplatin slows the progression of replication forks in vitro by an active mechanism that requires XRCC3. Studies with homologous proteins in yeast have suggested that XRCC3 functions by stimulating the DNA strand exchange activity of Rad51 (Sung *et al*., 1997, Tsutsui *et al*., 2000, Liu *et al*., 1998). In support of this, XRCC3 is required for the formation of normal levels of RAD51 nuclear foci following ionizing radiation or cisplatin (Bishop et al., 1998; Takata et al., 2001); such foci are believed to reflect the formation of Rad51 nucleoprotein filaments that initiate DNA strand transfer during recombination. Moreover, overexpression of Rad51 partially corrects the sensitivity of $XRCC3^{-1-}$ DT40 cells to ionizing radiation and cisplatin (Takata *et al*., 2001), consisting with XRCC3 promoting Rad51, or a Rad51-like, activity. XRCC3 was inactivated in human cells by gene targeting. Consistent with its role in homologous recombination, $XRCC3^{-1-}$ cells showed a two-fold sensitivity to DNA cross-linking agents, a mild reduction in sister chromatid exchange, impaired Rad51 focus formation and elevated chromosome aberrations. Furthermore, endoreduplication was increased five- seven-fold in the mutants. The T241M variant of XRCC3 has been associated with an increased cancer risk. Expression of the wild-type cDNA restored this phenotype, while expression of the variant restored the defective recombinational repair, but not the increased endoreduplication. RPA, a protein essential for homologous recombination and DNA replication, is associated with XRCC3 and Rad52. Over expression of RPA promoted endoreduplication, which was partially complemented by overexpression of the wild-type XRCC3 protein, but not by over expression of the variant protein. Over expression of Rad52 prevented endoreduplication in RPA-over expressing cells, in $XRCC3^{-1-}$ cells and in the variant-expressing cells, suggesting that deregulated RPA was responsible for the increased endoreduplication. These observations offer the first genetic evidence for the association between homologous recombination and replication initiation having a role in cancer susceptibility (Takashai *et al.,* 2003).

XRCC3 defective cells have an increased number of centrosomes, resulting in mis-segregation of chromosomes (Griffin *et al*., 2000), which is an important driving force in the etiology of cancer (langeaur *et al*., 1998). Although the XRCC3 T241M protein is functional for homologous recombination, it may still be defective in maintaining the correct number of centrosomes. Researchers compared centrosome number in a series of cell lines derived by transfection from the XRCC3-deficient irs1SF cell line (Tebbs *et al*., 1995), which itself originated from the wild-type AA8 CHO cell line (Fuller *et al*., 1988). The cell lines comprised irs1SF complemented with the human XRCC3 (WT), XRCC3 T241M or XRCC3 D213N cDNAs, expressed from the pcDNA3.1 vector stably integrated into the genome of irs1SF cells (Rafii *et al*., 2003). At least two independent transfected clones were examined in each case in order to avoid clonogenic variability, to identify the expressed XRCC3 protein, by western blotting. The irs1SF cell line shows no detectable XRCC3 protein, whereas all irs1SF clones expressing hXRCC3 variants produce an XRCC3 protein, showing that the human XRCC3 is expressed in every clone (Anna *et al., 2006*). Several studies have examined the association between the XRCC3 Thr241Met polymorphism and risk of cancer (Matullo *et al* ., 2001,Duan *et al*., 2002 , Smith *et al*., 2003, Misra *et al*., 2003,Winsey *et al*., 2003).

2.5.1 Functions of XRCC3 gene

The XRCC3 gene is mapped to 14q32.2 (Tebbs *et al*., 1995, Zou *et al*., 2009). The gene XRCC3 (X-ray cross complementing group 3) has the task of repairing damage that occurs when there is recombination between homologous chromosomes. Repair of recombination between homologous chromosomes plays an important role in maintaining genome integrity, although it is known that double-strand breaks are the main inducers of chromosomal aberrations. Changes in the XRCC3 protein lead to an increase in errors in chromosome segregation due to defects in centrosomes, resulting in aneuploidy and other chromosomal aberrations, such as small increases in telomeres. The XRCC3 (X-ray cross complementing group 3) gene is needed to repair damage caused when recombination occurs between homologous chromosomes (Loizidou *et al*., 2008, Andreassi *et al*., 2009). The repair of recombination between homologous chromosomes is a mechanism that fixes various types of DNA damage. The large number of repeated sequences can potentially lead

to a large number of undesirable interactions between chromosomes. The repair of recombination between homologues plays an important role in maintaining genome integrity. Although it is known that double-strand breaks are the main inducers of chromosomal aberrations, the mechanisms by which these are formed are still unresolved (Griffin *et al*., 2004). Deficiencies in the XRCC3 protein lead to an increase in errors in chromosome segregation due to defects in centrosomes, resulting in aneuploidy and other chromosomal aberrations such as small increases in telomeres (Thacher *et al*., 2005). XRCC3 is an important component in the repair machinery via homologous recombination. Changes in this machine due to the presence of polymorphisms lead to damage that results in tumor development (Jiao *et al.,* 2008). The XRCC3 gene has a sequence variation in exon 7 (C18067T), which results in an amino acid substitution at codon 241 (Thr241Met) that may affect the enzyme's function and/or its interaction with other proteins involved in DNA damage and repair (Matullo *et al*., 2001). The XRCC3 Thr241Met polymorphism (rs861539) located in exon 7 is characterized by the substitution of thymine (T) to cytosine (C) at codon 241, leading to a change of threonine (Thr) to methionine (Met), and could affect enzyme function and/or its interaction with other proteins involved in repair of DNA damage (Matullo *et al*., 2001, Jiao *et al*., 2008, Andreassi *et al.,* 2009). Pierce *et al*. (1999) demonstrated that cell lines defective in XRCC3 had a 25-fold decrease in homology-directed repair of DNA double-strand breaks. Mutant cell lines deficient in XRCC3 experienced high frequencies of spontaneous chromosomal aberrations (Tebbs *et al*., 1995, Liu *et al*., 1998, Fuller *et al*., 1998). In addition, these cell lines have shown an array of sensitivities to DNA damaging agents ranging from modest sensitivity, caused by alkylating agents and ionizing and ultraviolet radiation, to extreme sensitivity due to DNA crosslinking agent (Fuller *et al*., 1998, Caldecott *et al*., 1991). Shen *et al*. (2003) recently identified a C to-T substitution in exon 7 at position 18067 of XRCC3, a polymorphism that results in a threonine-to-methionine amino acid substitution at codon 241. The XRCC3 gene product is a member of the RAD51- related gene family, and directly interacts with Rad51 (Liu *et al*., 1998, Brenneman *et al*., 2000 and Schild *et al*., 2000). XRCC3 protein associates directly with DSBs and recruit Rad51 (Forget *et al.,* 2004) and XRCC3-Rad51 complex cooperatively modulate the progression of the replication forks on damaged chromos

omes (Henry *et al*., 2003). Deficiency of XRCC3 gene resulted in increased hypersensitivity to ionizing and UV radiations as well as mono and bifunctional alkylating agents (Xu *et al*., 2005, Alsbeih *et al*., 2007)

2.5.2 Role of XRCC3 gene in Homologous Recombination pathway.

Homologous recombination (HR) is essential for the accurate repair of DNA double-strand breaks (DSBs), potentially lethal lesions. HR takes place in the late S-G2 phase of the cell cycle and involves the generation of a single-stranded region of DNA, the intact strand as a template, branch migration and resolution. It is investigated that RecA/Rad51 family proteins play a central role (Fig.8). The product of the XRCC3 gene, paralogue of Rad 51 family, functions in the homologous recombination repair (HRR) for double-strand breaks (DSBs) and cross-link repair in mammalian cells (Dianov *et al*., 2003). During HRR the XRCC3 protein interacts with the Rad51C protein and possibly with theRad51 protein itself, enabling Rad51 protein multimers to assemble at the site of damage (Brenneman *et al*., 2002). Since XRCC3 protein performs a very important function in the HR DNA DSB repair pathway. In the HR pathway, XRCC3 is earlier than RAD51 to localize at sites of DSB damage, and subsequently functions to promote the assembly of active RAD51 repair complexes, namely RAD51 foci (Fig. 9) (Forget *et al*., 2004). Once RAD51 foci are formed, mammalian cells use the sister chromatid as the template of preference over a homologous or heterologous chromosome. Lack of the XRCC3 gene will result in decreased function of HR (Pierce *et al.,* 1999) and defects in chromosome segregation (*Griffin et al., 2000*). Hence, XRCC3 mediates RAD51-dependent HR necessary to repair DNA damage that may result in carcinogenesis if left unrepaired or misrepaired.

Homologous recombination repair (HR) plays an important role in the repair of DNA double strand breaks (DSBs) caused by ionizing radiations or from break down of stalled replication forks. Accurate DSB repair, using sister chromatid as template, is necessary for the maintenance of genomic stability, and defects in this process can lead to the introduction of mutations, chromosomal translocations, apoptosis, and cancer.

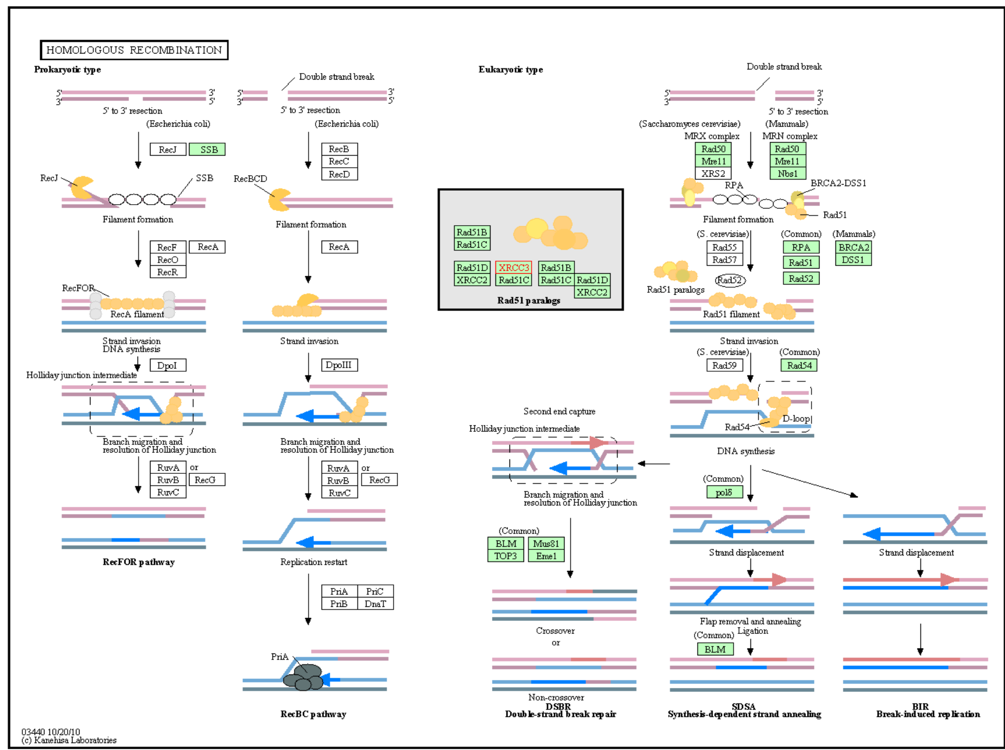


Fig. 8: Role of XRCC3 gene and Homologous Recombination repair, schematic illustration (source: Wikipedia)

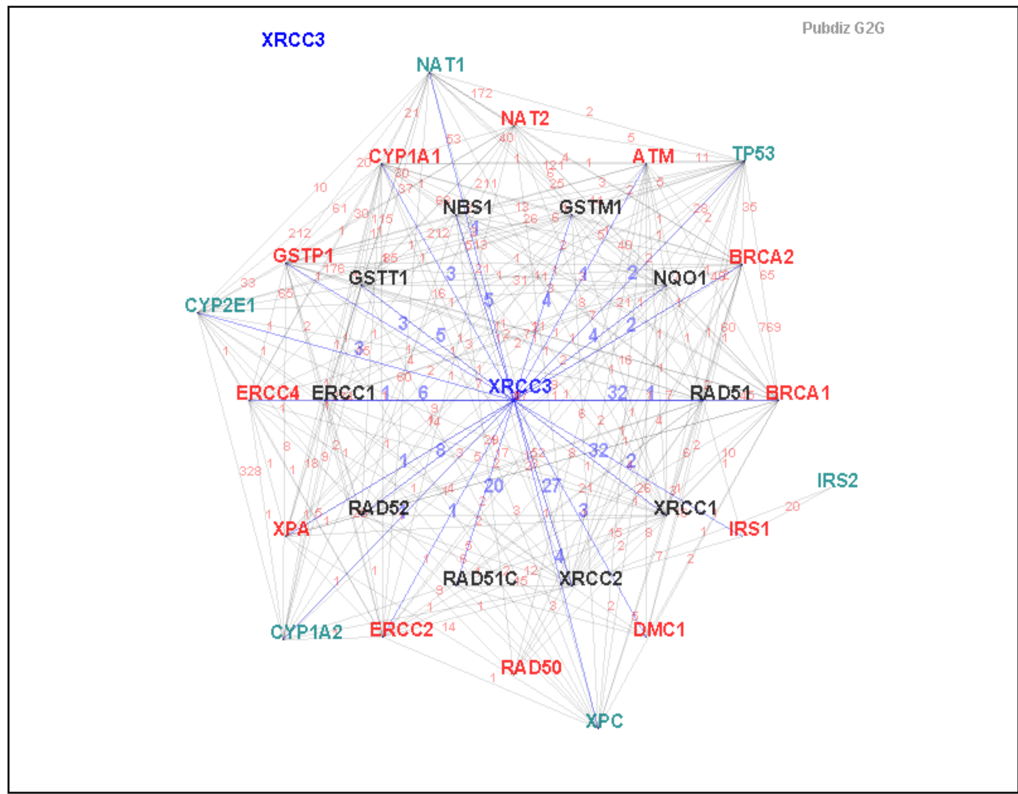


Fig. 9: Schematic description of repair pathways genes and interactions between XRCC3 gene and Rad51 family (source: Wikipedia)

2.6 Incidence and Mortality

Gastric cancer is the second-most common cancer among men and third-most among females in Asia and worldwide (Table. 1) (Ferlay *et al.,* 2008). Gastric cancer is one of the most common cancers worldwide with approximately 989,600 new cases and 738,000 deaths per year, accounting for about 8 percent of new cancers (Jemal *et al* .,2011). Approximately 21,320 patients are diagnosed annually in the United States, of whom 10,540 are expected to die (Table. 2) (Siegal *et al.,* 2011). Gastric cancer is less common in the United States and other Western countries than in countries in Asia and South America. Overall, gastric cancer incidence rates are decreasing. (Fig. 10). The worldwide incidence of gastric cancer has declined rapidly over the recent few decades (Haenszaz *et al.,* 1958*,* Munoz *et al.,* 1971*,* Hirayama *et al .,* 1975*,* Waterhouse *et al.,* 1976). Part of the decline may be due to the recognition of certain risk factors such as H. pylori and other dietary and environmental risks. However, the decline clearly began before the discovery of *H. pylori*. The decline first took place in countries with low gastric cancer incidence such as the United States (beginning in the 1930s), while the decline in countries with high incidence like Japan was slower. In the United Kingdom, there was a consistent decline in incidence of gastric cancer, with a reduction from 1.14 in 1971 to 1975 to 0.84 in 1996 to 2000 in men, and 1.18 in 1971 to 1975 to 0.81 in 1996 to 2000 in women (Fitzsimmons *et al.,* 2007). In China, the decline was less dramatic than other countries; despite an overall decrease in gastric cancer incidence, an increase has been observed in the oldest and the youngest group, and a less remarkable decline has been observed among women than in men (Jemal *et al.,* 2006). Of note is that the age of onset of developing gastric cancer in Chinese population is younger than that in the West. In the United States, risk factors for noncardia gastric cancer include male gender, non-white race, and older age (Schlansky *et al.,*2011). Between 1977 and 2006, the incidence rate for noncardia gastric cancer in the United States declined among all race and age groups except for whites aged 29 to 39 years for whom it increased (Anderson *et al.,* 2010). The rise in incidence of noncardia gastric cancer among those at 25 to 39 years is noteworthy since this may signal the introduction of new environmental factors. Gastric cardia cancer, which was once very uncommon, now constitutes nearly half of all stomach cancers among white males in the United States. Infection with *H. pylori* is the primary identified cause of gastric cancer. Other factors that increase the risk for gastric cancer include chronic gastritis,

older age, male sex, a diet high in salted, smoked, or poorly preserved foods and low in fruits and vegetables, tobacco, smoking, anemia, a history of stomach surgery for benign conditions, and a family history of stomach cancer (Forman *et al .,* 2006, Brenner *et al.,* 2009). It is estimated that 21,520 men and women (13,120 men and 8,400 women) will be diagnosed with and 10,340 men and women will die of cancer of the stomach in 2011 (Howalder *et al.,* 2011, Malik *et al*., 2011).

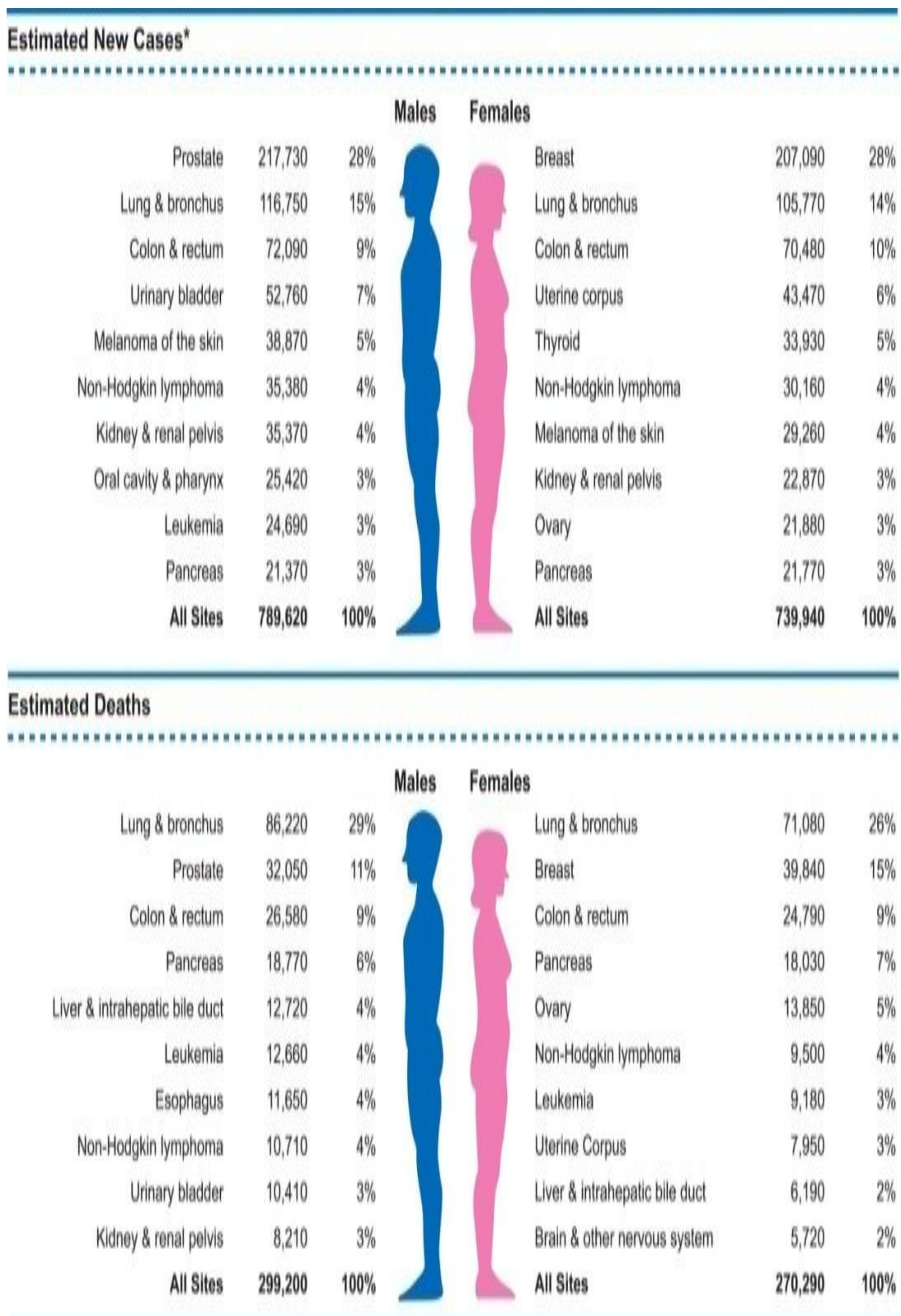
Gastric cancer is the most aggressive malignant tumors of gastrointestinal tract with diverse risk factors and incidence patterns. Within the Indian subcontinent, the valley of Kashmir presents a strikingly different picture, where incidence of gastric cancer has been reported to exceed 40% of all cancers and incidence is 3-6 times higher than various metropolis cancer registries in India (Khuroo *et al*., 1992). The people of the Kashmir valley have many unique dietary features which are different from rest of world. Salted tea used by people is prepared by using baking soda (sodium bicarbonate) along with common salt (sodium chloride) and boiled for few hours before consuming. Some of the genetic and environment factors have been reported to be associated with an increased risk of gastric cancer in Kashmir valley (Siddiqui *et al*., 1992, Malik *et al.,* 2011).

Table. 1: Stomach Cancer Incidence and Mortality Worldwide in 2008*.

Estimated numbers (thousands)	MALES		FEMALES	
	CASES	DEATH	CASES	DEATH
World	640	463	348	73
More developed regions	173	110	101	70
Less developed regions	467	353	246	202
WHO Africa region (AFRO)	10	9	8	8
WHO Americas region (PAHO)	54	40	35	27
WHO East Mediterranean region (EMRO)	15	14	8	7
WHO Europe region (EURO)	99	79	66	54
WHO South-East Asia region (SEARO)	39	37	28	25
WHO Western Pacific region (WPRO)	420	282	201	150
IARC membership (22 countries)	190	119	110	75
United States of America	13	6	8	4
China	315	231	148	121
India	**21**	**20**	**13**	**12**
European Union (EU-27)	50	37	32	24

*Source: (Globoscan., 2008)

Table 2: Incidence and mortality **2010** *(American Cancer Society)*

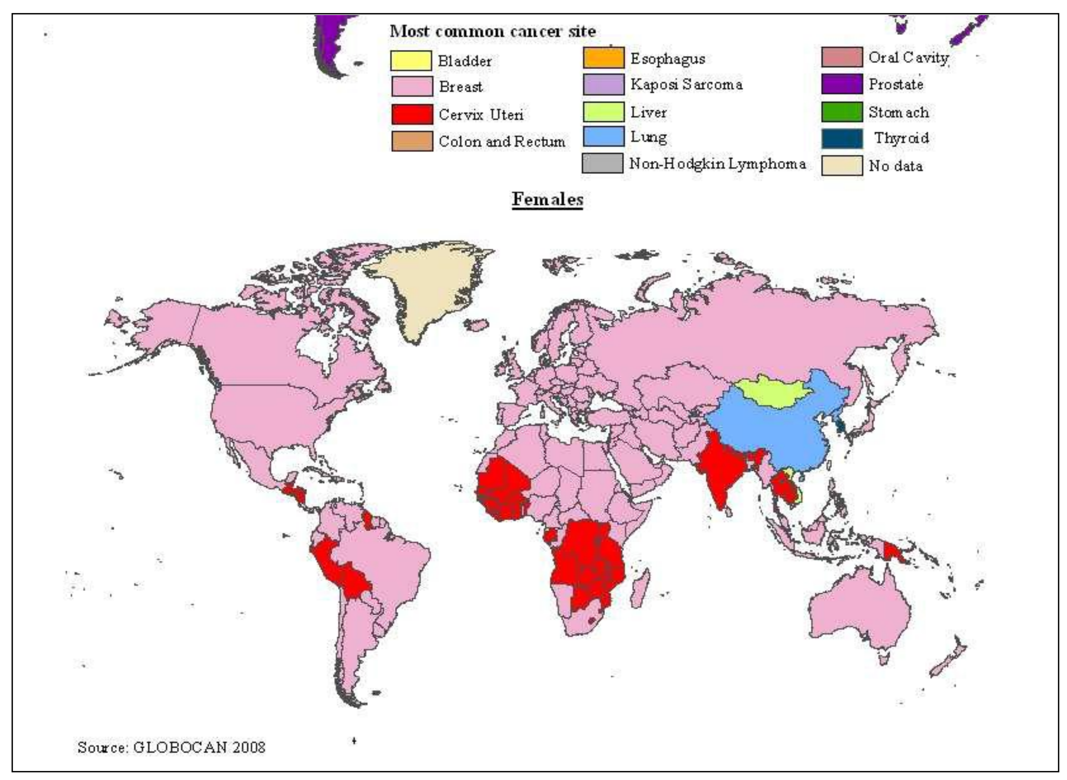


Estimated New Cases*

Males			Females		
Prostate	217,730	28%	Breast	207,090	28%
Lung & bronchus	116,750	15%	Lung & bronchus	105,770	14%
Colon & rectum	72,090	9%	Colon & rectum	70,480	10%
Urinary bladder	52,760	7%	Uterine corpus	43,470	6%
Melanoma of the skin	38,870	5%	Thyroid	33,930	5%
Non-Hodgkin lymphoma	35,380	4%	Non-Hodgkin lymphoma	30,160	4%
Kidney & renal pelvis	35,370	4%	Melanoma of the skin	29,260	4%
Oral cavity & pharynx	25,420	3%	Kidney & renal pelvis	22,870	3%
Leukemia	24,690	3%	Ovary	21,880	3%
Pancreas	21,370	3%	Pancreas	21,770	3%
All Sites	**789,620**	**100%**	**All Sites**	**739,940**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	86,220	29%	Lung & bronchus	71,080	26%
Prostate	32,050	11%	Breast	39,840	15%
Colon & rectum	26,580	9%	Colon & rectum	24,790	9%
Pancreas	18,770	6%	Pancreas	18,030	7%
Liver & intrahepatic bile duct	12,720	4%	Ovary	13,850	5%
Leukemia	12,660	4%	Non-Hodgkin lymphoma	9,500	4%
Esophagus	11,650	4%	Leukemia	9,180	3%
Non-Hodgkin lymphoma	10,710	4%	Uterine Corpus	7,950	3%
Urinary bladder	10,410	3%	Liver & intrahepatic bile duct	6,190	2%
Kidney & renal pelvis	8,210	3%	Brain & other nervous system	5,720	2%
All Sites	**299,200**	**100%**	**All Sites**	**270,290**	**100%**



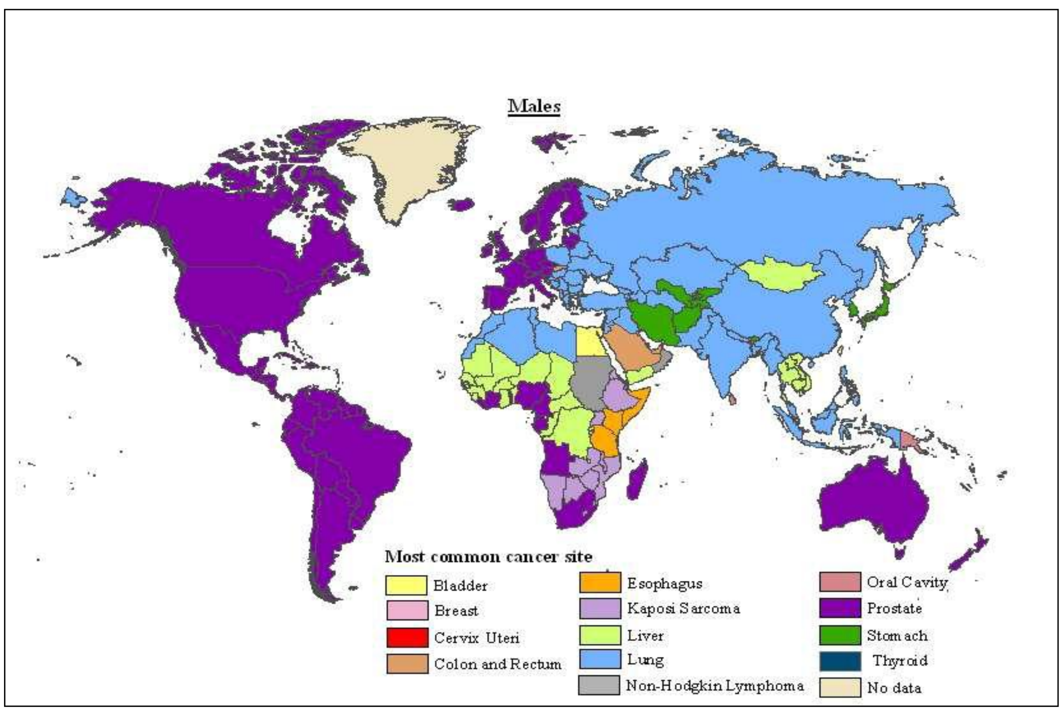


Fig. 10: Cancer Incidence and mortality (females and males) worldwide 2010 as given by Globocan.

2.7 XRCC3 polymorphism and risk of possible cancer

A common polymorphism in exon 7 of the XRCC3 gene results in an amino acid substitution at codon 241(Thr241Met) that may affect the enzyme's function. The XRCC3 variant allele has been identified in healthy individuals at a frequency ranging from 0.23 to 0.38 (Shen *et al.,* 1998, David-Beabs *et al.,* 2001), and has been associated with increased risk of melanoma (Winsey *et al.,* 2000), bladder cancer (Matullo *et al.,* 2001), breast cancer (Smith *et al.,* 2003) and lung cancer (Jacobsen *et al.,* 2004, Lee *et al.,* 2007) in a meta-analysis on Thr241Met reported that Met241 is associated with risk of developing breast cancer in Asian and Caucasian populations. The Thr241Met substitution is the most thoroughly investigated polymorphism in XRCC3 due to the C>T transition at exon 7. Functional data also suggest that the polymorphism may be associated with slightly decreased DNA repair capacity (Jara *et al.,* 2010). Kiuru *et al.* (2008) studied the XRCC3 Thr241Met polymorphism in samples of nervous system tumors and concluded that the genotype Thr/Met and homozygous Met/Met are associated with increased risk for developing this kind of tumors. Zhou *et al.* (2009) conducted a case-control study in a Chinese population on the XRCC3 Thr241Met polymorphism in 771 samples of gliomas and 752 control samples, concluding that the presence of genotype Met/Met may contribute to the development of gliomas. The XRCC3 Thr241Met polymorphism was analyzed in samples from breast cancer. The result demonstrated that the presence of the polymorphism does not interfere with disease development (Loizidou *et al.,* 2008).

Several studies have examined the relationship between XRCC3Thr241Met polymorphism and risk of certain cancers. Moreover there are numerous studies in some of which XRCC3 Thr241Met polymorphism have been a risk factor, in some of which it has been a protective factor and in some it does not have any association with the disease development. Studies about XRCC3 polymorphisms and their interaction with the related diseases were listed in (Table. 3), below. However the results from these previous studies are conflicting. For this reason, additional studies to address the role of XRCC3 Thr241Met polymorphism in human carcinogenesis are needed.

Table. 3. Genetic polymorphism of *XRCC3* Thr241Met gene and its interaction with various types of cancer**s.***

S.No	Cancer types	Association	No.of control	References
1.	Lung cancer	Increased risk	190	Wang *et al.*, 2003
2.	Bladder cancer	Increased risk	209	Stern *et al.*, 2002
3.	Acute Myeloid Leukemia	Increased risk	186	Seedhouse *et al.*, 2004
4.	Colorectal cancer	Increased risk	100	Krupa *et al.*, 2004
5.	Breast cancer	Increased risk	1826	Kuschel *et al.*, 2002, Smith *et al.*, 2003
6.	Acute lymphoblastic Leukemia	Protective role	193	Cihan *et al.*, 2010
7.	Bladder cancer	Protective role	214	Shen *et al.*, 2003
8.	Colorectal cancer	Protective role	128	Mort *et al.*, 2003
9.	Skin cancer	Protective role	873	Han *et al.*, 2004
10.	Supraglottic cancer	Protective role	172	Benhamou *et al.*, 2004
11.	Lung cancer	No association	272	Jacobsen *et al.*, 2004
12.	Gastric cancer	No association	166	Shen *et al.*, 2004
13.	Cutaneous Malignant Melanoma	No association	319	Duan *et al.*, 2002
14.	Colorectal cancer	No association	725	Tranah *et al.*, 2004

* (Source: Cihan *et al.*, 2010)

2.7.1 XRCC3 and its relation with Gastric cancer

DNA repair pathways are responsible for maintaining the integrity of the genome in face of environmental insults and general DNA replication errors, playing a role in protecting it against mutations that lead to cancer (Lindhal *et al.,* 2000). So, polymorphisms of DNA repair enzymes, which may alter the function or efficiency of the DNA repair, may contribute to an increased risk of environmental carcinogenesis (Mohrenweiser *et al*., 1998). These low-penetrance susceptibility genes have common variants and interact with environmental factors, contributing as a major factor to the populational incidence of cancer (Shields *et al*., 2000). The polymorphism in genes that participate in different DNA repair pathways, such as XRCC3 (Shen *et al*., 1998), have been identified and related to cancer susceptibility. Protein XRCC3 functions in the DNA double-strand break (DSB) and cross-link repair (Thompson *et al*., 2002) and interacts and stabilizes Rad51 (Schild *et al*., 2000), one of the key components of the homologous repair (HR) pathway. The HR pathway uses a second intact copy of a homologous chromosome as a template to copy the information lost at the DSB site, resulting in a high-fidelity process and preventing chromosomal aberrations (Christmann *et al*., 2003). The main polymorphism in this gene involves the change of threonine (Thr) to methionine (Met) at codon 241 in exon 7 (Shen *et al*., 1998). Little is known about the functional consequences of this variation, although some studies observed a positive relation between the Thr241Met polymorphism and an increased risk for skin (Winsey *et al*., 2000), bladder (Matullo *et al*., 2001), breast (Smith *et al*.,2003) and lung (Jacobsen *et al*., 2004) cancers. So far, the investigations about interactions between XRCC3 polymorphism and environmental carcinogenesis have produced scarce and conflicting results (Ratnasinghe *et al*., 2004, Duan *et al*., 2002, Shen *et al*., 2004, Sanyal *et al*., 2004), showing the functional complexity of these variants, that can include their interaction with environmental factors, thus modulating the susceptibility to cancer. Regarding gastric cancer, only a few studies were conducted to investigate its association with XRCC3 variants (Shen *et al*., 2000, Ratnasinghe *et al*., 2004, Shen *et al*., 2004, Fang *et al*., 2010). Currently there are no reports on the association between *XRCC3* polymorphisms and Gastric cancer risk.

2.7.2 Related risk factors

Gastric carcinogenesis is a multistep process composed of genetic and epigenetic alterations (Tahara *et al*., 1993). Gastric cancer can develop in any part of the stomach and may spread throughout the stomach and to other organs; particularly the oesophagus and the small intestine. As per Cancer, World Health Organization (Feb 2006), Stomach cancer causes nearly one million deaths worldwide per year. In the global incidence stomach cancer is second most common tumour (Chan *et al*., 2006). India, overall, is deemed to be a low incidence (<6.7 /100,000) country but there are regions, like Kashmir, with a particularly high incidence (Sipponen *et al*., 1983, Khuroo *et al*., 1992). Clinical experience has revealed very high prevalence of gastric cancer in Kashmir. It is suspected that several risk factors are involved including diet, gastritis, smoking, intestinal metaplasia and Helicobacter pylori infection. It is more common in men. Kashmir valley is one of the high incidence areas (Khuroo *et al*., 1992) where different environmental and dietary habits play an overwhelming role in the development of gastric cancer. These include intake of sun-dried vegetables of Brassica family (Hakh), pickled vegetables (Anchar) and hot salted tea, which contains potentially high content of carcinogenic compounds like nitrosamines (Kumar *et al*., 1992). Personal habits like smoking of hukka/cigarette increases the risk of developing gastric cancer (Khuroo *et al*., 1992). In Kashmir, the most important specific food habit is consumption of large quantities of hot salted tea. The use of sodium bicarbonate at the time of boiling the tea leaves and the further addition of common salt to the prepared tea cause one to suspect that the tea does more than cause thermal injury to oesophageal epithelium. Common salt (NaCl) is a well-known irritant of gastric epithelium and has been considered a risk factor for gastric cancer (Khuroo et al., 1992).The presence of N-nitroso compounds (found in most of the customary dietary items of a native Kashmiri) in the stomach has been incriminated as a possible etiological factor in the genesis of gastric cancer (Mirvish *et al*., 1972, Siddiqi *et al* 1992). As per Census of India 1981, Series 8, Jammu and Kashmir; one of the factors include the peculiar geography of the valley (situated at an altitude of 1800-2400 m above the sea level), and severe cold winter may have a bearing on the etiology. Gastric cancer most often develops in the context of chronic inflammation, named "chronic gastritis" in the stomach. However, inflammation in this organ is by a crucial agent i.e bacterium, *Helicobacter pylori* (*IARC., 1994*)

2.7.3 Gastric cancer and its association with genetic factors

Globally literature suggests that polymorphism in genes involved in inflammation viz interleukin (Hold *et al*., 2007) toll-like receptor 4 (Magnusson *et al*., 2001), human leukocyte antigen (Li *et al*., 2005),metabolic phase I enzyme viz CYP1A1 (Gonzalez *et al*., 2002), metabolic phase II enzyme viz GSTM1, GSTT1 (Glutathione-S-transferase) (Shen *et al*., 2001) and NAT1, NAT2 (N-acetyltransferase), DNA repair Viz XRCC1 (Malik *et al*., 2011) are involved in development of gastric cancer. Indian studies on this topic are limited because of inadequate sample size, improper study designs and inability to controls for confounders. Only small numbers of polymorphism were investigated in all these studies. Most of the Indian studies have used RFLP method for genotyping while studies conducted outside India have used Taqman and Illumina assays for genotyping. Evidence for a genetic predisposition to gastric cancer comes from both epidemiological studies and case reports of gastric cancer families. Systematic case-control and cohort analyses of gastric cancer patients have shown that the risk of gastric cancer in first-degree relatives is increased 2–3-fold (Woolf *et al.,* 1954, Videbaek *et al*., 1954, Macklin *et al*., 1960, Zangheri *et al.,* 1990, La *et al*., 1992, Goldgar *et al*., 1994, Nagase *et al*., 1996, Hoshino *et al*., 1985, Palli *et al*., 1985, Karner *et a*l., 1997). An association between gastric cancer and other cancers (colorectal and CNS tumours) has been observed in some of these studies (Goldgar et al., 1994, Karner et al., 1997). These associations suggest the presence of predisposition genes with pleiotropic effects. The risk of gastric cancer in relatives of patients has been shown to be dependent upon histology (Palli *et al*.,1985). Relatives of patients with intestinal disease have a 1.4-fold increase in risk, compared with a 7.0-fold increase in risk in relatives of patients with diffuse disease (Palli *et al*., 1985). This suggests that differences in histology reflect, in part, a greater hereditary basis in diffuse gastric cancer.

2.8 Aim of This Study

As per Cancer, World Health Organization (Feb, 2006), gastric cancer causes nearly one million deaths worldwide per year. In the global incidence stomach cancer is second most common tumour (Chan *et al*., 2006). In India it is believed to be a low incidence (<6.7 /100,000) country but there are regions, like Kashmir, with a particularly high incidence (Sipponen *et al*., 1983, Khuroo *et al*., 1992). Observation

of association between some cancer types and DNA repair defects suggested the DNA repair genes as candidate cancer susceptibility genes. Homologous recombination repair is the most important mechanism for the sake of the cell. It is well established that individuals having a modified ability to repair double strand breaks are at an increased susceptibility to cancer. Therefore, polymorphisms in genes encoding DSB repair molecules have strong relevance in determining susceptibility to cancer.

As described in more detail previously, XRCC3 *is* susceptible enzyme due to the dual roles in repair of double strand DNA breaks. Genetic polymorphisms altering the activity or the structure of *XRCC3* may modify the individual's susceptibility for the gastric cancer. Polymorphisms of *XRCC3* have been widely studied in relation to various cancer types as risk modifiers. However, so far, there have been no reports evaluating the clinical significance of XRCC3 genetic polymorphisms for the risk of gastric cancer. Therefore, this study focused on the effects of *XRCC3* genetic polymorphisms, alone, as risk modifier for the development of gastric cancer and therefore the aims related with the present study included:

- Investigation of association of *XRCC3* Thr241Met (codon241) polymorphism in control and case group, and determination of the effects of this polymorphisms on the risk of development of gastric Cancer (if any).
- Investigation of the interaction between *XRCC3* Thr241Met genetic polymorphisms with non-genetic factors such as age of patients, gender, smoking status of patients, residence, salty food, *Helicobacter pylori* (if any) etc.

3.1 MATERIAL

3.1.1 STUDY POPULATION

Subjects were recruited at Sheri- Maharaja Hari Singh Hospital (SMHS), Srinagar Kashmir. The recruitment process was initiated following approval by Departmental ethical committee of Biochemistry Government Medical College (GMC) Srinagar. The diagnosis of gastric cancer was based on the standard histopathalogical criteria. Controls were recruited from the different wards of Sheri- Maharaja Hari Singh Hospital (SMHS) and Associated Government Medical College (GMC) Hospitals, following the referral pattern of sex and age matched patients. None of the controls had a personal history of malignancy.

- **SUBJECTS**

Criteria adopted for selecting the cases and controls

The criteria for including or excluding a subject in the study were formulated prior to the commencement of the study.

- **Cases**

All the histopathologically confirmed Gastric cancer patients were included irrespective of cancer stage or age. In our case control males, cases were 50 male patients and 40 female patients.

Exclusion criteria

Under the following conditions the patients were not recruited in the study;

- Patients suffering from any disease that affected their life style.
- Patients who had received prior chemo or radiotherapy.
- Patients not belonging to Kashmir valley.
- Who did not agree to participate

Inclusion Criteria

- Histopathologically proven gastric cancer patients.
- Patients who were willing to take part in the study.
- Patients belonging to Kashmir valley.

➢ Controls

Controls were matched to the cases by age and gender.

Exclusion criteria

- ➢ Patients who never suffered from any kind of malignancy.
- ➢ Patients who did not agree to participate.

Inclusion criteria

- ➢ Residents of the Kashmir valley.
- ➢ Matched gender ratio with the cases.

3.1.2. COLLECTION OF BLOOD SAMPLES

Individuals who gave consent to participate in this case-control study were enrolled at the SMHS. Blood sample was collected from both the cases and the controls. After proper consent from each subject, 3-4 ml of blood was collected in EDTA vial. The samples were stored at -80^0 C until processed.DNA was extracted from peripheral leukocytes according to Phenol-Chloroform method (Sambrook and Russel, 2001).

Note: Chemicals used and preparation of reagents are illustrated in Appendix.

3.2. METHODS

3.2.1. DNA extraction from Blood

1. To 3 ml of blood, 9 ml of erythrocyte lysing buffer was added.
2. The contents were shaken gently, incubated for 30 min on ice, and centrifuged at 4000 rpm for 10 min.
3. The supernatant (blood waste) was removed and the pellet was resuspended in 9 ml of erythrocyte lysis buffer and centrifuged for 10 min at 4000 rpm.
4. The supernatant was discarded. 5 ml of SE-buffer, 20 µl proteinase K (10mg/ml) and 100 µl 20% SDS were added to the pellet. The contents were shaken gently and incubated overnight at 37^0C in a water bath/Incubator. During this step the white blood cells membranes are denatured and DNA goes out in solution.
5. Next day, equal volume of Tris-equilibrated (TE) Phenol was added to the contents and shaken gently for 10 min. The tubes were centrifuged at 3000 rpm for 5 minutes.

6. The supernatant was aspirated and transferred into a new tube. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the contents. The emulsion was then centrifuged for 10 min at 2000 rpm with minimal extracted into the supernatant and proteins separated into the lower phase.
7. The aqueous phase was removed using a Pasteur pipette without disturbing the protein interface and was transferred into a new tube to which equal volume of chloroform/isoamylalcohol (24:1) were added. The tubes were shaken for 10 min and centrifuged at 3000 rpm for 5 min.
8. The upper aqueous phase (containing DNA) was transferred into a clean and sterile conical centrifuge tube using a sterile Pasteur pipette, followed by the addition of 300 µl of chilled 3M sodium acetate (pH 5.2) and an equal volume isopropanol along the sides of the tube.
9. DNA was precipitated by gentle swirling of the tube and observed visually as a white thread like strand that was spooled out using a cut-tip and transferred into a 1.5 ml eppendorf tube.
10. The DNA was washed twice in 70% ethanol by inversion to clean it from any remaining salts and the tube was centrifuged at 11000g for 4 min. The supernatant was discarded without disturbing the DNA pellet.
11. After discarding the supernatant the pellet was air dried from excess ethanol by leaving the tubes open and inverted.
12. The dried pellet was re-suspended in 300µl of DNA storage buffer and left overnight at 37^0C for complete dissolution.
13. DNA purity and concentration was determined spectrophotometrically (pharmacia, Gene Quant) by measuring the absorbance at 260nm.Purity was assessed by the $A_{260/280}$ ratio.
14. The quality of extracted DNA was determined by electrophoresis, on 1% agarose gel.
15. DNA was dissolved in TE-storage buffer at -20^0C for longer storage and shelf life.

3.2.2. DETERMINATION OF CONCENTRATION, PURITY AND QUALITY OF GENOMIC DNA

➢ ***Qualitative Analysis***

The quality of the genomic DNA was examined by gel electrophoresis using 0.8 % agarose gel. Two microlitre of each DNA sample was mixed with 1µl of 1X DNA

loading dye (1X loading dye consists of 4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose in1ml water) and was loaded in the gel. Electric current was applied at 20 volt until DNA entered in to the gel and was raised to 50 volt for rest of the run. Run was stopped when the dye had travelled nearly 2/3rd of the gel. Gel was visualized by a Gel doc system (AlphaimagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system.

➢ ***Quantitative Analysis***

The quantity of the DNA was estimated by making appropriate dilutions to determine the optical density (OD) at 260nm and 280 nm by double beam spectrophotometer (Spectron 2206) and the concentration was determined using equation:

DNA (µg/ml) = A_{260} x 50 x dilution factor

The ratio of A_{260}/A_{280} was calculated and the DNA samples for which the ratio was 1.7-1.9 was kept for the future use. The DNA was stored at 4°C for a short time but the vials were kept at -20°C for longer duration storage.

3.2.3. Agarose Gel Electrophoresis

- The edges of clean, dry glass plate were sealed with tape to form a mold, and were set on a horizontal section of the bench.
- Sufficient electrophoresis buffer (1xTAE) was prepared to cast the gel and to fill the electrophoresis tank.
- Eight hundred milligrams of agarose was dissolved in 100 ml of 1x TAE buffer and heated to boil in oven until a clear transparent solution was formed.
- After removing from the oven the solution was allowed to cool to 50-60°C and then a small volume (5µl) of ethidium bromide was added as visualizing agent.
- The warm agarose was poured into the mold. An appropriate comb was positioned for forming the sample slots in the gel when the agarose was added to the mold.
- The gel was allowed to set completely (30-45 min), then a small amount of TAE buffer was poured on top of the gel and the comb was carefully removed. TAE buffer was poured, after removing tape also, and gel was mounted in the tank.
- Sample was mixed with few drops (2-3 µl) of 6X gel loading buffer.

- Sample was loaded slowly and carefully into the slot of submerged gel using disposable loading tips.
- The lid of gel tank was closed and electric leads connected so that the DNA would migrate toward positive anode (red lead).Voltage gradient of 1-5 V/cm was applied (distance measured from cathode to anode). The gel was allowed to run until the bromophenol blue and xylene cyanol migrated an appropriate distance through the gel.

Pattern of separation was visualized by a Gel doc system (AlphaimagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system. Samples containing high molecular weight DNA with no fragmentation/ shearing and without any apparent contamination or streaking were selected for further analysis.

3.3 Primers for genotyping

Primers used throughout the study were selected by literature search and were derived from known sequences of human. The primer pairs were purchased from (MERCK, Germany). Primers stocks were brought to 100 pmol/µl concentration and stored at -20 ^{0}C. Aliquots of 6.5 pmol/µl concentration were prepared and used for PCR.

3.3.1. GENOTYPING OF XRCC3 GENE POLYMORPHISM

Once it was confirmed that the genomic DNA is present and concentration and purity is also desirable, the desired fragment of DNA i.e. exon 7 containing the codon 241 in XRCC3 gene, were amplified by Polymerase Chain Reaction (PCR). The standard protocol for PCR was used; however the technique was standardized or optimized for available environmental conditions. After standardizing all the parameters of technique like varying annealing temperature, dNTP, primer and template concentration, regions containing the codons 241 in XRCC3 gene were amplified. PCR was performed in total volume of 25µl. The PCR reactions were composed of 50-150 ng genomic DNA, 0.1mM dNTPs, 6.2pmoles/µl of each primer, 1.5mM $MgCl_2$ and 1U/µl of Taq polymerase in 1x PCR buffer as shown in (Table.4,5). Reaction mixture in PCR tubes was gently mixed and placed in a twenty-five well automated thermal cycler (Appendorf) for amplification after properly placing the tubes tightly within the wells and closing the lid. The program with suitable set of temperatures was run. During this

procedure precautions were taken to keep the Taq polymerase active by not only preparing the reaction under $4^{o}C$ temperature but also adding the various reaction components as quick as possible the PCR conditions were selected after extensively standardizing all the PCR parameters. Negative controls in all PCR assays consisted of a similar reaction mixture with the template replaced with sterile water. The primer pairs used for amplification were as follows:

For codon 241 (exon 7)

Forward primer: **5' - GCTGTCTCGGGGCATGGCTC- 3'**

Reverse primer: **5' - ACGAGCTCAGGGGTGCAACC- 3'**

The sequence of the amplified fragment, location of the SNP site and sequence of the recognition site for the restriction enzyme were given in (Fig. 6)

Table 4: Volume and final concentration of different reagents used in PCR

Reagent*	Volume required	Final concentration
1. PCR buffer (10X)	2.5 µl	1X
2. dNTP mix	0.5 µl	10mM
3. Forward primer	0.5 µl	20pmoles/µl
4. Reverse primer	0.5 µl	20pmoles/µl
5.Taq DNA polymerase	0.75 µl	1 U/ul
6. Genomic DNA	2.5 µl	50-100 ng
7. Milli Q *w*ater	17.75µl	-
Total volume	**25.0 µl**	

* see appendix for these reagents

Lyophilized primer stocks were diluted first to 100pmol/µl concentration and then to 20pmol/µl using miliQ water. PCR buffer contained Tris-Cl, KCl, $(NH_4)_2SO_4$ and 15mM $MgCl_2$; pH 8.7. Amplification and specificity of amplicon obtained in the PCR reaction was analyzed by agarose gel electrophoresis on 2% gel. After the electrophoresis, the gel was visualized on UV-illuminator and photographed on a Gel Doc (Pharmacia).

The concentration of amplified PCR products was approximated by comparison with the corresponding bands in 100bp ladder. However, the total amount of 100bp DNA ladder in one lane was 200ng, representing approximate concentration of 33ng, 8.8ng, 6.6ng, 4.4ng and 2.4ng respectively.

Table 5: PCR cycling parameters

Step	Temperature (^{O}C)	Time	Cycles
1. Initial Denaturation	94	10 min	1
2. Denaturation	94	30 sec.	30
3. Annealing	**66.5**	45 sec	
4. Extension	72	1 min	
5. Final Extension	72	10 min	1
			32 cycles

3.3.2. Restriction digestion

The PCR product 208 b.p was digested with specific restriction enzymes for detecting the codon 241 polymorphism of the XRCC3 gene. The standard protocol for restriction digestion was used. Ten microliters of the PCR products were digested separately with 5 units (0.5 microlitres) of ***Nla* III** for codon 241. The reaction mixture included 2 µl of 10x buffer and 18 µl of Mili Q water. The mixture was incubated at 37°C for 24 h. The products were then resolved on 3% agarose gels. DNA molecular weight marker of 100 bp was used to assess the size of the PCR–RFLP products.

3.3.3. DNA Sequencing of XRCC3 gene PCR products

Ten percent of samples of both gastric cancer and healthy controls were sent for DNA sequencing (SciGenomics labs pvt. Ltd, Cochin).

3.4. Statistical Analysis

Results were statistically analyzed and data was expressed as mean±SD. Allele and genotype frequencies were compared between groups using the χ^2-test. The association between XRCC3 genotype and the risk of Gastric cancer was estimated by calculating odds ratio (OR) and their 95% confidence intervals (95% CI). A P value of <0.05 was used as a criterion for statistical significance. For the analysis, statistical software GraphPad Prism version 5.0 was used.

4.1. General characteristics of Study Population

In the present study 80 blood samples from gastric cancer patients (males and females) and 70 blood samples from healthy controls were used. General characteristics of the gastric cancer patients and controls are given in table 6.

A total of 150 subjects were taken for the study from OPD/IPD Sections of SMHS Hospital Srinagar. The genotyping was carried out at Department of Biochemistry SMHS Hospital Srinagar. Among 150 subjects 80 were histopathalogical confirmed gastric cancer cases and 70 were healthy controls with age and sex matched. Among 80 cases. 50 were ≥50 years and 30 were ≤ 50 years. Similarly, in 70 healthy controls, 45 were ≥50 years and 25 were ≤ 50 years age. Gender wise, among 80 cases, 45 were males and 35 were females. In healthy controls, 55 were males and 15 were females . Mean age was 52.5 years for cases and 53.2 years for healthy controls; in the range of 30-60 years for both cases and controls and sex-wise (56.2% males, 43.75% females in cases; 78.57 males, 21.43 females in controls) and these differences were not stastically significant, suggesting that the frequency matching was adequate. In addition there were no significant differences between cases and controls by non-genetic factors (Smoking, residence, salty tea consumption, family history of cancer).

Table 6: General characteristics of study population.

Variables		Cases (80)	Controls (70)	P value
Age	≤ 50	50 (62.5)	45 (64.28)	
	≥ 50	30 (37.5)	25 (35.72)	0.95
Gender	Males	45 (56.25)	55 (78.57)	0.006
	Females	35 (43.75)	15 (21.43)	
Smoking status	Smoker	50 (62.5)	40 (57.14)	0.61
	Non-smoker	30 (37.5)	30 (42.85)	
Residence	Rural	45 (56.25)	40 (57.14)	
	Urban	35 (43.73)	30 (42.85)	0.95
Salty tea	Yes	50 (62.5)	45 (64.28)	
consumption	No	30 (37.5)	25 (35.72)	0.95
Pickle	Yes	45 (56.25)	30 (42.85)	
consumption	No	35 (43.75)	40 (71.43)	0.14
H. Pylori	positive	45 (56.25)	20 (28.57)	
infection	negative	35 (43.75)	50 (71.43)	0.001
***X*-Ray radiation**	Yes	48 (60.0)	25 (35.71)	
lifetime/once	Never	32 (40.0)	45 (64.28)	0.005
Family history	Yes	12 (15.0)	3 (4.28)	
of cancer	No	68 (85.0)	67 (95.71)	0.05

4.2. Evaluation of DNA concentration and purification

The genomic DNA was extracted from the whole blood samples of cases and control patients. The integrity, concentration and purification of the genomic DNA was checked not only by UV- spectrophotometry but also by agarose gel electrophoresis, by analyzing 3-4μl of genomic DNA on the 0.8 % agarose gel (as shown in Fig. 9), which reflected the intactness of genomic DNA, because the genomic DNA was restricted to the wells with no smear in the gel suggesting that DNA was not degraded. The results showed that DNA concentration of the samples ranged from 300ng/μl to 1200 ng/μl which was sufficient enough to proceed for the other phases of the study. The purity of DNA was determined by A_{260} / A_{280} ratio and it was found that the ratio in all DNA preparations was in the range of 1.73-1.85. Agarose gel electrophoresis also demonstrated that DNA samples had high molecular weight without degradation as shown in (Fig. 11).

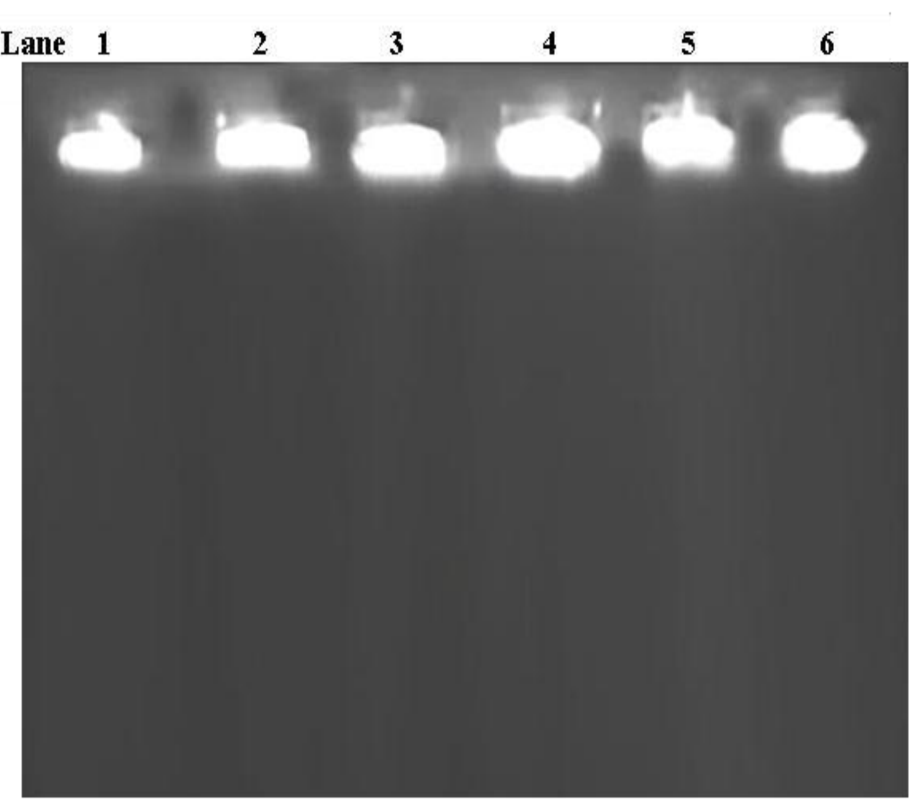


Figure 11: Representative gel picture showing the integrity of DNA analyzed by agarose gel electrophoresis (0.8%). Lane no.1- 4 represents DNA samples isolated from gastric cancer cases and lane no. 5, 6 represents DNA samples isolated from controls.

4.3. Amplification of codon 241 region of XRCC3 gene

The genotyping of XRCC3 gene polymorphism was done by PCR. A primer sets were used to amplify the region containing the polymorphism in the XRCC3 gene. 80 genomic DNA samples from gastric cancer patients (both males and females) and 70 from normal subjects were randomly taken as DNA templates. The reaction conditions and PCR program which was used for amplification is described in methodology section. During PCR amplification, when all the PCR parameters were standardized and optimized, exon containing polymorphism was amplified. Using a primer pair, an amplicon representing exon 7 of codon 241 was produced. After efficient amplifications, 5-6μl of PCR product was analyzed on the 2.0% agarose gel. The amplified product was then visualized under UV exposure, using the ethidium bromide as visualizing agent. The result of PCR of exon 7 is shown in (Fig. 12). A single band of 208 bp was visible and represented exon 7 respectively 9 out of 80 genomic DNA from cases and 10 out of 70 genomic DNA from controls were not amplified properly and thus could not get desired results.

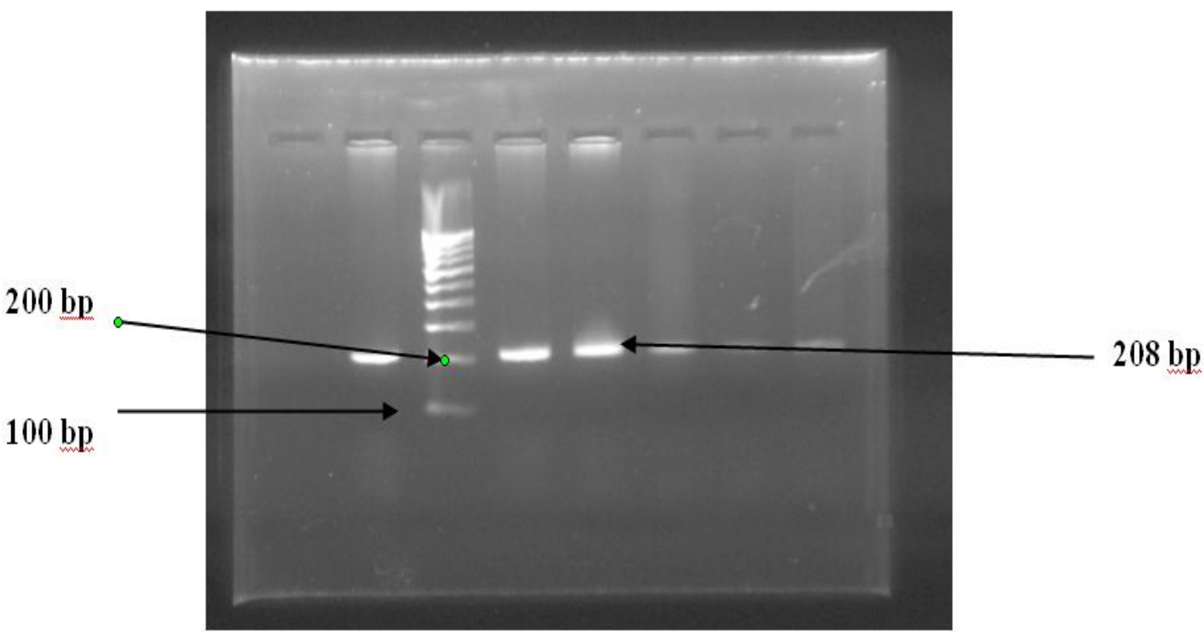


Fig. 12: Representative gel picture showing PCR results of *XRCC3* gene exon7. Lane no. 1, represents blank whereas lane 2, 3, 7, represents 208 bp PCR amplicon of GC cases and lane 4, 5, 6 represent 208 bp PCR amplicon of controls. Lane M contains 100 bp DNA ladder.

4.4. Restriction digestion for the codon polymorphism of the XRCC3 gene

For the detection of polymorphism in codon 241 of XRCC3 gene PCR product, restriction digestion was performed, which was digested with a restriction enzyme *Nla* III. The enzyme *Nla* III recognizes the wild type alleles of codon 241. The PCR product of 208 bp was digested for codon 241 polymorphism by the standard restriction digestion protocol discussed in methodology in both GC Cases and controls. The product was then resolved on 3% agarose gels. Among 80 GC Cases 60 samples remains uncut (homozygous wild) by *Nla*111 restriction enzyme, 10 were digested (heterozygous) having three bands 208bp, 120bp and 88 bp, only 1 sample was digested into two bands 120 bp and 88 bp (mutant homozygous). Same was done in 70 controls were 52 samples remains uncut (homozygous wild) by *Nla*111 restriction enzyme, 8 were digested (heterozygous) into three bands 208bp, 120bp and 88 bp, and none shows mutant homozygous condition (Fig. 13) . The fidelity of restriction enzyme was cross checked in order to rule out the possibility of enzymatic inactivity. The results thus obtained were further validated by the sequencing report of samples that showed no variation (Fig. 14a-15b). The XRCC3 gene was monomorphic for suspected polymorphism in our population as no major mutant homozygous condition was observed in either cases or controls.

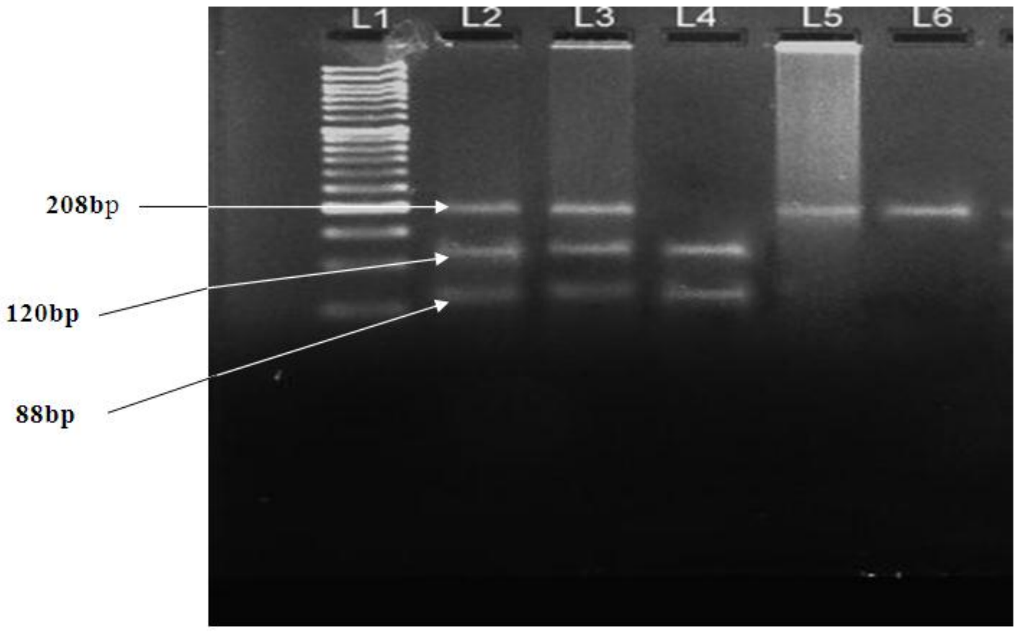


Fig. 13 Representative gel picture of XRCC3 Exon7 gene restriction digestion by *NLA*III restriction enzyme, on agarose gel 3%.

L1-Represents Ladder 50 bp
Lane 2-Represents heterozygous genotypes (Three bands at 208bp,120bp, 88 bp)in Controls.
Lane 3 -Represents heterozygous genotypes (Three bands at 208bp,120bp, 88 bp)in Cases
Lane 4-Represents mutant homozygous genotype (Two bands 120bp, 88 bp) in cases
lane 5-Represents Wild homozygous genotypes (one band at 208 bp) in cases.
Lane 6-Represents Wild homozygous genotypes (one band at 208 bp) in Controls.

Sequencing result for XRCC3 gene exon7:

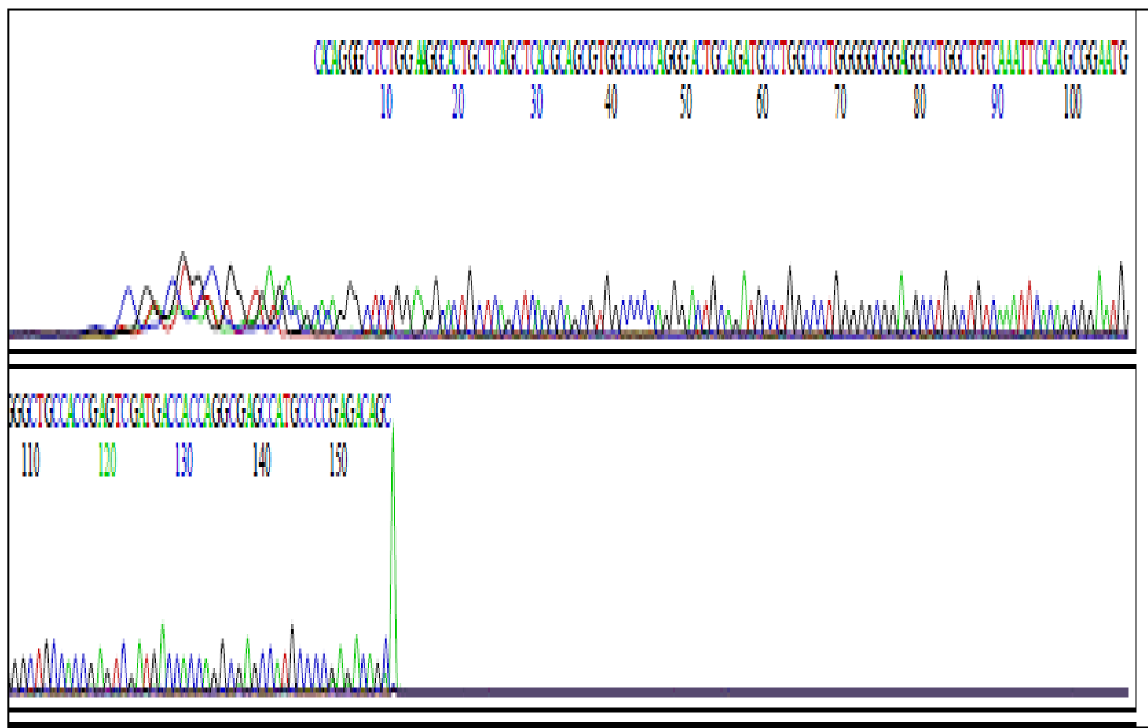


Fig. 14 (a): Representative chromatogram of direct sequencing for XRCC3 gene exon7 241 codon in controls.

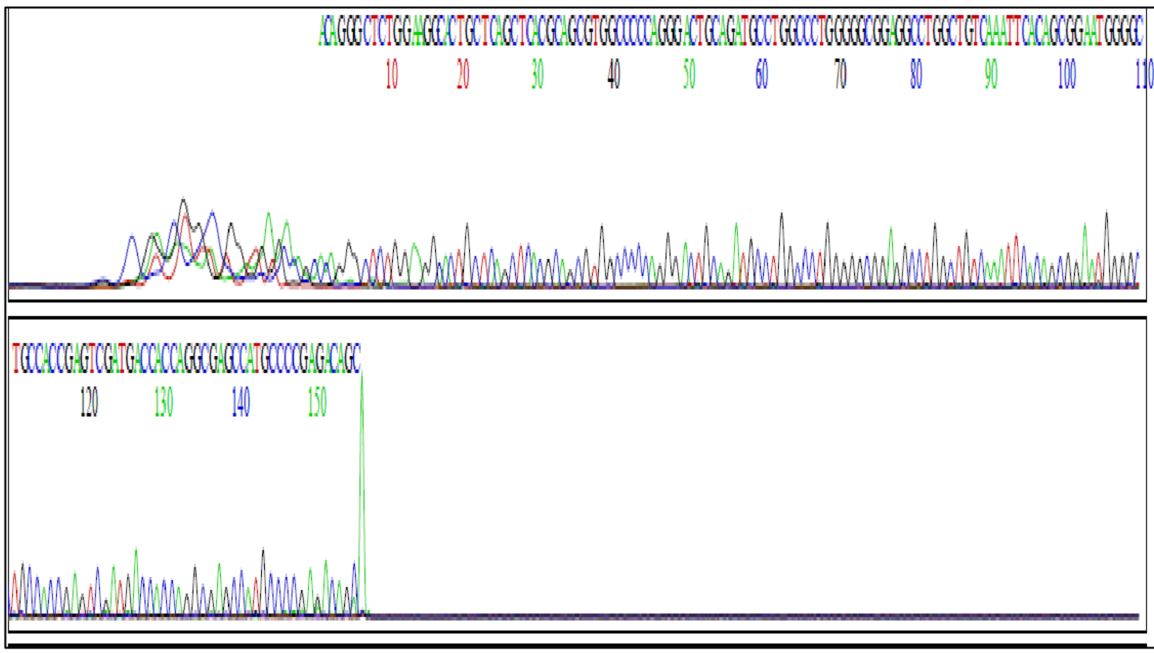


Fig. 14 (b): Representative chromatogram of Direct sequencing for XRCC3 gene exon7 241 codon in GC cases..

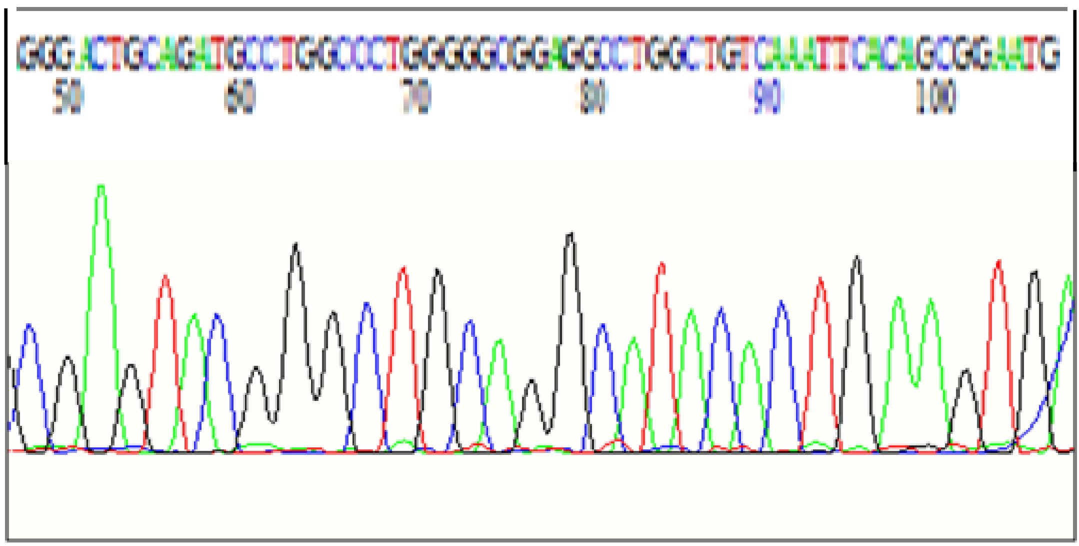


Fig. 15 (a): Partial sequence electropherogram showing wild type homozygous XRCC3 exon 7 241 codon in normal controls.

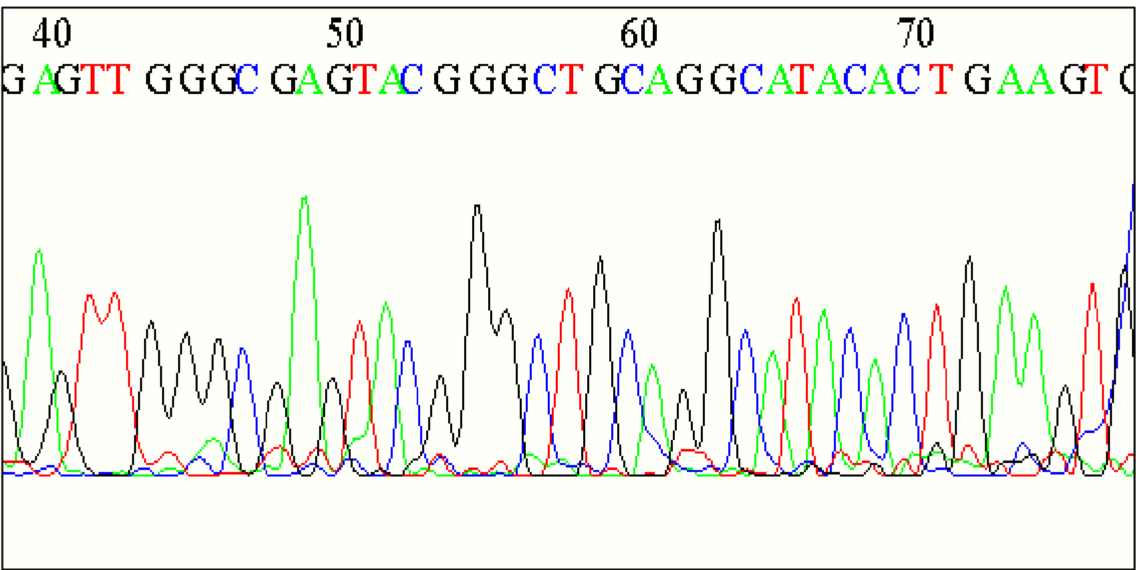


Fig. 15 (b) : Partial sequence electropherogram showing wild type homozygous XRCC3 exon7 241 codon in GC cases.

Table. 7. Genotypic and allelic frequencies of XRCC3 gene codon 241 among case and controls and their association with risk of gastric cancer

Gene	Variants	Cases (n=80)	Controls (n=70)	χ^2	OR (95%CI)	p value
XRCC3 (codon 241)	Thr/Thr	60 (75%)	52 (74.2%)	-	1	referent
	Thr/Met	10 (12.5%)	8 (11.4%)	0.009	1.08 (0.39 - 2.49)	0.92
	Met/Met	1 (1.25%)	0	0.006	2.60 (0.10 – 65.33)	0.93
	Thr	130 (91.5%)	112 (93.3%)	-	1	referent
	Met	12 (8.7%)	8 (6.66%)	0.08	1.13 (0.58 – 2.1)	0.72
	Thr/Met + Met/Met	11 (13.75%)	8 (11.42%)	0.01	1.19 (0.44 - 3.18)	0.89
	Missing result	9	10			

As shown in (Table.7) and histogram (Fig. 16) , no significant differences were observed for either the frequency of XRCC3 241Thr/Met genotype or the frequency of 241Met allele between cases and controls (12.5% of 241Thr/Met in cases, and 11.4% in controls, **P=0.92**; 8.7% of Met allele in cases and 6.6% in controls, **P=0.72**). Compared with 241Thr/Thr wild-type genotype, the XRCC3 241Thr/Met was not associated an increased risk of gastric cancer in the logistic regression analysis (adjusted **OR=1.08:95%CI=0.39-2.49,P=0.92).** Because, the XRCC3 241Met/Met genotype was infrequent in this Kashmiri population, it was combined with the XRCC3 241Thr/Met Genotype. However, the combined genotype (Met/Met and Thr/Met) in (Table. 7), was also not associated with the risk of gastric cancer (**OR=1.19;95% CI=0.44-3.18,P=0.89).** The dichotomized genotypes (XRCC3 241Thr/Met or Met/Met vs Thr/Thr) were further examined individually for subgroups of the variables listed in (Table. 8) and adjusted odd ratio with 95% confidence intervals are presented in (Table. 8) , were p valve was greater than **0.05**, that means association insignificant in these subgroups.

Table . 8. Stratification analysis of XRCC3 genotype frequencies of non-genetic factors in gastric cancer.

Variable	Cases=80				Controls=70				OR* 95%CI	P-Value
	n	Thr/Thr	Thr/Met	Met/Met	n	Thr/Thr	Thr/Met	Met/Met		
Age										
>50	50	32(64.0)	15(30.0)	1(2)	45	36(80.0)	8(17.7)	0	2.38(0.85-6.69)	0.20
<50	30	24(80.0)	4(13.3)	0	25	13(52.0)	7(28.0)	0	0.31(0.07-1.26)	0.18
Sex										
Male	45	28(62.2)	13(28.8)	1(2)	55	42(76.3)	12(21.8)	0	1.62(0.64-4.07)	0.42
Female	35	29(82.8)	5(14.2)	0	15	9(60.0)	3(20.0)	0	0.51(0.10-2.60)	0.71
Smoke										
Yes	50	30(60.0)	15(30.0)	1(2)	40	31(77.5)	7(17.5)	0	2.21(0.79-6.19)	0.19
No	30	26(86.6)	3(10.0)	0	30	20(66.6)	8(26.6)	0	0.28(0.06-1.23)	0.16
Salty Tea										
Yes	50	33(66.0)	14(28.0)	1(2)	45	36(80.0)	7(15.5)	0	2.18(0.78-6.07)	0.20
No	30	25(83.3)	4(13.3)	0	25	16(64.0)	8(32.0)	0	0.32(0.08-1.24)	0.17
Pickle consumer										
Yes	45	29(64.4)	13(28.8)	1	30	21(70.0)	8(26.6)	0	1.17(0.41-3.34) 0.76(0.21-2.67)	0.96

No	35	29(82.8)	5(14.2)	0	40	31(77.5)	7(17.5)	0		0.91
Residence										
Urban	45	32(71.1)	12(26.6)	1(2)	40	29(72.5)	9(22.5)	0	1.20(0.44-3.28)	0.90
Rural	35	26(74.2)	6(17.1)	0	30	22(73.3)	6(20.0)	0	0.84(0.23-3.0)	0.95

*ORs were adjusted for age, sex, smoking, residence, tea consumption, H. pylori infection and family history of cancer in a logistic regression model. Thr/Thr (1.00) was taken as referent.

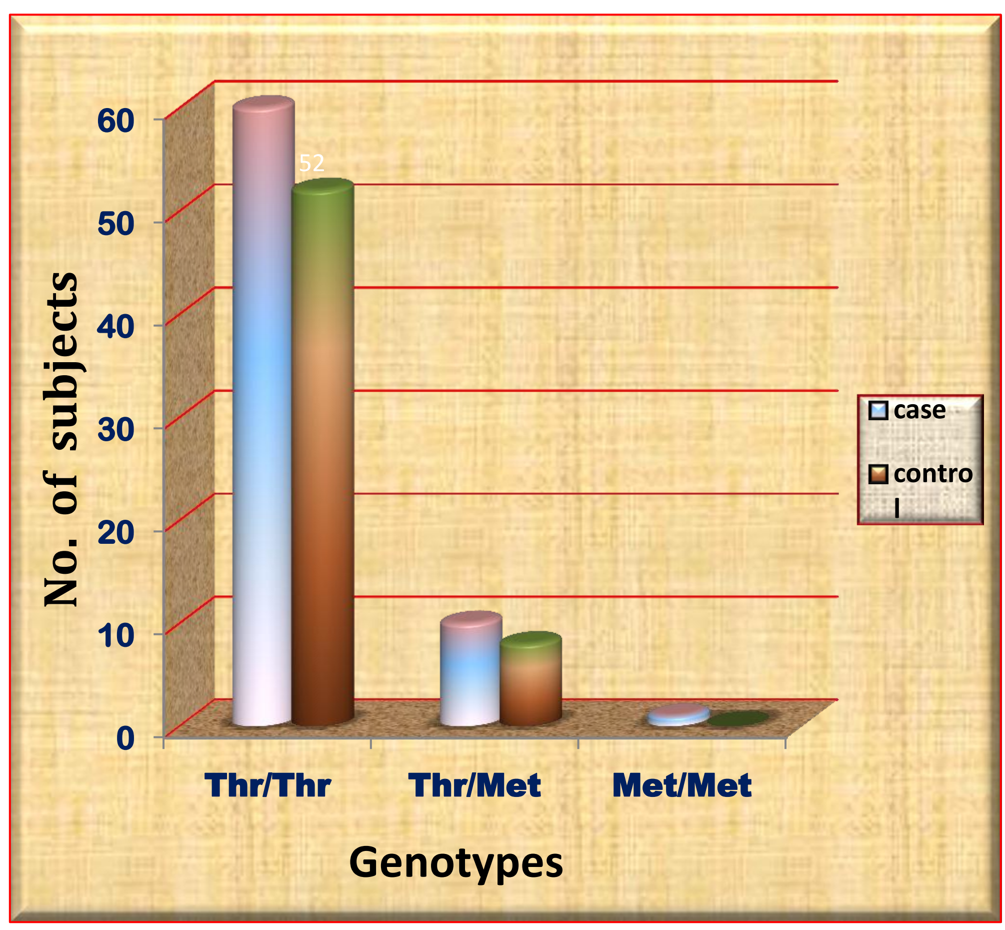


Fig. 16: Histogram showing the distribution of XRCC3 genotypes in cases and control population.

Gastric cancer is the second most frequent malignancy worldwide accounting for 9.9% of all cancer incidences and 12.1% of all cancer deaths. Gastric cancer, can develop in any part of stomach and may spread throughout the stomach and other organs of gastrointestinal tract like, esophagus, liver, colon etc. GC costs 80,000 deaths worldwide per year (Parkin *et al*., 2010). Therefore, prognosis for gastric cancer patients remains poor as most patients are diagnosed in advanced stages. Studies have shown incidence rates in men are twofold than those in women. In the Kashmir valley, gastric cancer has emerged as the most common cancer, forming about 25% of all cancers (Parkin *et al*., 2001). Kashmir valley, has greater malignancy rate and the incidence rates in southern district of Kashmir are higher (Khuroo *et al*., 1992). Genetic polymorphism in DNA repair genes influences individual variation in DNA repair capacity, which has been associated with risk of developing cancer (Boer *et al*., 2002, Ruzzo *et al*., 2007). Xenobiotics (nitrates, nitrosamines, nitrites, heterocyclic amines and polycyclic hydrocarbons), ionizing radiations (UV,IR, X-Ray), life style factors (smoking, tobacco eating, alchol drinking, salty tea with sodium bicarbonate) and environmental chemical agents cause double strand DNA –breaks in some candidate genes (Siddiqi *et al*.,1992, Malik *et al*., 2011).

The XRCC3 repair genes have been reported to play an essential role in homologous recombination repair (HRR) in mammalian cells (Cui *et al*., 1999, Hoeijimakers *et al.,* 2001). XRCC3 is located on chromosome 14q32.3. In HR pathway, XRCC3 is structurally related to RAD51 gene family to localize at sites of DSB damage and subsequently functions to promote the assembly of active RAD51 repair complexes, namely RAD51 foci (Ohgaki *et al*., 2003, Sathornsumetee *et al*., 2007). Once RAD51 foci are formed, mammalian cells use the sister chromatids as the template of preference over a homologous chromosome. Lack of the XRCC3 gene will result in decreased function of HR (Vangent *et al*., 2001, Sathornsumetee *et al*., 2007) and defects in chromosome segregation (Thompson *et al*.,2001). Hence, XRCC3 gene mediates RAD51 dependent HR necessary to repair DNA damage that may result in carcinogenesis if left untreated or misrepaired. Carriers of the variant allele of XRCC3 Thr241Met had relatively high DNA adduct levels in lymphocyte DNA, indicating that this polymorphism has been associated with relatively low DNA repair capacity (Griffin *et al*., 2004). Therefore, XRCC3 gene has been of considerable interest as a candidate susceptibility gene for cancer. The XRCC3 gene has a sequence variation in

exon7, which results in an aminoacid substitution at codon 241(Thr241Met) that affects the enzymes function and its interaction with other proteins involved in DNA damage and repair (Matullo *et al*., 2001). Our aim was to study the XRCC3 exon7 (Thr241Met) single nucleotide polymorphism (SNP) in gastric cancer risk.

In the present hospital based case-control study, of XRCC3 gene codon 241 polymorphism (Thr241met; C/T Transition) in 80 histopathologically gastric cancer patients and 70 healthy controls has been analyzed at Department of Biochemistry, GMC Srinagar. Very few studies have investigated the role of polymorphisms of the DNA repair genes XRCC3 in the risk of gastric cancer. A case-control study was conducted to investigate the association between the polymorphism of XRCC3 Thr241Met with the risk of gastric cancer in a Kashmiri population. This polymorphism results in amino acid substitution of DNA repair genes involved in HRR. DNA repair pathways play a vital role in maintaining genetic integrity and it is becoming clear that defects in repair pathways are connected to many different types of diseases. DNA repair systems maintain genomic integrity, in the face of environmental insults, cumulative effects of age and general DNA replication errors. In our case control study, a single nucleotide polymorphism (SNP) in the XRCC3 gene and its association with gastric cancer was investigated in total 150 subjects, among 80 were gastric cancer cases and 70 were apparently normal controls. The polymorphism assessed, was not present in codon 241. Each polymorphism in the 241 codon of the human XRCC3 gene was composed either of two types of alleles – the wild type or polymorphic variant type with different RFLP size distributions. Thr/Thr was the wild type allele present in codon 241 and Thr/Met was a heterozygous variant present in this particular codon and Met/Met represents homozygous mutant. Out of these alleles, the frequency of homozygous mutant allele Met/Met was lower for codon 241 (8.7% vs. 6.6%) in patients as compared to control subjects. In codon 241 polymorphism, out of 80 gastric cancer subjects, 1 case was found to be homozygous for Met/Met polymorphic allele, 10 were heterozygous while 60 were homozygous for Thr/Thr wild allele as compared to 0, 8 and 52 in case of 70 normal subjects respectively. In this codon 241 polymorphism, we found that number of Met alleles were present equally less (8.7% vs. 6.6%) in cases as compared with controls. Cases with codon241 Thr/Thr homozygous variant had an slight increased frequency of 75% vs. 74.2% in controls but a slight increased frequency was also observed in cases with heterozygous variant

Thr/Met of codon241 (12.5% Vs. 11.4%) as compared with controls (OR= 1.08, 95% CI = 0.39 - 2.49, P = 0.92), however it was not statistically significant. In the case of the homozygous variants of codon241 Met/Met genotype, 1.25% in cases was observed, as compared to healthy controls 0% (OR = 2.60, 95% CI = 0.10 – 65.33, P = 0.93), which was also statistically insignificant. In all the three variant forms, the Thr/Thr wild type allele was taken as the referent category. Moreover the Met allele of codon241 showed a slight increased frequency in cases (8.7% vs. 6.6%) in comparison with controls (OR= 1.13, 95% CI = 0.58 – 2.1, P = 0.72), this also having a statistically insignificant result. This proves, there is no association of XRCC3 Gene in gastric cancer risk. A statistically insignificant interaction between non-genetic factors (age, sex, smoking status, residence, pickle consumption, radiation exposure, family history of cancer) was also observed. P value was ≥0.05 that means association insignificant (Table. 8).

In this case control study, it was observed that most of the cases belong to above 50 years of age rather than with below 50 years of age. We calculated the total number of polymorphic variants in case of below 50 years old (Thr/Thr as well as Met/Met) was calculated, as their individual genotype was less in number. However, the result was not statistically significant. Moreover, distribution of patients into various subtypes of gastric cancer also indicated that majority of patients suffered from squamous cell and adeno carcinoma.

In this population-based case-control study in Kashmiri subjects, it was found that a C toT variant (Thr241Met) of DNA repair gene XRCC3 was not associated with risk of developing gastric cancer, suggesting that the variant genotype of XRCC3 codon241 may not play a major role in the etiology of gastric cancer. To the best of our knowledge, this is the first report on this XRCC3 Thr241Met polymorphism in a Kashmiri population. It was found that the frequency of 241 Met allele was significantly lower in this Kashmiri population than that reported in Caucasians (G.David *et al.*, 2001). Several studies have examined the association between the XRCC3Thr241Met polymorphism and risk of cancers (Matullo *et al.*, 2001, Kuschel *et al.*, 2002, Winsey *et al.*, 2000, Duan *et al.*, 2002, Ster *et al.*, 2002). However, other studies found that the XRCC3 241Met variant allele was not associated with risk of gastric, lung, colon, head/neck in Chinese, European, Brazillian population (G David *et al.*, 2001, Shenhongbin *et al.*, 2004, Improtal *et al.*, 2009, Fang *et al.*, 2010). Genetic

polymorphisms often vary between ethnic populations. In the 70 healthy controls of Kashmiri subjects, we did not observed the XRCC3241Met/Met homozygous genotypes and the frequency of 241Met allele was 6.6%. Both XRCC3 codon 241 varaiant genotypes and allele frequencies in this Kashmiri populations were significantly lower than those reported for the control subjects in previous studies in Caucasians (David *et al*., 2001, Matullo *et al*., 2001, Kuschel *et al*., 2002, Winsey *et al*., 2000, Duan *et al*., 2002, Stern *et al*., 2002).

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Conclusion

There are lot of contradictions with the gene XRCC3 in different cancers with different ethnicity. In India, no reports of any study of this gene XRCC3 exon 7 (Thr241Met) in gastric cancer are reported. This study from north-India's Kashmir valley, is a first report of its kind that the XRCC3 gene has no association with gastric cancer risk. Similar observations are also reported from, China, Europe, Italy and Brazil (G David *et al*., 2001, Shenhongbin *et al*., 2004, Improtal *et al*., 2009, Fang *et al*., 2010). Therefore, lack of association between the XRCC3241Met variant and risk of gastric cancer is not surprising. One possible explanation is that the 241met variant in this low-penetrance gene may not be an important determinant in the etiology of gastric cancer. It is also likely that many variants or common susceptibility polymorphisms, not just single one, of DNA repair genes may jointly contribute to the susceptibility of gastric cancer. Therefore, it is important to include more variants in the genes that participate in the same DNA repair pathways in order to identify genetic markers as well as gene-gene and gene-environment interactions that may predict individual susceptibility to gastric cancer. Further study on larger samples is needed not in gastric cancer alone but in different cancers as well.

APPENDIX

CHEMICALS AND REAGENTS
CHEMICALS

Chemical Name	Company
Absolute ethanol	BENGAL CHEMICALS
Acetone	GALAXO LABORATORIES
Agarose	MP BIOMEDICALS
Ammonium chloride	BDH
Ammonium acetate	BDH
Bromophenol blue	SARABHAI M CHEMICALS
Chloroform	THOMAS BAKERS
De Ionized water	ALFA LABORATORIES
Ethidium bromide	SRL
Ethyl acetate	MERCK
Ethylene diamine tetra acetate (EDTA)	LOBA CHEMIE
Formaldehyde	GALAXO LABORATORIES
Glacial Acetic acid	MERCK
Hydrochloric acid	S D FINE CHEMICALS
Hydrogen peroxide	MERCK
8-Hydroxyquinoline	CDH
Isoamyl alcohol	BDH
Isopropanol	THOMAS BAKERS
Magnesium chloride	MERCK
Methanol	SARABHAI M CHEMICALS
Phenol	SRL
Potassium acetate	QUALIGENS
Potassium bicarbonate	QUALIGENS
Potassium chloride	LOBA- CHEMIE
Potassium hydroxide	S D FINE CHEMICALS
2-Propanol	MERCK
Sodium acetate	SARABHAI M CHEMICALS
Sodium azide	LOBA CHEMIE
Sodiun bisulphate	LOBA CHEMIE
Sodium carbonate	FIZMERCK
Sodium chloride	MERCK
Sodium dodecyl sulphate	MP BIOMEDICALS
Sodium hydroxide	HIMEDIA
Sodium hydrogen carbonate	LOBA- CHEMIE
Sodium phosphate dibasic	LOBA- CHEMIE
Sodiun thiosulfate	LOBA CHEMIE
Sucrose	QUALIGENS
Sulfuric acid	MERCK

TE buffer	SRL
Tris base	SIGMA CHEMICAL COMPANY
Tris HCL	HIMEDIA
Triton X 100	S D FINE CHEMICALS

ENZYMES

Taq polymerase	FERMENTAS / BIOTOOLS
Proteinase K	ZYMO RESEARCH
NLA III	FERMENTAS

MISCELLANEOUS MATERIAL

100bp DNA ladder	FERMENTAS / BIO ENZYME

PCR REAGENTS

10 X Buffer (with $Mgcl_2$)	BIOTOOLS
dNTPs	CINNAGEN
Primers	MERCK GERMANY

DNA storage buffer:

0.5 M EDTA	0.01 ml
1 M Tris	0.5 ml

Final volume was made 50 ml with sterile distilled water.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

Agarose 1 % / 2%/3%:

Agarose	0.5g / 1.0g
Buffer	50ml
Ethidium bromide	10µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. Ethidium bromide was then added to the solution during its cooling just before being poured into the casting tray.

Bromophenol Blue:

Bromophenol Blue	0.4g
Sucrose	20.0g

Bromophenol blue was dissolved in 100ml of distilled water.
From the above stock solution 31.25ml was taken and sucrose was added. Final volume was made 50ml with distilled water.

Ethidium Bromide

Ethidium bromide	10mg

Ethidium Bromide was dissolved in 1ml of distilled water. The solution was stored in a dark bottle at 4˚C.

50-X TAE (pH 8.0) STOCK SOLUTION:

Tris base	242g

0.5M EDTA	100ml
Glacial acetic acid	57.1ml

Final volume was made 1000ml with distilled water. This is stock solution.

1-X TAE (pH 8.0) WORKING SOLUTION:

50-X TAE	20ml

Final volume was made 1000ml with distilled water.

Reagents for PCR:

Stock

Deoxyribose Nucleotide Triphosphate (dNTP) 100mM each dATP, dGTP, dCTP and dTTP.

Taq polymerase (5U/µl)

10X Taq buffer (16 mmol/L Ammonium sulphate; 67 mM/L Tris- HCL, pH 8.8; 10 mM/L 2-Mercaptoethanol); 6.7 mM/ L $MgCl_2$)

Primers: 100pM in sterile deionised water (Genescript)

100bp DNA ladder (0.5µg/µl)

50 bp DNA ladder(0. 5µg/µl)

Reagents for RFLP:

MILLI Q, NLA III (Restriction enzyme) and buffer.

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