ANALYSES OF MUTATION IN CELL CYCLE GENES ASSOCIATED WITH GASTRIC CANCER IN MIZO POPULATION

Thesis submitted in fulfilment of the

Requirements for the degree of

Master of Philosophy

in Biotechnology

by

RAVI PRAKASH YADAV

Registration No and Date: MZU/M. Phil. /229 of 22.05.2015

To

Department of Biotechnology, Mizoram University,

Aizawl, Mizoram 796004

India. 2015

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11 December 2015

Declaration of the Candidate

I, Ravi Prakash Yadav, hereby declare that the subject matter of this thesis entitled "**ANALYSES OF MUTATION IN CELL CYCLE GENES ASSOCIATED WITH GASTRIC CANCER IN MIZO POPULATION**", is record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University, for the award of the degree of Master of Philosophy in Biotechnology.

(Ravi Prakash Yadav)

(Head)

Department of Biotechnology

(Prof. N. Senthil kumar)

Supervisor

CERTIFICATE

This is to certify that the thesis entitled **"ANALYSES OF MUTATION IN CELL CYCLE GENES ASSOCIATED WITH GASTRIC CANCER IN MIZO POPULATION"** to the Mizoram University for the award of the degree of Master of Philosophy in Biotechnology by **Ravi Prakash Yadav** *Registration No.* ***MZU/M. Phil. /229 of 22.05.2015***, Research Scholar in the Department of Biotechnology, is a record of original research work, based on the results of the experiments and investigations carried out independently by him during the period from 2014-2015 of study under my guidance and supervision and has not been previously submitted for the award of any degree in any Indian or foreign University.

It is further certified that the scholar fulfils all the requirements as laid down by the university for the purpose of submission of M. Phil. thesis.

Date :

Place : Aizawl, Mizoram ***Supervisor name & signature***

Course work certificate

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Master of Philosophy First Semester Award-Sheet

The following is the assessment record of ***Ravi Prakash Yadav****, Roll No.* ***BT/M. Phil/14/01****, Registration No.* ***6741 of 2014*** *in the M. Phil. First Semester Examination held in December,* ***2014***

SEMESTER EXAMINATION								
LS-501 Research Methodology		LS-502 Instrumentation: Tools & Techniques		LSBT-503(A) Molecular Evolution and Phylogenetics		Total Grade Point	Grade Point Average	Grade
Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
8.2	'O'	7.4	'O'	6.9	'A'	22.5	7.5	'O'

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EVALUATION : *The following indicates the corresponding grades of grade points :*

'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

The performance of the scholar shall be evaluated in the following grades : 'O' – Outstanding, 'A' and 'B' with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to 'O' grade, 6.00-6.99 corresponding to 'A' grade, 5.00-5.99 corresponding to 'B' grade. Those securing less than 5.00 points shall be graded as 'C'. A scholar will be eligible for the award of M. Phil Degree if he/she secures at least a 'B' grade in each course.

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Acknowledgement

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Dated:
Place: Aizawl, Mizoram **Ravi Prakash Yadav**

Abbreviations

CI	Confidence Interval
CV	Coefficient of variation
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
FFPE	Formalin fixed paraffin embedded
GC	Gastric Cancer
HP	*Helicobacter pylori*
IARC	International Agency for Research on Cancer
Kb	Kilobases
MDM2	Mouse double minute 2
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*)
mM	Milimolar
MMR	Mismatch Repair
MSI	Microsatellite Instability
OR	Odds ratio
PBS	Phosphate buffered saline
P21	cyclin-dependent kinase inhibitor 1A
PCR	Polymerase chain reaction
PI	Propidium Iodide
SDS	Sodium dodecyl sulphate
SNPs	Single nucleotide polymorphisms
SPF	S-phase fraction
TAE	Tris-acetate-ethylenediamine tetracetate (Sodiun salt)
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris- borate- ethylenediamine tetracetate (Sodium salt)
TE	Tris- ethylenediamine tetracetate (Sodium salt)
Tm	Melting temperature
Tris	Tris (hydroxymethyl) aminomethane
U	Units
UTR	Untranslated region
µg	Microgram
µM	Micromolar
µl	Microlitre

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Introduction and Review of Literatures

Cancer

Cancer is a disease characterized by the uncontrolled growth and division of cells within the body, usually leading to the formation of a tumor. In many countries, cancer has been considered as the second leading cause of death after heart diseases (Xiaomei *et al.,* 2006).

Gastric Cancer

Gastric cancer is defined as any malignant neoplasm that arises from the region extending between the gastroesophageal junction and the pylorus. Approximately, 95 percent of stomach tumours originate in epithelial cells and are designated as adenocarcinomas. The progression from normal epithelial cells to tumor cells stepwise process involves the following stages: superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, and carcinoma (César *et al.,* 2002).

Molecular epidemiology of gastric cancer

Gastric cancer is the fifth most commonly diagnosed cancer. Despite marked decreases in incidence over the last century, particularly in developed countries, Gastric cancer (GC) is still the third leading cause of cancer death worldwide with highest estimated mortality rates in Eastern Asia (Ferlay *et al.,* 2015). More than 70% of cases (677,000 cases) occur in developing countries (456,000 in men, 221,000 in women), and half the world's total occurs in Eastern Asia (mainly in China) (GLOBOCAN 2012). Age-standardized incidence rates are about twice as high in men as in women, ranging from 3.3 in Western Africa to 35.4 in Eastern Asia for men, and from 2.6 in Western Africa to 13.8 in Eastern Asia for women. According to a survey by Dikshit *et al.,* 2012, it has been seen that 1,22,429 studies showing death due to cancer and stomach cancer is contributing in men (25,200 [12·6%]) and in women (27,500 [14·1%]).

Incidence of gastric cancer varies among countries and even in different regions of the same country (Torres *et al.,* 2013). India is a pluralistic, multilingual, and multi-ethnic society. Mizoram state has the highest rate of stomach cancer incidence in India (Rao *et al.,* 1998; Phukan *et al.,* 2004). The age-adjusted rates for only the Aizawl district of the Mizoram state are 55.4 and 24.4 per 10^5 populations in males and females, respectively (NCRP, 2013). Hospital-based data from

Mizoram have shown gastric cancer to be the most common cancer accounting for 30 % of all cancer cases.

The high prevalence of gastric cancer in Mizoram might be attributed to peculiar dietary habits, chronic *H. pylori* infection and heavy addiction to smoking and alcohol. In Mizoram, very few studies have been carried out so far to detect the genetic (Ihsan *et al.,* 2011; Malakar *et al.,* 2012) and environmental risk factors associated with stomach cancer.

Types of Gastric Cancer

Several classification systems have been proposed, but, the most commonly used are those of the WHO and Laurén (1965), which describes two main histological types, diffuse and intestinal. The intestinal (well differentiated) type is characterized by large, distinct cells with large, irregular nuclei and the cells form glandular tubular-like structures. The diffuse (undifferentiated) type is more infiltrative, lacks the characteristic tubular gland-like structures and consists of small single cells in a non-organised pattern (Laurén, 1965). The intestinal type is related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, whereas the diffuse type usually originates in pan-gastritis without atrophy (Correa, 1992). The intestinal type is more common in males, blacks, and older age groups, whereas the diffuse type has a more equal male-to-female ratio and is more frequent in younger individuals. The worldwide incidence of intestinal type is decreasing, whereas incidence of diffuse type gastric carcinoma, particularly the signet ring type has been increasing (Henson *et al.*, 2004).

Pathogenesis of Gastric cancer

Pathogenesis of gastric cancer involves multiple risk factors including dietary habits, infections, occupation, genetic and preneoplastic, most of which action the gastric mucosal microenvironment over a prolonged time period. A ‘precancerous cascade’ is formed as a resultant of sequential changes in the gastric mucosa that precede the development of invasive cancer (Correa, 1992), where normal gastric mucosa is transformed by chronic atrophic gastritis and develops multifocal atrophy and intestinal metaplasia, followed by the appearance of dysplasia and finally invasive carcinoma. Although gastric cancer is one of the most common malignancies worldwide, its pathogenesis and the molecular genetic events that contribute to its development are poorly understood.

Aetiology and Risk Factors of Gastric Cancer

Most gastric cancers have sporadic origin and their etiology is complex, combining dietary, environmental, infectious and metabolic risk factors. The heterogeneous geographic distribution of these cancers can be largely accounted for by variations in these factors and their combinations.

As a result of variety of exogenous and/or endogenous factors that induce genetic alterations, the above mentioned sequential changes occur in gastric mucosa over a period of many years. Some environmental risk factors such as cigarette smoking, *Helicobacter* infection, salt-preserved foods, dietary nitrite, and low intake of fruits and vegetables (Brenner *et al.,* 2009) were identified, and recent developments in molecular genetics showed that the accumulation of multiple genetic alterations, including alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules and growth factor/receptor systems (Brenner *et al.,* 2009). *H. pylori* was categorized by the World Health Organization (WHO) as a class I carcinogen due to its significant role in the pathogenesis of gastric adenocarcinoma (Kandulski *et al.,* 2008). With rapid progress in molecular biology, the number of polymorphic genes that modify the effects of identified or suspected carcinogens is increasing. There is evidence that genetic polymorphisms modulate the effects of environmental factors in the development of cancer (Gianfagna *et al.,* 2008).

Diet and its role in Gastric Cancer

In India, areas of high incidence are generally rural, mountainous areas with limited resources and poor dietary diversity. Foods and beverages containing carcinogenic chemicals such as N-nitroso compounds and aromatic amines play a crucial role in tumour growth. These compounds induce alkylation and cause DNA damage (Lijinsky, 1999). Additionally, low or poor ingestion of fruit and vegetables and a high intake of salty foods increase the risk of gastric carcinogenesis. The method of food preservation and storage plays an important role in cancer development. Food smoking, as a method of preservation and less refrigeration is an important contributing factor (Yalcin, 2009). Additionally, the use of salt when preserving food contributes towards gastric carcinogenesis by degrading the mucosal layer and causing inflammation in the stomach (Tsugane and Sasazuki, 2007). Numerous studies have reported the correlation between diet and gastric cancer (Ngoan *et al.,* 2002; Kelley and Duggan, 2003; De Stafani *et al.,* 2004). It was shown that pickled, salted and fatty foods significantly enhanced the risk of cancer development. In previous studies, it have shown that consumption of alcohol, tobacco and different food and lifestyle habits

are important risk factors for stomach cancer in addition to *H. pylori* infections (García-G *et al.*, 2012). A study from Mizoram reported higher rate of infection by *H. pylori* in stomach cancer patients. The significant interaction between *H. pylori* infection and smoked, salted meat (OR-1.9) and sa-um which is locally made dish (OR-2.1) was observed (Parkin, 2006). Mizoram is ethnically different from other parts of India showing different lifestyle and dietary habits, as the Mizo people consume many uncommon foods which includes smoke and sun dried salted meat and fish, soda (alkali), traditional fermented food etc. (Phukan *et al.,* 2005, 2006). N-Nitrosamines present in diet and tobacco are well-recognized carcinogens involved in cancer development and progression in various sites, including the oesophagus and stomach (IARC, 2000). Earlier, in many epidemiological studies from India a positive association of dietary items containing substantial amount of N-nitroso compounds with stomach cancer have been reported (Rao *et al.,* 2002; Phukan *et al.,* 2006; Sumathi *et al.,* 2009). A hospital based matched case control, study was conducted by Phukan *et al*. (2006) showed an elevated risk of stomach cancer in case of frequent consumption of sa-um and smoked dried salted meat and fish. Soda (alkali), used as a food additive, also increase the risk of stomach cancer. *H. pylori* infection was not found to be an independent risk factor for carcinogenesis of stomach cancer, however, when *H. pylori* infection interacted with consumption of sa-um or smoked dried meat, it showed a significant association in Mizo population.

Smoking and alcohol intake in gastric cancer

Consumption of a tobacco smoke–infused water "Tuibur" and smoked meat have been linked to the high rates of Stomach cancer in Mizoram. The toxic nature of tuibur was studied by In vitro studies (Mahanta *et al.,* 1998). Smoking as a variable risk factor for stomach cancer has also been reported from India (Dikshit *et al.*, 2012). A potential causal role of tobacco in causation of pre-cancerous lesions, in a high-risk area of China, where smoking was found to nearly double the risk of transition to gastric dysplasia (Piazuelo *et al.*, 2011). Another study carried out in the United States revealed that current smokers had 2.3 times increased risk of dying from stomach cancer compared with non-smokers (Piazuelo *et al.*, 2011). A hospital-based case-control study was carried out by Phukan *et al.* (2005) to identify the influence of tobacco use on the risk of developing stomach cancer in Mizoram. The risk of stomach cancer was significantly elevated among current smokers but not among ex-smokers. Mizo people are also having the habit of chewing betel quid, containing fresh betel nut, slaked lime wrapped in betel leaf. Chewing tobacco and areca nut both contain known carcinogens, most notably the nitrosamines, for example N'-nitrosonornicotine (NNN) and 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in chewing tobacco (IARC, 2000). Alcohol

intake and cigarette smoking have also been implicated in the development of stomach cancer. A direct correlation was observed between consumption of alcohol and tobacco and the risk of gastric cancer (Sjödahl *et al.,* 2007).

Genetic factors

The development of gastric cancer is a complex, multistep process that underlie the process of progression from pre-neoplastic lesions to cancer and metastatic disease involving multiple genetic and epigenetic alterations. Genetic instabilities namely; germline and somatic mutations, microsatellite instability and polymorphisms contribute to the risk of malignant formation. These changes include targeted mutations (single base substitutions, insertions, deletions) that either activate genes, rearrangements, translocations, loss of alleles and amplifications that collectively modify the structure and activity of a number of genes results in gain or loss of entire chromosomes, aneuploidy which results in gain or loss of entire chromosomes and massive, multi-site genomic rearrangements, etc.

There is increasing interest in genetic polymorphisms and mutation studies as causes for gastric cancer, probably due to advances in DNA-analysis technologies (El-Omar *et al.,* 2004). Individual variations in cancer risk have been associated with specific genes polymorphisms and mutations. Polymorphisms and mutations in a wide variety of genes may modify the effect of environment (González *et al.,* 2002); these gene-environmental interactions may explain the high variation in the incidence of gastric cancer around the world (Ames *et al.,* 1993). Abnormalities in cell-cycle regulators are also involved in the development and progression of gastric cancers through unbridled cell proliferation (Yasui *et al.,* 2001). Specifically, genetic alterations and abnormal expression of various cyclins and cyclin-dependent kinases (CDKs), as well as CDK inhibitors, play a role in gastric cancer pathogenesis. There are three main classes of genes involved in cancer development: oncogenes, tumor suppressor genes and stability genes.

The majority of human malignancies develop due to genetic instabilities (Langauer *et al.,* 1998). These genetic instabilities involve DNA damage and repair. DNA repair is a mechanism that corrects errors such as repeat sequences, mismatches and strand breaks which may occur during DNA replication (Madhusudan and Middleton, 2005). The two important repair mechanisms are the nucleotide excision (NER) and (MMR) mismatch repair (Langauer *et al.,* 1998). When DNA repair genes accumulate mutations it gives rise to the development of cancer through this mechanism (Hoeijmakers, 2001).

p21 gene

Maintaining genomic integrity is a fundamental goal of the cell. Therefore, precise checkpoints exist throughout the cell cycle to ensure DNA is replicated without mutations and abnormal cells cannot proliferate. p21 is a major regulator of the G1/S checkpoint, preventing inappropriate DNA replication in S phase. In response to various stimuli (oxidative stress, DNA damaging agents), p21 can become activated.

p21 has been reported to have multiple roles within the cell including cell cycle regulation, senescence, apoptosis, DNA repair and differentiation (Decesse *et al.,* 2001). The p21 (WAF1/CIP1) gene consists of three exons of 68, 450 and 1600 bp and encodes a 21 kDa protein of 164 amino acids.

Mutations and deletions of the p21 gene have been rare in human cancers suggesting that p21, if involved in tumorigenesis, may be exerting itself mainly on the expression level rather than on the gene level (Bahl *et al.,* 2000). However, p21 polymorphisms have been observed in various cancers. The polymorphic variants have been reported to occur more frequently in cancer patients than in healthy individuals suggesting a role in increased susceptibility to the development of some types of cancers (Mousses *et al.,* 1995). p21 induction, regulated through p53 -dependent and -independent pathways, is essential for the onset of cell cycle arrest in DNA damage response and cell senescence (Gartel *et al.,* 1999). Previous studies have shown dual role for p21 as both a tumor suppressor and an oncogene (Roninson, 2002; Gartel, 2009).

According to Liu *et al*. (2001) in gastric adenocarcinoma, p21 (WAF1/CIP1) (18) was expressed in 40% of the patients, and its expression was considered a positive prognostic factor (Liu *et al.,* 2001). Different SNPs such as the p21 exon 2 codon 31 polymorphism, which produces variant proteins with serine (AGC) or arginine (AGA), may also affect gene expression. Aberrant expression of p21 has rarely been evaluated in gastric cancer formation. Both p21 and p53 gene functions are closely associated with apoptosis, but resistance to apoptosis in gastric cancer cells with elevated p21 levels is independent of p53 status, and an alternative upstream haem oxygenase-1 (OH-1) pathway had been proposed. Different SNPs such as the p21 exon 2 codon 31 polymorphism, which produces variant proteins with serine (AGC) or arginine (AGA), may also affect gene expression. Studies indicate that the upregulation and over expression of p21 typically leads to cell cycle arrest, and that the protein product of p21 is a crucial governing mechanism for regulation a cell's entry into the cell cycle (Cheng *et al.,* 2000). p21 is also important in its

regulation of the overall quality of DNA and has been thought in some cases to prevent accumulation of genetic errors in DNA (Viale *et al.,* 2009).

p21, also called cyclin-dependent kinase inhibitor 1A (CDKN1A, Cip1, Waf1; gene location 6p21.2) is a transcriptional target of p53 by which it is activated (el-Deiry *et al.,* 1993). However, there are also p53 independent pathways of p21 induction (Elbendary *et al.,* 1994). p21 inhibits the phosphorylation of cyclin-cdk2 or cyclin-cdk4 complexes and induces cell cycle arrest at the G1/S checkpoint (Sheikh *et al.,* 1995). Evidence is increasing that p21 is also a major inhibitor of p53-induced as well as p53-independent apoptosis, but why this is the case is unknown (Gartel and Tyner, 2002). p21 protein is produced in normal gastric mucosa and is lost in gastric cancer (Xie *et al.,* 2004). There are less known mutations of the p21 gene (Park *et al.,* 1998). The role of p21 for survival prediction is still unclear (Tsihlias *et al.,* 1999). In gastric cancer, patients that lack p21 expression in the tumour may have a favourable prognosis (Okuyama *et al.,* 2002) but, again, this has not been the case in all studies (Al-Moundhri *et al.,* 2005). Although mutations in p21 are infrequent in human cancers (Tsumanuma *et al.,* 1997), a number of studies have shown that p21 may act to either promote or suppress certain cancer types.

mdm2 gene

Mouse double minute-2 (mdm2) is a negative regulator of tumour suppressor p53. mdm2 is one of the central nodes in the p53 pathway and can control p53 protein levels and activity. The mdm2 gene is composed of 2 known promoters and 12 exons. mdm2 is also an E3 ubiquitin ligase and promotes proteasome-mediated p53 degradation, maintaining low basal levels of p53. Overexpression of mdm2 has also been correlated with the cyclin-dependent kinase inhibitor p21. In breast cancer cells, overexpression of mdm2 correlates with lack of p21 expression. p53 gets activated in response to cellular stress, act as a principal mediator to initiate multiple cellular functions such as DNA repair, cell cycle arrest and apoptosis (Miller *et al.,* 2005). Most of cancers had exhibited inactivation or attenuation of p53 gene expression either due to mutations or abnormal expression of p53 regulators, such as mdm2 (Michael and Oren 2002). mdm2 not only inhibits the transcriptional activity of p53 gene but also acts like E3 ubiquitin ligase to stimulate the nuclear export and degradation of p53 protein (Dingding and Wei *et al.,* 2012). A study conducted on colorectal cancers in association with mdm2-309 T>G polymorphism and mdm2 gene amplification had revealed that 9% of 284 colorectal cancer had amplified mdm2.There was no correlation observed between -309SNP and gene amplification (Oda *et al.,* 2000). It was reported

that in a small dataset, the mdm2 gene was frequently amplified, and that this gene is associated with several cancers (Marchetti *et al.,* 1995).

mlh1 Gene

DNA repair plays a critical role in protecting the genome of the cell from the insults of cancer causing agents. Genetic polymorphisms in DNA repair genes may influence individual variation in DNA repair capacity, which may be associated with the risk of developing cancer. An inability to identify and repair the "mismatched DNA" makes it more likely that mutations will accumulate and set the stage for the development of cancer (Burt and Neklason, 2005; Kaz and Brentnall, 2006; Borelli *et al.,* 2012). mlh1 gene normally function as mismatch repair genes, with the role of maintaining the microsatellite stability, because of their role in identifying and excising single-base mismatches and insertion-deletion loops that may arise during DNA replication. mlh1 is located at chromosome 3p21 and contains 19 exons that span over 58 kilobases, with the mlh1 protein containing 756 amino acids. mlh1 has also been found to be epigenetically silenced in cases of HNPCC (Gazzoli *et al.,* 2002).

Cell cycle

The cell cycle is a ubiquitous, complex process involved in the growth and proliferation of cells, organism development, regulation of DNA damage repair, tissue hyperplasia as a response to injury, and diseases such as cancer. The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and the production of two daughter cells (Schafer, 1998). Not surprisingly, defects in cell cycle regulation are a common cause of the abnormal proliferation of cancer cells, so studies of the cell cycle and cancer have become closely interconnected, similar to the relationship between studies of cancer and the cell signalling pathways (Cooper and Hausman, 2006).

The actively dividing cancer cells also express large and chromatin-rich nuclei as a sign of an aberrant amount of chromosomes, which can be assessed by DNA flow cytometry. DNA aneuploidy is associated with the carcinogenesis and prognosis of gastric cancer.

Healthy human cells have 23 pairs of chromosomes, i.e., a total of 46, and are called diploid. A disturbed number of chromosomes or chromosome sets is called aneuploidy. Aneuploidy is a good marker of a malignant cell population and an abnormal amount of DNA in solid tumours is a specific sign of neoplasia (Barlogie *et al.,* 1983). Several studies have reported that aneuploidy is a sign of a poor prognosis in gastric cancer (Michels *et al.,* 2004). The S-phase fraction (SPF) is a

measure of the percentage of cells in the DNA synthesis phase of the cell cycle. A high SPF is apparently a sign of poor prognosis of gastric cancer patients (Russo *et al.,* 2001).

Although gastric cancer is one of the most common malignancies worldwide, its pathogenesis and the molecular genetic events that contribute to its development are poorly understood. In Mizoram a very few studies have been carried out so far to detect the genetic (Ihsan *et al.,* 2011; Malakar *et al.,* 2012) and environmental risk factors associated with stomach cancer. A case-control study for the high prevalence of gastric cancer in Mizoram has been attempted in order to identify the mutations in cell cycle genes p21 and mdm2 and DNA damage repair gene mlh1 and to identify the correlation of these mutations with environmental and dietary factors that seem to play a role in gastric cancer aetiology.

Aim and Objectives

The primary aim of this study was to determine the prevalence of p21, mdm2, mlh1 gene mutations in gastric cancer patients seen in the Mizo population and to correlate these findings with the demographic/clinicopathological data. The mutational data was obtained using PCR and sequencing techniques.

The objectives of this study was to:

- To study the Somatic mutations and Germ line in p21, mdm2, mlh1 genes in gastric cancer patients from Mizoram.

- To study the correlation between gene mutations and cell cycle progression.

Materials & Methods

Ethical statement

Ethical approval for this study was obtained from the Institutional Review Board (IRB) of the Civil hospital [B.12018/1/13-CH(A)/IEC] and Mizoram University, Aizawl, India. All volunteers were fully informed about the study and participated with their full consent. The ethical approval letter is attached in Appendix 1.

Study Population

This study included a cohort of 40 pathologically confirmed cases of gastric cancer. Gastric cancer samples were collected from Mizoram State Cancer Research Institute, Zemabawk, Civil Hospital and Genesis Diagnostic Centre, Aizawl, Mizoram. All blood and tissue with adjacent normal samples for this study were taken from gastric cancer patients. Fourty Blood samples of healthy persons (age, gender, dwelling and smoking matched) with no signs of any malignancy or any other disease were collected as controls. The information on demographic features such as age, gender, dietary habits, familial incidence of cancer, addiction to smoking and alcohol were collected by in-person interview using a structured questionnaire.

Sample collection/storage

1 ml of peripheral blood was obtained from each subject in sterile 1.5 ml microfuge tubes containing 10 µl of 0.5 M EDTA (pH 8.0) as an anticoagulant and stored at -20°C till use. Approximately 500 mg of surgically resected tumour tissue and adjacent normal tissue at a distance of 5 cm from the tumour site were collected directly into sterile vials containing chilled PBS (pH 7.4)/Formalin embedded frozen tissues and frozen at -80°C for molecular investigations. Histopathological report of the collected tumour tissues was obtained from the Mizoram State Cancer Research Institute, Civil Hospital and Genesis Diagnostic Centre. Histopathologicaly confirmed gastric cancer tissues and corresponding normal tissues were used for the study.

Extraction and Quantitation of genomic DNA

Genomic DNA extraction by FFPE (formalin fixed paraffin embedded) samples

Genomic DNA was extracted by FFPE tissues following the modified protocol of salting out method (Ghatak *et al*., 2014). Deparaffinization was carried out by adding 1 ml of xylene to the tissue section in each microfuge tube followed by vigorous vortexing for 10 min. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the deparaffinization steps were repeated once again, followed by rehydration through subsequent washings with 100%, 90%, and 70% absolute ethanol diluted in Nuclease free DEPC treated water, respectively. The remaining tissues were collected after centrifugation at 12,000 rpm for 5 min after each step. After a 70% ethanol wash, the sample was suspended in 10 ml glycine-Tris ethylenediamine tetra-acetic acid buffer (100 mM glycine, 10 mM Tris-HCl - pH 8.0, 1 mM EDTA) and incubated at 55ºC in a shaking water bath for 30 min with 20 µl of proteinase K (10 mg/ml) and DTT (2 mM). The homogenate was washed with 1X PBS Buffer, followed by resuspension in 600 µl of cell lysis buffer (500 mM Tris pH 9.0; 20 mM EDTA; 100 mM NaCl) with 10 µl of Proteinase K (10 mg/ml) and 20 µl of 100 mM dithiothritol (DTT) followed by incubation at 56ºC for 1 h. The sample was soaked in TE9 buffer (500 mM Tris pH 9.0; 20 mM EDTA; 100 mM NaCl) at 37°C for 2 h, with single buffer change. Tissues were then minced thoroughly and 1 ml lysis buffer plus [50 µl 20% SDS, 0.5% TritonX 100, 15 µl dithiothreitol (8 mg/ml)], and 10 µl proteinase K (10 mg/ml) were added and incubated at 55°C in a water bath for 1 h. Further, 20 µl each of SDS and proteinase K were added and incubated for 1 h at 65°C in a water bath. Centrifugation was performed at 10,000 rpm for 15 min, the supernatant was taken in a separate 2 ml tube and DNA was extracted twice with phenol, chloroform, Isoamyl alcohol (25:24:1), and once with chloroform washing. DNA was precipitated by adding double the amount of ice cold isopropyl alcohol and 3 M sodium acetate (1/10 of total volume) to the supernatant followed by 2 h precipitation at −20°C. The solution was centrifuged at 10,000 rpm for 10 min at 4°C to pellet out the DNA which was subsequently washed with freshly prepared 70% ethanol, air dried, resuspended in 80 µl 1X TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) and frozen at 4°C or -20°C for storage.

Genomic DNA extraction by Blood

Genomic DNA was isolated form the blood following the standard protocol (Ghatak *et al*., 2013). Lymphocytes from whole blood were separated by lysing the red blood cells (RBCs) using a hypotonic buffer (ammonium bicarbonate and ammonium chloride; Himedia) with minimal lysing effect on lymphocytes. Three volumes of RBC lysis buffer was added to blood sample and mixed by vortexing and inverting thoroughly for 5 min and centrifuged (Eppendorf 5415R) at 20,000 g for 10 min. The supernatant was mostly discarded, leaving behind 1 ml to prevent loss of cells. To the pellet, 3 vol. RBC lysis buffer was added, and vortexing, inverting, and centrifuging steps were repeated two to three times until a clear supernatant and a clean white pellet were obtained. After the final wash, the supernatant was discarded completely, and the pellet was resuspended in 500 µl PBS, followed by addition of 400 µl cell lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5) and 10 µl proteinase K (10 mg/ml stock; Himedia). The sample was vortexed to dissolve the pellet completely and incubated for 2h at 56°C in a water bath (CW-30G; Jeio Tech) for lysis. An equal volume of phenol (equilibrated with Tris, pH 8) was subsequently added to the tube and mixed well by inverting for 1 min. The tube was centrifuged at 10,000 g (at 4°C) for 10 min, and the aqueous upper layer was transferred to a fresh tube containing equal volumes (1:1) of phenol and chloroform: isoamyl alcohol (24:1). The tube was mixed by inverting for 1 min and centrifuged for 10 min at 10,000 g (at 4°C). The supernatant was then transferred to a fresh tube, and 10 µl of 10 mg/ml RNase A (Fermentas, Thermo Scientific) was added. The sample was incubated at 37°C for 30 min before an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting the tube for 1 min and centrifuging at 10,000 g (at 4°C) for 10 min. The supernatant was transferred to a fresh tube, and twice the volume of absolute alcohol (Merck) was added and inverted gently a few times and chilled at -20°C, followed by centrifugation at 10,000 g at 4°C for 20 min. The supernatant was discarded, 250 µl 70% ethanol was added, and the pellet was tapped gently, followed by centrifugation at 10,000 rpm for 10 min and decanting the supernatant gently. The pellet was air-dried in a laminar air flow, and the dried pellet was resuspended in 50 µl nuclease-free water or 1 X TE buffer and frozen at 4°C or -20°C for storage.

Quantitation of DNA

Agarose Gel Electrophoresis

The quality of the DNA obtained from the tissue specimens and blood samples was analyzed on 0.7% (w/v) ultrapure agarose gel for DNA quality checking. A 3 µl DNA mix (3 µl DNA, 3 µl D.W

and 1 µl loading dye) was loaded directly into the wells of the ultrapure agarose gel. The DNA was electrophoresed for 30 minutes at 80 voltage and visualized using a Gel Documentation system (G-Box; Syngene, UK).

Spectrophotometric Analysis

The DNA concentration in each of the 86 samples was determined using Qubit® 3.0 Fluorometer.

Polymerase Chain Reaction (PCR)

PCR is a technique that allows for the amplification of a specific segment of DNA. The following sections will describe the methods used for PCR amplification in this study. The primers used in this study were designed using Primer3 software and Oligoanalyzer. The Oligoanalyzer software was utilized to analyse primer properties such as hairpins, self and cross dimers. These are primer secondary structures produced as a result of self-complementarity within the primers. Since these secondary structures may interfere with amplification, they should be avoided during primer design. The primers were checked for specificity of binding using the UCSC in Silico PCR tool at the UCSC Genome browser. The primers were designed to include portion of the intron/exon boundaries. The sequence and PCR conditions for the primers used in this study are given in Table 1.1. The newly synthesized PCR primers ("oligos") were provided dried down in lyophilized form. A 100 mM stock solution of each primer was prepared by adding the appropriate amount of sterile ddH_2O according to the manufacturer's instruction sheet. Then, a 10 mM working solution of each primer was prepared by adding 10 µl from 100 mM stock solution and bringing it up to 100 µl with sterile ddH_2O. Finally, the stock solutions were stored at -80°C while the working solutions were stored at -20°C.

PCR conditions for DNA amplifications

PCR was carried out in 25 µl total reaction volume [containing 100 ng template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 1 unit Taq DNA polymerase (MBI Fermentas, Hanover, MD)]. The PCR program on the thermal cycler (Vapo.protect; Eppendorf) was as follows: an initial denaturation step at 94 ºC for 5 min, followed by 40 cycles, each consisting of 1 min denaturation at 94ºC, 1 min annealing at different exons of different genes (Table 1.1), 1 min extension at 72ºC, and a final 7 min extension at 72ºC.

Agarose Gel Electrophoresis of PCR Products

The PCR amplification products (10 µl) were subjected to electrophoresis (Bio-Rad) on 1.2% agarose gel in 1X Tris-acetate-EDTA buffer at 80 V for 30 min and stained with ethidium bromide (Himedia) and images were obtained in gel documentation (G-Box; Syngene, UK) system. A 1KB+ DNA ladder (Invitrogen, USA) was used to size the bands in the gel.

Table 1.1 Sequence of the primers used for exon amplification of the p21, mdm2 and mlh1 gene.

Gene Name	Exon	Primer (5' -3')	Annealing Temp. (°C)	Expected Product size (bp)
p21	2	5'- GTTGACATTAGCTTGCCCTTC -3' 5'- TCTGAGAATCCTGGTCCCTTAC -3'	59	741
	3	5'-GGGTGCGGTGATGGATAAA-3' 5'-CGGGATGAGGAGGCTTTAAATA-3'	60	541
mdm2	2	5'-TCGGAAAGATGGAGCAAGAAG-3' 5'-CGTGACCTTTACCCTGAACTC-3'	58	537
	8	5'-GGAAACAGATACAGAGGTCAAGAG-3' 5'-CTATGAAATCCTCAAGTCCACAAAC-3'	60	382
mlh1	8	5'-AGTTTGCTGGTGGAGATAAGG-3' 5'-ACAAGCCTGTGTATTTGAC-3'	56	307
	16	5'-CTTCACCCTTCCCATCCTTTC-3' 5'-CTCCTGAGCAGCTTGGATTAC-3'	59	643

Sequencing of PCR Products

For the characterization of DNA mutations for the investigated genes, direct sequencing was performed on the relevant DNA fragments. Direct sequencing of the DNA fragments containing DNA mutations was performed using the same primer sets used for PCR amplification. All PCR products were sequenced from the opposite direction to ensure reading accuracy. Sanger sequencing was performed at SciGenom Labs Private Ltd., Cochin, India.

Mutation Analysis of Sequenced PCR Products

After sequencing, data analysis was undertaken to determine if there were any variants present. The sequencing data were analyzed using FinchTV software. Sequences and chromatograms obtained were examined using chromas software version 2.13 and aligned by BLAST (http://www.ncbi.nlm.nih.gov/blast). The DNA sequences were compared with the published databases like Genecard, Ensembl (p21- HGNC: 1784 Entrez Gene: 1026 Ensembl: ENSG00000124762 OMIM: 116899 UniProtKB: P38936, mdm2- HGNC: 6973 Entrez Gene: 4193 Ensembl: ENSG00000135679 OMIM: 164785 UniProtKB: Q00987, mlh1- HGNC: 7127 Entrez Gene: 4292 Ensembl: ENSG00000076242 OMIM: 120436 UniProtKB: P40692).

All the sequences containing the mutation were evaluated for their potential pathogenicity using the following algorithms: DNA baser version 3.5.4.2, Codon Code aligner version V.4.2.2, Mutation taster [www.mutationtaster.org/], PolyPhen-2 [http://genetics.bwh.harvard.edu/pph], SIFT [http://sift.jcvi.org], Mutation Assessor [http://mutationassessor.org/], SNPs3D [http://www.snps3d.org/], pMUT [http://mmb2.pcb.ub.es:8080/PMut/], Condel [http://bg.upf.edu/condel/analysis]. The MEGA Align algorithm were used at two depths of alignment [Cancer to Normal and Normal to database sequences]. The effect of mutation on splice sites were identified by using tools human splicing finder (http://www.umd.be/HSF/), ESE finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home). To find the protein stability and the scores for free energy alterations for changing the single site mutation, the sequences were submitted to Project Have yOur Protein Explained (HOPE; http://www.cmbi.ru.nl/hope/home) for prediction of structural variation between wild and mutant type amino acids, which provides

the 3D structural visualization of desire proteins and the results by using UniProt servers (Venselaar *et al.,* 2010).

Multifactor dimensionality reduction analysis

The multifactor dimensionality reduction (MDR) is a nonparametric, genetic model-free method and was applied to identify high-order gene-gene and gene-environment interactions associated with gastric cancer risk. The test is used to overcome the limitations encountered by parametric methods like LR due to small sample size. In the present study, MDR software package (MDR 3.0.2) was used to generate a best one-dimensional multifactor model to classify and predict GC susceptibility. The best model was selected based on maximum cross-validation consistency (CVC) and testing balance accuracy (TBA). The MDR permutation results were considered to be statistically significant at the 0.05 level.

Interaction entropy graphs

Interaction graphs were built to visualize and interpret the results obtained from MDR. Entropy estimates were used to determine the information gain about a class variable (e.g., case-control status) from merging two variables together. Entropy estimates are useful for building interaction graphs facilitating the interpretation of relationships between variables.

Flow Cytometric Analysis

DNA content analysis was done by flow cytometer following a modified method (Victorzon *et al.,* 1996) of Hedley *et al*. (1983).

Isolation of nuclei

The DNA content of tumour cells was measured by flow cytometry using FFPE tumour tissue. Following pathology review (to choose the block with the highest proportion of tumour cells), a single 30-µm-thick section was cut. Sections were de-waxed in xylene and rehydrated in graded alcohols. The sections were washed in distilled water (twice) and then digested at 37 °C in 1 ml of 1.5% pepsin (p-7000; Sigma Aldrich, Gillingham, UK) in 0.9% NaCl (pH 1.5) for 2 h with intermittent vortex mixing. Following digestion, the sections were vigorously vortexed for 60 s to release the nuclei. The suspended nuclei were counted using Automatic cell counter TC20 (BioRad) to ensure that there were more than 10^6 cells/ml nuclei present. The tissues were then passed through a 70-lm nylon filter (Becton Dickinson)

and centrifuged at 239 g for 5 min. The supernatant was removed and the pellet was re-suspended and washed twice in PBA (0.1% BSA in PBS) to remove residual pepsin. The cell pellet then was re-suspended in the staining solution in the flow cytometer tubes.

Prior to analysis by flow cytometry, the suspended nuclei were stained by incubation for 30 min with propidium iodide and RNAs (both sigma) at a respective final concentration of 1 and 10 mg/ml in PBA. Flow cytometry was performed on a BD FACSCalibur, USA flow cytometer using performed against PE colour beads Area (PE-A) VS Width (PE-W).

Data analysis was performed using the ModFit LT software (DNA Modeling System) version 2.0 (Verity Software House, Inc.). Classification of DNA histograms was done according to the recommendations of the International Society of Analytical Cytology without knowledge of the clinical outcome (Hiddeman *et al.,* 1984; Shankey *et al.,* 1993). The upper limit for the coefficient of variation (CV) of the internal control (diploid peak) was 8.0%. Tumors with more than one peak were considered DNA aneuploid. The SPF was calculated using a rectangular model (Wingren *et al.,* 1988).

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Ibe´rica, Madrid and Spain) and SYSTAT 13.0 (Systat Software Inc., USA). Observed frequencies of mutations in gastric cancer patients were compared to controls using chi-square when expected frequencies were small. The chi-square test was used to verify whether mutation distributions were in Hardy-Weinberg equilibrium. Odds ratio at 95% confidence intervals (95% CI) and P values were computed by Binary Logistic regression. The independent sample Student's t-test was applied to check association between cases and controls. The differences were considered statistically significant when P values were <0.05.

Results and Discussion

It is widely accepted that genetic and environmental factors are major etiologic factors for gastric cancer. Among the genetic factors, cell cycle regulatory genes such as p21 and mdm2 and DNA mismatch repair gene (mlh1) were found associated with various cancers including gastric cancer, could play a role in the development of gastric cancer for this population (Gratel *et al.*, 1999). Cell cycle control is crucial for normal cell growth and differentiation. In the present study, we investigated the spectrum of mutations in genes p21, mdm2 and mlh1 among gastric cancer patients from a high risk area- Mizoram. The study also attempted to establish a potential association of these gene variations with demographic and dietary factors. In this study, we investigated the role of cell cycle regulatory and MMR gene mutations in diffuse type gastric cancer. To the best of our knowledge, the present study results are the first report from Mizoram population suggesting the implication of genetic alterations and its correlation with cell cycle in gastric cancer development.

Demographic and clinicopathological characteristics of study participants

A total of 40 gastric cancer cases were enrolled in the study. All the GC samples were of diffused type adenocarcinoma according to the Lauren classification system. Our study consisted of 12 male patients and 28 female patients. The median age was 58.7 ± 9.76 for the patients and 52.18 ± 12.35 for the controls. The frequency distributions of selected characteristics of the patients and controls are presented in Table 1.2. The results indicated that smoked meat /vegetable [16.214 (2.746 – 95.749); P=0.002] and oral snuff [10.496 (2.410 - 45.710); P=0.002] are significant factors for high risk of GC.

Previous studies have shown that the aetiology of gastric adenocarcinoma has also been shown to be associated with other environmental and genetic factors (Crew and Neugut, 2006). The high prevalence of gastric cancer in Mizoram has been attributed to peculiar dietary habits (viz. high consumption of smoked meat, salt-preserved foods, dietary nitrite, traditional fermented food and heavy addiction to various tobacco products and alcohol (Phukan *et al.,* 2005, 2006).

Earlier, in many epidemiological studies from India a positive association of dietary items containing substantial amount of N-nitroso compounds with stomach cancer have been reported (Rao *et al.,* 2002; Phukan *et al.*, 2006; Sumathi *et al.*, 2009). Polycyclic aromatic hydrocarbons such as benzo[a] pyrene formed in smoked food have been correlated in many areas of the world with high stomach cancer rates (Jedrychowski, 2003; Wogan *et al.,* 2004). A hospital based matched case control, study was conducted by Phukan *et al.* (2006) showed an elevated risk of stomach cancer in case of frequent consumption of sa-um and smoked dried salted meat and fish. Soda, an alkaline preparation frequently used as food additives was significantly associated with increased risk of stomach cancer in Mizoram (Phukan *et al.,* 2006). Smoking as a variable risk factor for stomach cancer has also been reported from India (Dikshit *et al.*, 2011). A potential causal role of tobacco in causation of pre-cancerous lesions, in a high-risk area of China, where smoking was found to nearly double the risk of transition to gastric dysplasia (Piazuelo *et al.*, 2010). A hospital-based case-control study was carried out by Phukan *et al.* (2005) to identify the influence of tobacco use on the risk of developing stomach cancer in Mizoram. A direct correlation was observed between consumption of alcohol and tobacco and the risk of gastric cancer (Sjödahl *et al.,* 2007). A study from Mizoram reported higher rate of infection by *H. pylori* in stomach cancer patients. The significant interaction between *H. pylori* infection and smoked, salted meat and sa-um which is locally made dish was observed (Parkin, 2006). The association between the risk of gastric cancer and unique dietary has been controversial for decades. However, the reason for disparities is still not yet known, although recent research has suggested that genetic factors may be the reason for differences in gastric cancer susceptibility of different populations (Yan *et al.*, 2015). Also family history of other cancers are found to be associated with the increased risk of gastric cancer in this population which may be because of their genetic make-up and inheritance of faulty genes, which makes them predisposed to cancer (Yaghoobi *et al.*, 2010). The Mizo population is of mongoloid origin and are different from the rest of India in terms of their diet, lifestyle, and geographical distribution (Ghatak *et al.*, 2014). Also in other mongoloids like Japanese, consumption of meat daily among women was found to increase the risk of gastric cancer by 6.5 fold (Santarelli *et al.*, 2008). In another study a potential causal role of tobacco was observed in high-risk area of China, where smoking was found to nearly double the risk of transition to gastric dysplasia (Piazuelo *et al.*, 2013).

Table 1.2 Demographic and clinical characteristics of the samples.

Demographic factor	[a]HC (n=40)	[b]GC (n =40)	[c]ORs (95% CI)[d]	P value
Age years ± SD (range)	52.18 ± 12.35	58.7 ± 9.76	-	
Sex Male	12 (30%)	28 (70%)	-	
Female	28 (70%)	12 (30%)		
Saum	29 (72.5%)	37 (92.5%)	0.979 (0.346 - 2.770)	0.968
High Salt Intake	30 (75)	31 (77.5%)	0.507 (0.077 - 3.340)	0.480
Smoked Meat/Vegetable	**25 (62.5%)**	**38 (95%)**	**16.214 (2.746 – 95.749)**	**0.002**
Pickle	22 (55%)	23 (57.5%)	0.340 (0.108 - 1.072)	0.065
Tuibur consumption	14 (35%)	17 (42.5%)	0.755 (0.350 - 1.631)	0.475
Cigarette smoking	24 (60%)	29 (72.5%)	2.091 (0.810 - 5.400)	0.127
Oral Snuff	**6 (15%)**	**20 (50%)**	**10.496 (2.410 - 45.710)**	**0.002**
Tiranga/Gutkha	8 (20%)	4 (10%)	8.954 (0.816 - 98.308)	0.073
Kuhva	15 (37.5%)	17 (42.5%)	1.094 (0.394 - 3.036)	0.864
Family history of gastric cancer	5 (12.5%)	7 (17.5%)	2.148 (0.579 - 7.970)	0.253
Family history of other cancers	5 (12.5%)	3 (7.5%)	1.011 (0.369 - 2.769)	0.983

[a] HC – Healthy Control
[b] GC – Gastric Cancer
[c] OR – Odd ratio
[d] 95% CI – 95 % Confidence Interval

Quantitative and Qualitative Evaluation of DNA Isolated From Subjects

The DNA, which was checked on 0.7 % ultrapure agarose gel stained with ethidium bromide, was found to be good in quality. No smears under the DNA bands appeared indicating that the DNA is not gradated. The wells of the gel are clear, indicating minimum protein contamination (Fig. 1.1).

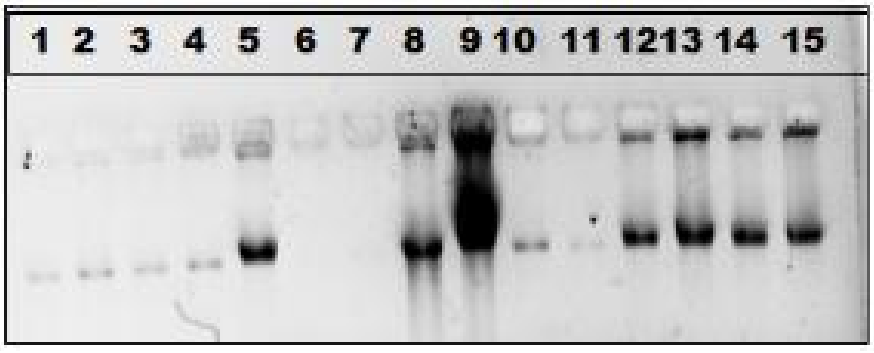

Figure 1.1: DNA Checking on 0.8 % gel for samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15.

The concentration of the DNA in the 80 samples was kind of good and enough for the subsequent PCR reactions. The DNA concentration in the samples (GC as well as Normal samples) ranges from 5.92 - 138.46 ng/ul (The mean of the DNA concentration is 37.68 ng/ul). The 260/280 ratios were in most of the DNA samples above 1.7, some samples had a ratio of 1.3 but it didn't interfere with PCR reactions.

PCR and Gel Electrophoresis

As described in previous sections, PCR was carried out for 80 samples with different pairs of primers to amplify selected exons and exon-intron boundaries were investigated (Fig. 1.2, 1.3, 1.4).

The following figures are gel electrophoresis pictures for PCR reactions of some samples.

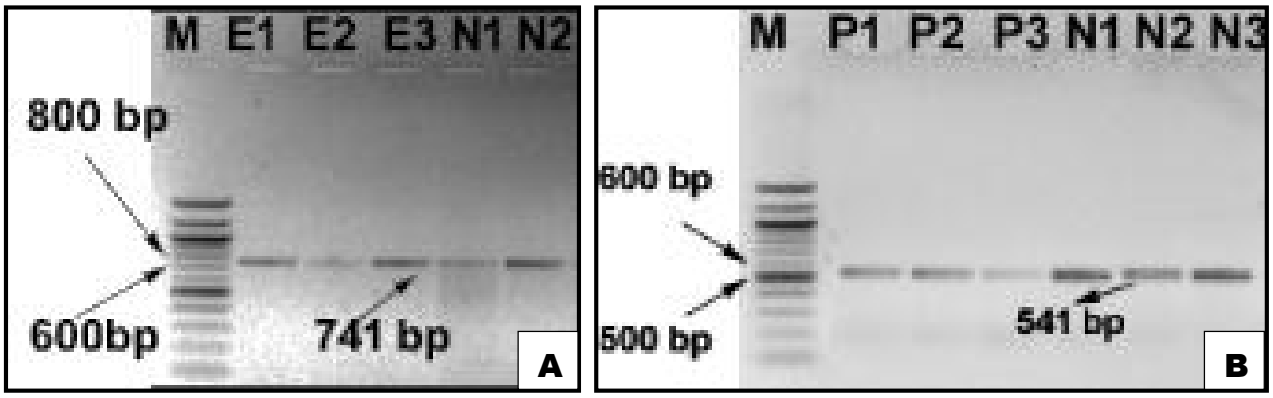

Figure 1.2: Gel electrophoresis image of the PCR products of mdm2 gene A.) Exon 2 B.) Exon 8. Lanes M: 100 bp DNA ladder (Invitrogen, USA), D1, D2, D3, D4, D5 and T1, T2, T3 = GC samples, N1, N2 = controls.

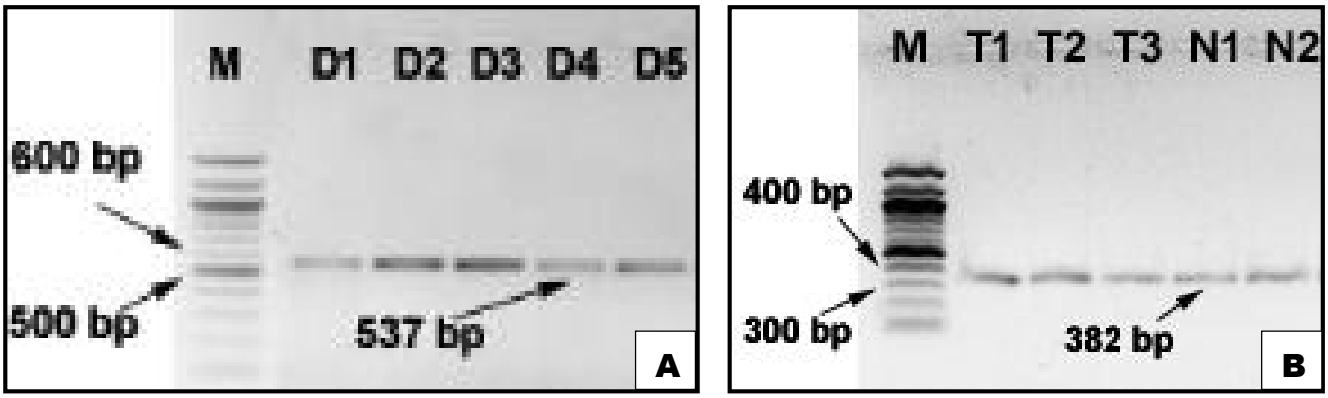

Figure 1.3: Gel electrophoresis image of the PCR products of p21gene A.) Exon 2 B.) Exon 3. Lanes M: 100 bp DNA ladder (Invitrogen, USA), E1, E2, E3 and P1, P2, P3 = GC samples, N1, N2, N3 = controls.

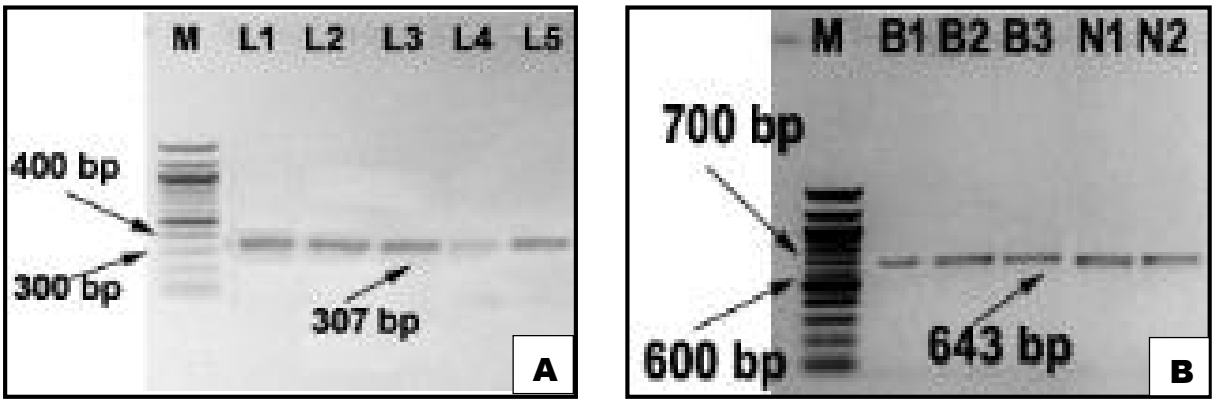

Figure 1.4: Gel electrophoresis image of the PCR products of mlh1 gene A.) Exon 8 B.) Exon 16. Lanes M: 100 bp DNA ladder (Invitrogen, USA), L1, L2, L3, L4, L5 and B1, B2, B3 = GC samples, N1, N2 = controls.

DNA Sequencing of the PCR amplified fragments

Tumor Frequencies and Distribution

p21 Gene Mutation

The molecular analysis revealed total 5 mutations in P21 gene (Table 1.3). In the intron 2 36651738G>A, 36651804A>T, 36651825G>T were identified in 5% of total GC samples. All the mutations were found to be novel (not reported previously in the database). The mutations were on single base change type. Splice site changes were identified as a result of 36651738G>A mutation, it is also predicted that this might affect the protein features. Splice site donor is marginally increased (wt: 0.8042/mu:0.8719). No changes in potential splice site changes were identified due to 36651804A>T. 36651825G>T is affecting the splice site donor to increase marginally (wt: 0.8725/mu:0.9361) (Fig. 1.6). The p21 Cdk-inhibitor gene is located at 6q21.2, and its expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and -independent mechanisms by a variety of transcription factors that are induced by a number of different signaling pathways (Gartel *et al.,* 1999). Previous studies demonstrated that FOXA2, Transcription Factor activation of p21 transcription via direct binding to the p21 promoter and affects the activity of p21 gene, which results in cell cycle arrest at the G1 phase and inhibition of cell proliferation in p53-deficient cell (Joo-Hee *et al.,* 2014).

3'UTR region of p21 showed 2 known (previously reported) mutations 36653580C>T in 10% and 36653597C>T 5% GC samples (Fig 1.6). These mutations are affecting the splice site change by acting after stop codon, , and as this polymorphism lies in a crucial region for cell differentiation, proliferation may increase cancer risk by altering messenger RNA stability, which, in turn, may affect protein expression and activity (Campbell *et al.*, 2009). Mutations or single nucleotide polymorphisms (SNPs) in the p21 gene may result in alteration of p21 expression and/or activity, thereby modulating susceptibility to cancer (Ma *et al.*, 2011; Gravina *et al.*, 2009; Keshava *et al.*, 2002). Splice site acceptor is marginally increased in both the cases. 36653580C>T polymorphism is thought to cause a functional change in p21, and as this polymorphism lies in a crucial region for cell differentiation, proliferation may increase cancer risk by altering messenger RNA stability, which, in turn, may affect protein expression and activity (Rastinejad *et al.,* 1993; Wang *et al.,* 2012).

Table 1.3: Nucleotide changes in the p21 gene (exon 2 and exon 3).

Position	Nomenclature of mutation	Frequency of mutation (%)	Novel/Reported	Effect of mutation by Mutation Taster
Intron 2	36651738G>A	5	**Novel (Not reported in the database)**	Polymorphism (Single base change) Protein features (might be) affected Splice site changes (Donor marginally increased wt: 0.8042/mu:0.8719)
	36651804A>T	5	**Novel (Not reported in the database)**	Polymorphism (Single base change) No abrogation of potential splice site
	36651825G>T	5	**Novel (Not reported in the database)**	Polymorphism (Single base change) Donor marginally increased (wt: 0.8725/mu:0.9361)
3'UTR	36653580C>T	10	Reported	Polymorphism (Single base change) Splice site changes (Splice site change occurs after stop codon, Acceptor marginally increased, wt:0.53/mu:0.64)
	36653597C>T	5	Reported	Polymorphism (Single base change) Splice site changes (Splice site change occurs after stop codon, Acceptor marginally increased, wt:0.5311/mu:0.5459)

mlh1 Gene Mutation

A known exonic variant 37053568A>G (rs1799977) observed in 10% of the study participants (40 GC) (Fig. 1.6) with the replacement of isoleucine to valine in codon 219 (219I>V) in exon 8 of MLH1 gene (Campbell *et al.,* 2009). SIFT/PolyPhen2 analysis showed that the mutation is of benign, tolerated and of neutral type. Mutation Taster analysis showed that the stability of protein is disturbed by alteration in helix structure (212-220) of protein. The splice site acceptor is marginally increased (wt: 0.7064/mu:0.7505). The mlh1 I219V polymorphism, located in exon 8 at nucleotide position 655 (A>G), was shown to be associated with an increased risk for childhood acute lymphoblastic leukemia (Mathonnet *et al.,* 2003). Few studies have found a significant association between the I219V homozygous variant (GG) and an increased risk of breast cancer (Listgarten *et al.,* 2004). mlh1 655 A>G (I219V) has been reported as a common polymorphism in Western populations, with a G allele frequency of more than 30% (Raptis *et al.,* 2007; Christensen et al., 2008). However, in the current study it was only detected in 7 of 236 gastric cancer patients and in 12 of the 240 healthy individuals. In gastric cancer patients the G allele frequency was 1.5%, lower than in controls who demonstrated a frequency of 2.5%, similar to data in Eastern Asians where the G allele frequency is reported to be approximately 2% (Mann *et al.,* 2008). This is indicative of an ethnic difference in the frequency of this polymorphism. The mlh1 c.655A>G mutation was reported 224 times in the InSiGHT database and 251 times in the LOVD database. The significance of the sequence alteration is controversial as the c.655A>G variant is considered a polymorphism (dbSNP1799977) and was reported in HapMap database (www.hapmap.org). mlh1 c.655A>G is in a conserved region of exon 8, and both alleles result in non-polar and pH-neutral amino acids. Functional analyses suggest that the variant has efficient DNA repair activity (Trojan *et al.,* 2002; Raevaara *et al.,* 2005) and binding properties to PMS2 are similar to the wild type (Kondo *et al.,* 2003). Reduced mlh1 protein associated with c.655A>G polymorphism was also documented among sporadic CRCs in Korean population (Kim *et al.,* 2004). Many studies reported to occur of mlh1 c.655A>G in association with CRC risk at frequency ≥ 1% (Campbell *et al.,* 2009 ;Listgarten *et al.,* 2004;Nejda *et al.,* 2009). This variant is not only involved in colorectal cancer, but also in some other types of cancers. mlh1 c.655A>G is associated with an almost five fold increased risk of ulcerative colitis (Bagnoli *et al*., 2004), and 6- to 16-fold increased risk of acute lymphoblastic leukemia when combined with known genotypes of increased susceptibility to leukemia (Mathonnet *et al.,* 2003). The variant may be associated with the young-onset of

lung cancer, especially in histological squamous cell type (An *et al.,* 2008), and may influence the onset of prostate cancer (Fredriksson *et al.,* 2006). Also the Ile219Val polymorphism may be linked with greater mutation frequency (deletions or substitutions rather than insertions) (Hutter *et al.,* 2002). However, the recent qualitative classification by calibration in silico Tools defined the mlh1 (p.Ile219Leu) missense substitution as non-pathogenic (Thompson *et al.,* 2013). Some studies also show no association between mlh1 Ile219Val polymorphisms and hereditary colorectal cancer development (Mei *et al.,* 2006; Blanco *et al.,* 2008).

37088831C>G variant in intron 16 of mlh1 gene was identified in 5% of GC samples. It is a novel (not reported in database) was observed. It is found to affect splice site by increasing the acceptor marginally (wt: 0.5187/mu: 0.5635).

Prediction of structural variation between wild and mutant type amino acids was carried out by HOPE analysis showing that the mutant type is smaller in size than the wild type, affects the intramolecular and external interactions due to clashes (Fig. 1.5).

Table 1.4: Nucleotide changes in the mlh1 gene (exon 8 and exon 16).

Position	Nomenclature of mutation	Frequency of mutation (%)	AA change	PolyPhen-2/SIFT /PROVEAN	Novel/Reported	Effect of mutation by Mutation Taster
Exon 8	37053568A>G	10	219I > V (**A**TC>**G**TC)	(0.018) Benign/ Neutral/Tolerated	Reported	In the protein structure helix (212-220) might be lost Splice site changes (wt: 0.7064/mu:0.7505, Acceptor marginal increased)
Intron 16	37088831C>G	5	-	-	Novel (Not reported in the database)	Polymorphism (wt: 0.5187/mu: 0.5635, Acceptor marginal change)

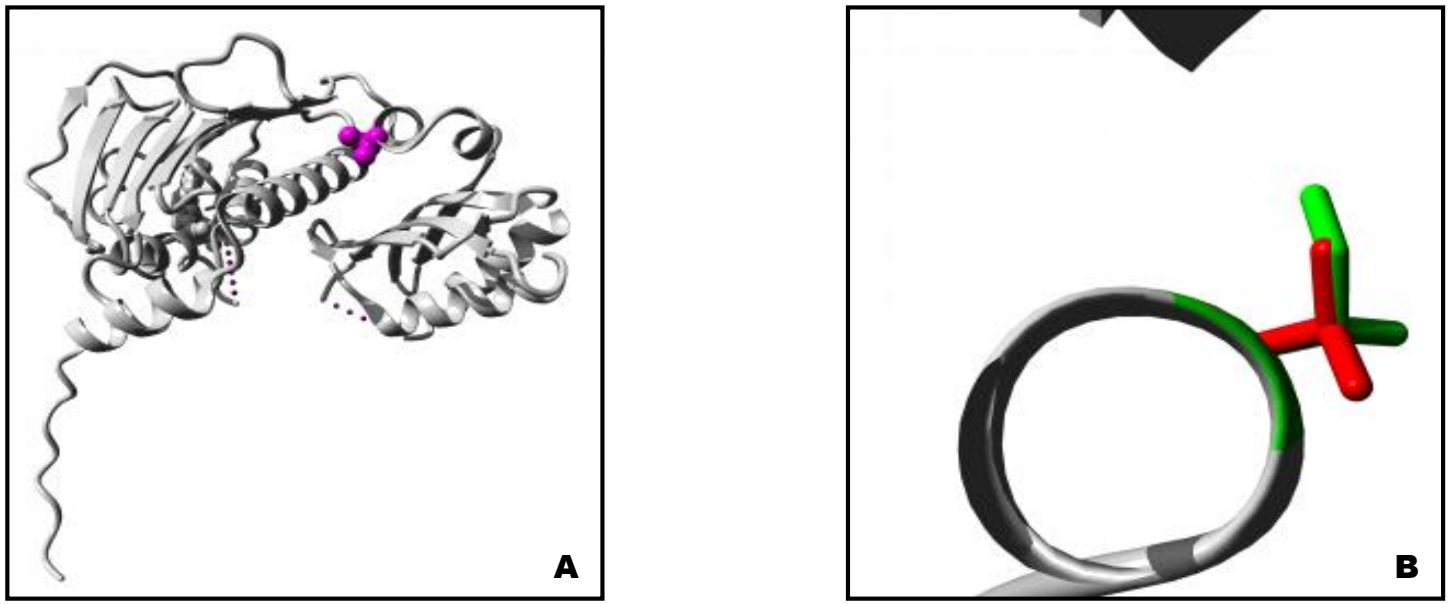

Figure 1.5: 3-D structure of the mutation in gastric cancer samples. A.) Complete chain of protein and the pink spheres and rest of the protein is shown in grey representing the site of mutation (219I > V). B.) Mutation of Isoleucine to Valine at position 219 due to 37053568A>G Wild-type and mutant side chain are shown in green and red, respectively.

mdm2 Gene Mutation

In the present study MDM2 gene had no significant mutations in GC samples. According to the previous studies mutations and polymorphisms were identified in various exons of mdm2 gene in esophageal and gastric cancer (Marchetti *et al.,* 1995). The known variant of mdm2, rs2279744 may independently influence susceptibility to gastric cancer in Chinese population (Elingarami *et al.,* 2015). Chua *et al.* (2010) stated that mdm2 SNPs was not associated an increased GC risk in Korean population, which represents the geographical variation in occurrence of GC. Our study is in concurrence with the study. This can be explained due to the difference in the genetic pool and other conditions between Mizo population and other populations.

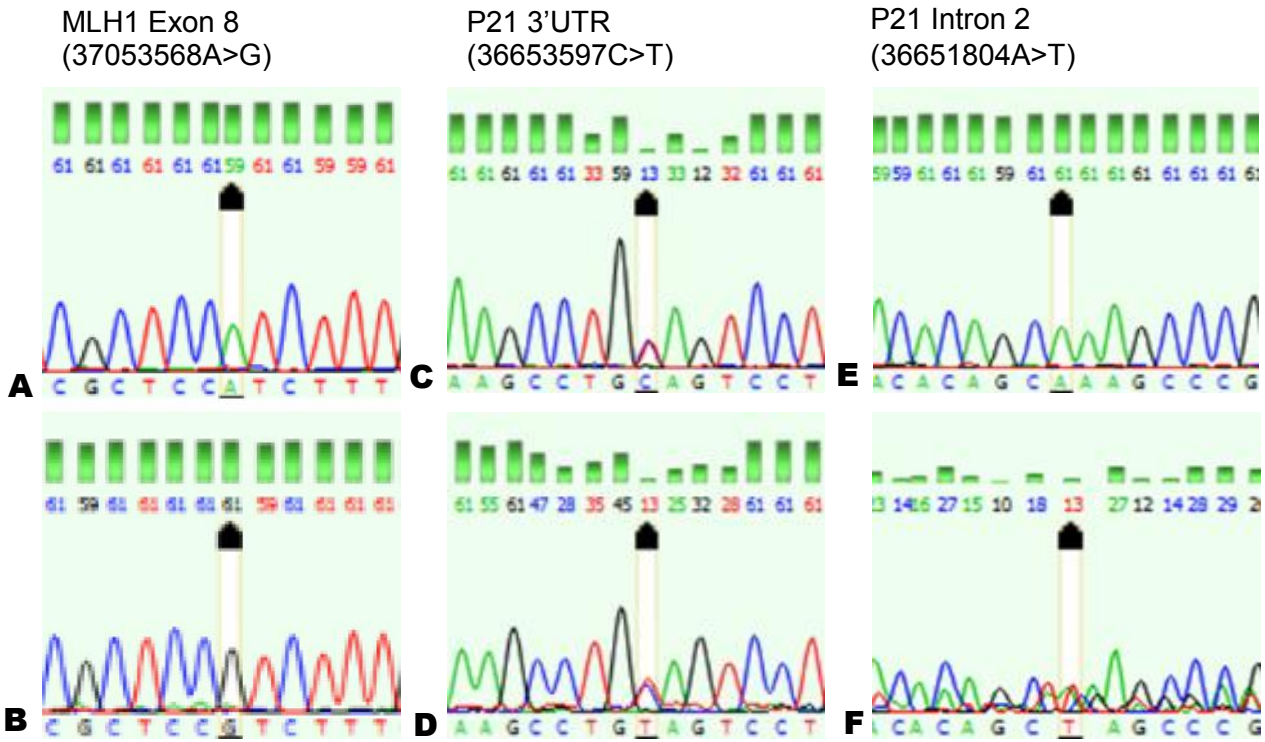

Figure 1.6: Chromatogram of the different gene sequences from GC samples (B, D, F) compared with Healthy samples (A, C, E).

Splice site analysis by ESE splice Finder of mutations

The effect of mutations in the splice site were identified by Human splicing finder (HSF). To predict the consequences of mutations associated with a splicing defect, we used all the intronic mutations that disrupt the 5'ss or the 3'ss and results in exon skipping and/or activation of a cryptic splice site was used. Branch Point sequences represent another essential splicing signal. When a mutation is localized in proximity of the 5' of the 3'ss, its potential effect on a BP sequence should be examined especially when a nucleotide located at less than 85bp from the 3'ss is targeted. When using the HSF algorithm, the threshold for 5' and 3'ss is 67 with a pathogenic CV of -10% except for position +4 where it is -7%. The new HSF algorithm to define consensus values (CV) of 50ss or 30ss was created to maximize the difference between wild-type (wt) active sites and mutant inactive sites. Thus, strong sites presented a CV higher than 80 and less strong sites a CV ranging between 70 and 80. Only a minor fraction of active sites showed a CV between 65 and 70. Since a mutation can result not only in the disruption of a 50ss or a 30ss, but also in the creation of a new splice site, HSF evaluates the 'creation of cryptic splice sites'. The analysis revealed that 36653580C>T in 3'UTR of p21 branch point is changing with a CV variation of -1.53 due to which the motif might get abolished leading to splicing defect. 36653580C>T polymorphism is thought to cause a functional change in p21, and as this polymorphism lies in a crucial region for cell differentiation, proliferation may increase cancer risk by altering messenger RNA stability, which, in turn, may affect protein expression and activity (Rastinejad *et al.*, 1993; Wang *et al.*, 2012). Similarly for mlh1 intron 16 37088831C>G mutation the CV variation is -4.94 conferring to splicing defect. Mutations located in the introns of mismatch repair genes can interfere with splicing and cause aberrant spliced mRNA transcripts leading to non-functional mismatch repair proteins (Petersen *et al.*, 2013) (Table 1.5).

Table 1.5: Effect of mutations in splicing.

Gene	Mutation	Position	WT Branch Point Motif	Mutant Branch Point Motif	WT CV	Mutant CV	CV variation (%)
p21	Intron 2	36651738G>A	AAGCAGG	AAGCAAG	9.95	39.57	29.62
		36651804A>T	AGCAAG	AGCTAG	61.53	65.52	3.99
		36651825G>T	ATAGTGT	ATATTGT	6.23	21.31	15.08
	3'UTR	36653580C>T	CGCCCAC	TGCCCAC	86.39	84.86	-1.53
		36653597C>T	CTGCAGT	CTGTAGT	16.53	25.56	9.03
mlh1	Exon 8	37053568A>G	CTCCATC	CTCCGTC	46.15	53.04	6.89
	Intron 16	37088831C>G	TTGACAG	TTGAGAG	47.68	42.74	-4.94

Multi dimensionality reduction (MDR) analysis

Multi dimensionality reduction (MDR) analyses were performed to explore the potential gene–gene and gene-environment interaction. In the present study for the entire dataset, smoked meat is the best one factor model found statistically significant ($p<0.0001$) with a CVC of 8/10 and testing accuracy of 0.6653. The combination of smoked meat and oral snuff was found to be the best two-factor model which was also the best overall model with a CVC of 10/10 and TBA of 0.7389 ($p<0.0001$). The best model was selected based on the highest CVC and TBA value among all the models. The combination of sa-um, smoked meat, and tuibur was found to the best three-factor model with a CVC of 3/10 and TBA of 0.4042 ($p<0.0001$) (Fig. 1.7, Table 1.6). The previous statistical analysis results were reproduced in MDR analysis also.

Table 1.6: Summary of multifactor dimensionality reduction analysis for GC risk prediction.

Best Interaction Model	TBA	CVC	P-Value
Smoked Meat	0.6653	8/10	<0.0001
Smoked Meat, Oral Snuff	0.7389	10/10	<0.0001
Sa-um, Smoked Meat, Tuibur	0.4042	3/10	<0.0001
TBA - Test balance accuracy; CVC - Cross-validation consistency			

Interaction entropy graph

Interaction entropy graphs were created using MDR results, for better verification and visualization of interactions between gene and environment factors. In interaction entropy graph, smoked meat showed the highest independent effect (20.56 %) and also had moderate synergistic interaction with sa-um (0.43 %). Oral snuff (8.19 %) also explained considerable entropy independently. Earlier, in many epidemiological studies from India a positive association of dietary items containing substantial amount of N-nitroso compounds with stomach cancer have been reported (Sumathi *et al.*, 2009). Polycyclic aromatic hydrocarbons such as benzo[a] pyrene formed in smoked food have been correlated in many areas of the world with high stomach cancer rates (Yeh et al., 2009). Soda-an alkaline preparation,

frequently used as food additives was significantly associated with increased risk of stomach cancer in Mizoram (Phukan *et al.*, 2006). Oral snuff and other tobacco products contains carcinogens like the nitrosamines which acts as cofactor for pathogenesis of GC (IARC, 2007).

Interaction between mutations and significant demographic factors

An unconditional binary logistic regression model was used to estimate the association between gene mutations and risk of GC (Table 1.7). The oral snuff and mutations in p21 gene 3' UTR (OR, 9.256; 95% CI, 1.842-46.509; P < 0.007) were found associated with increased risk of GC. Similarly, smoked meat/vegetable and p21 intron 2 (OR, 4.149; 95% CI, 0.970-17.738; P < 0.050) were also found to be significantly associated with increased risk for GC (Table 1.7). However, the other genes does not show any association with the demographic factors.

Table 1.7: Interaction between mutations and significant demographic factors.

Factor	Gene Name	Position	[a]ORs (95% CI)[b]	P value
Oral Snuff	p21	Intron 2	1.025 (0.253 - 4.150)	0.972
		3' UTR	9.256 (1.842 - 46.509)	0.007
	mlh1	Exon 8	0.956 (0.183 - 4.986)	0.958
		Intron 16	1.732 (0.143 - 20.956)	0.666
Smoked meat/vegetable	p21	Intron 2	4.149 (0.970 - 17.738)	0.050
		3' UTR	0.728 (0.182 - 2.909)	0.653
	mlh1	Exon 8	1.510 (0.324 - 7.043)	0.600
		Intron 16	1.386 (0.109 - 17.543)	0.801

[a]OR – Odd ratio
[b]95% CI – 95 % Confidence Interval

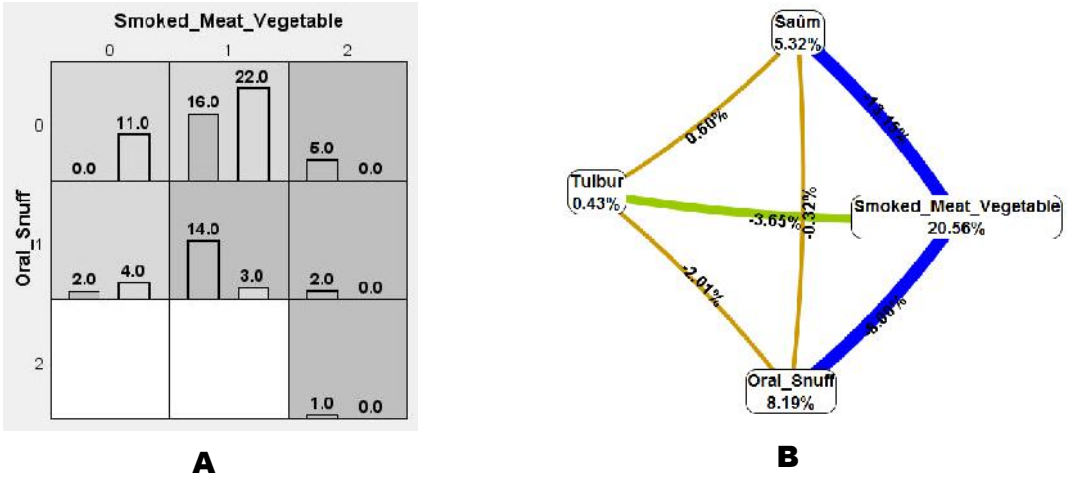

Figure 1.7: Multifactor dimensionality reduction (MDR) analysis to find interaction between genetic and environmental factors. A.) The summary of the two factor model (smoked meat and oral snuff) predicted by MDR is represented in the graph. For Smoked meat and oral snuff 0 represents less consumption, 1 represents moderate consumption and 2 represents high consumption. B.) Interaction entropy graph to find the gene-environment interaction with GC risk. The percent of the entropy for independent factors as well as their interactions are represented in the graph where positive percentage of entropy denotes synergistic interaction while negative percentage denotes redundancy. The red colour indicating a high degree of synergistic interaction, orange a lesser degree whereas; gold represent midpoint, blue represents the highest level of redundancy followed by green.

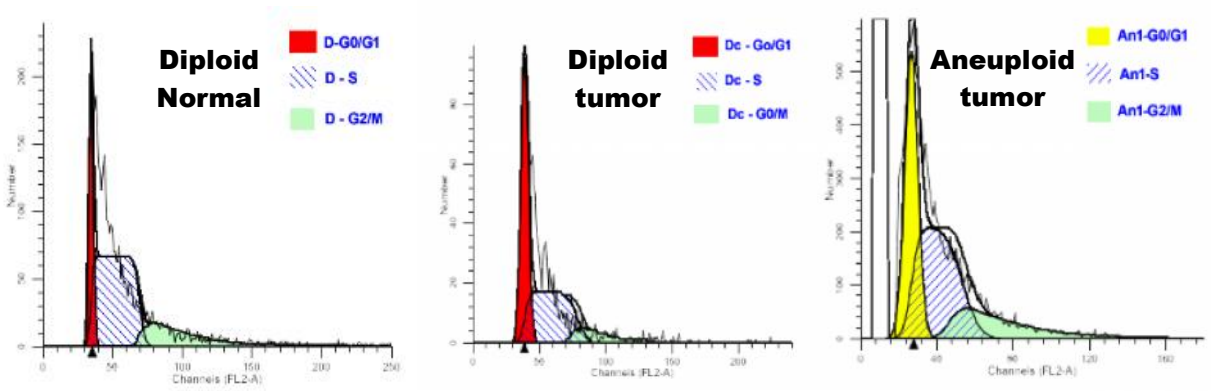

Figure 1.8: DNA content analysis in gastric cancer samples measured by the flow cytometric profile.

DNA content analysis

Flow cytometric analysis (FCM) analysis besides giving kinetic estimates such as the fraction of cells in S-phase (SPF), can subdivide neoplasms into DNA diploid or DNA aneuploid tumors based on the presence of different sub-populations in different phases of cell cycle. In previous study DNA aneuploidy has been reported in 40-50% of GC tumours (Yonemura *et al.,* 1992; Brito *et al.,* 1993). Flow cytometric analysis predicted DNA aneuploid in 07 (17.5%) and diploid in 33 (82.5%) diploid tumor samples (Fig. 1.8). A high S phase fraction (SPF) was observed in GC samples (51.24 - 72.09) compared to controls (32.45 - 44.12). Deregulation of cell cycle events leads to uncontrolled cell proliferation and a high S phase fraction is a hallmark of gastric cancer (Baba *et al.*, 2002). According to the previous study the p21 variant genotypes has been found to have an impact on cell cycle control induced by DNA damage caused by carcinogens in tobacco related product (Flejou *et al.,* 1993). Arrest of G2/M phase was observed in case of aneuploidy. DNA aneuploidy has been reported in 40-50% of tumours (Nanus *et al.,* 1993; *Quirke et al.*, 2005). One study reported aneuploidy in 76% (25 of 33) of adenocarcinomas arising in the gastric cardia, compared with 30% (8 of 27) of adenocarcinomas arising in the gastric antrum (Flejou *et al.,* 1994).

Table 1.8: **Distribution of different phase fraction according to DNA content status.**

Sample No.	Ploidy Status	Ploidy No. (%)	G0/G1 phase			S phase			G2/M phase	
			Low (%)	Moderate (%)	High (%)	Low (%)	Moderate (%)	High (%)	Low (%)	High (%)
Healthy (40)	Diploid	40 (100)	1 (2.5)	13 (32.5)	26 (65)	38 (95)	2 (5)	0	3 (7.5)	37 (92.5)
Cancer (40)	Diploid	33 (82.5)	18 (54.5)	15 (45.4)	0	0	3 (9.09)	30 (90.9)	28 (84.8)	5 (15.1)
	Aneuploid	7 (17.5)	0	1 (14.2)	6 (85.7)	0	1 (14.28)	6 (85.7)	7 (100)	0

The molecular analysis revealed total of five mutations in p21 gene among which three novel/ unreported were identified in intron 2 36651738G>A, 36651804A>T, 36651825G>T, whereas in mlh1 gene two variants were identified viz. one in exon 8 (37053568A>G; 219I > V) and other in intron 16 (37088831C>G) among which intronic variant is novel (37088831C>G). No mutations were identified in mdm2 gene. Flow cytometric analysis predicted DNA aneuploid in 07 (17.5%) and diploid in 33 (82.5%) tumor samples. The G2/M phase was significantly arrested in aneuploid gastric cancer samples whereas high S-phase fraction was observed in all the gastric cancer samples. In MDR analysis, the best model for gastric cancer risk was two-factor model of oral snuff and smoked meat. The interaction between identified mutations and the significant demographic factors, smoked meat/vegetable and oral snuff predicted that oral snuff is significantly associated with p21 3'UTR mutations. This study demonstrated that variation in cell cycle regulatory gene (p21) and mismatch repair gene (mlh1) and with environmental factors might modulate the susceptibility of gastric cancer by changing the DNA content level abnormally in tumors in the Mizo ethic population.

Conclusion and Summary

Pathogenesis of gastric cancer is a complex interplay between environmental and genetic factors in the development of gastric cancer. The process is multifactorial and requires mutations in somatic cells and subsequent alterations of morphology and growth pattern, ultimately resulting in transformation, local invasion, and metastasis. Although a number of factors probably influence an individual's predisposition to gastric cancer and course of progression to gastric cancer, gene mutations is among a feature that links this cancer to many other types of malignancy. The research on the role of cell cycle gene mutations and polymorphisms in gastric carcinoma is still evolving. Despite a major decline in the incidence and mortality over several decades, gastric cancer is still the fifth most common cancer and the third most frequent cause of cancer death in the world (Torre *et al.*, 2015). Mizoram shows the highest incidence of GC with an age-adjusted rate (AAR) of 50.6 and 23.3 per 10^5 populations in male and female, respectively (NCRP, 2013). Mizoram comprises of a distinct ethnic population with peculiar dietary habits such as extensive consumption of smoked meat/ Vegetables, Sa-um (fermented pork), nitroso salts and heavy addiction to different tobacco products (viz. tobacco infused water-tuibur, local cigarette- meiziol, Zarda pan, oral snuff and betel quid (betel nut, slaked lime wrapped in betel leaf) chewing) and heavy alcohol consumption (Phukan *et al.*, 2006).

This thesis addresses the role of mutations in cell cycle (p21 and mdm2) and DNA damage repair (mlh1) genes in gastric cancer patients and is correlated with various life style habits. The work basically describes the role of p21, mdm2 and mlh1 gene mutations and alterations in cell cycle DNA content.

Although gastric cancer is one of the most common malignancies worldwide, its pathogenesis and the molecular genetic events that contribute to its development are poorly understood. In Mizoram, very few studies have been carried out so far to detect the genetic and environmental risk factors associated with stomach cancer. In the present study, a case-control study for the high prevalence of gastric cancer in Mizoram has been attempted in order to identify the mutations in cell cycle genes p21 and mdm2 and DNA damage repair gene mlh1 and to identify the correlation of these mutations with environmental and dietary factors that seem to play a role in gastric cancer aetiology.

The work done in the study and findings can be briefly described as follows:

1. Demographic study identified that smoked meat /vegetable (OR-16.214; 95% CI-2.746 – 95.749; P-0.002) and oral snuff (OR-10.496; 95% CI-2.410 - 45.710; P-0.002) are the major risk factors for GC for our study population.
2. The molecular analysis revealed total of five novel mutations in p21 gene.
3. In mlh1 gene, two variants were identified:- one in exon 8 (37053568A>G; 219I > V) and other in intron 16 (37088831C>G), among which intronic variant is novel (37088831C>G). The exonic variant (37053568A>G; 219I > V) is of non-pathogenic type.
4. Flow cytometric analysis revealed high S-phase in gastric cancer samples. 17.5% of the samples were identified with aneuploidy with G2/M phase arrested.
5. The mutations in cell cycle regulatory genes is affecting the normal pathway of cell division which might lead to gastric cancer.
6. No mutations were identified in the mdm2 gene in this population.
7. The analysis revealed that in the 36653580C>T in 3'UTR of p21, branch point is changing with a CV variation of -1.53 due to which the motif might get abolished, leading to splicing defect. Similarly, for mlh1 intron 16, in the 37088831C>G mutation the CV variation is -4.94 conferring to splicing defect.
8. The MDR analysis showed that the combination of smoked meat and oral snuff is the best two-factor model which was also the best overall model with a CVC of 10/10 and TBA of 0.7389 ($p<0.0001$). In interaction entropy graph, smoked meat showed the highest independent effect (20.56 %) and also had moderate synergistic interaction with sa-um (0.43 %).
9. The oral snuff and mutations in p21 gene 3' UTR (OR, 9.256; 95% CI, 1.842-46.509; $P < 0.007$) was found associated with increased risk of GC. Similarly, smoked

meat/vegetable and p21 intron 2 (OR, 4.149; 95% CI, 0.970-17.738; P < 0.050) was also found to be significantly associated with increased risk for GC.

10. Flow cytometric analysis predicted DNA aneuploid in 07 (17.5%) and diploid in 33 (82.5%) tumor samples. Significantly higher S phase fraction (SPF) with arrested G2/M phase was observed in all the GC samples.

In summary, our findings indicate that cell cycle regulatory gene (p21) and mismatch repair gene (mlh1) are more predisposed to variation in gastric cancer for Mizo population and may play an important role in tumorigenesis by the aberrant distribution of DNA content in the phases of cell cycle in tumor cells. Ethnicity and dietary habits are acting as crucial covariates, suggesting that the mutations have different penetrance according to ethnicity, dietary and life style habits. This study could afford early recognition of patients at risk of developing micro- or macroscopic, pathological lesions as well as the introduction of preventive measures. Due to the complexity with multiple genetic and environmental factors in the development of gastric cancer, large population studies are required that will be able to overcome the limitation of sample size and encompass all the variables including association with mutations in genes for DNA repair genes, cell cycle regulatory genes and cell cycle study, exposure to environmental factors, ethnic and demographic features.

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Appendix

Ethical Approval Letter

IEC, CIVIL HOSPITAL, AIZAWL.
COMMUNICATION TO THE PRINCIPAL INVESTIGATOR/GUIDE BY THE MEMBER SECRETARY, INSTITUTIONAL ETHICS COMMITTEE

No.B.12018/1/13-CH(A)/IEC Dated: 18th of April, 2014

To,

Dr. N Senthil Kumar
Prof. and H.O.D in Department of Biotechnology,
Mizoram University.

The Institutional Ethics Committee in its meeting held on 14th of October 2014, has reviewed and discussed your application to conduct the clinical trial/project entitled; **Mutational Analysis of Oncogenes and Tumor Suppressor Genes in Gastric Cancer Patients of Mizo Tribal Group.**

Sponsored by : **Department of Biotechnology, New Delhi.**

Code no. : _____

The following documents were reviewed:

a. Trial Protocol (including protocol amendments)/project, dated 14th of April, 2014.
b. Investigator's Brochure, dated 14th of April, 2014.
c. Patient Information Sheet and Informed Consent Form (including updates if any) in Hindi, English and/or vernacular language.
d. Proposed methods for patient accrual including advertisement (s) etc. proposed to be used for the purpose.
e. Current CV of investigator from outside Civil Hospital, Aizawl.
f. Insurance Policy/Compensation for participation and for serious adverse events occurring during the study participation.
g. Investigator's Agreement with the Sponsor,
h. Investigator's Undertaking.
i. Ethics Committee Proforma.
j. DCGI approval letter/submission letter, if any.
k. Civil Hospital, Aizawl Case Report Form
l. Any other/additional documents

Decision of Committee: **Approved/NotAprroved**

18/4/14.

(DR. C. LALCHHANDAMA)
Member Secretary
Institutional Ethics Committee

Secretary
Institutional Ethical Committee
Civil Hospital, Aizawl

List of Publications

Published

1. **Ravi Prakash Yadav,** Kalibulla Syed Ibrahim, Guruswami Gurusubramanian, N. Senthil Kumar (2014) In silico docking studies of non-azadirachtin limonoids against ecdysone receptor of *Helicoverpa armigera*. Medicinal Chemistry Research 24:2621-31. ***(Impact factor: 1.41)***

2. Lalro Kimi, Souvik Ghatak, **Ravi Prakash Yadav**, Jeremy L Pautu, Lalhma Chhuani, Doris Lallawmzuali, Nachimuthu Senthil Kumar (2015) Relevance of GSTM1, GSTT1 and GSTP1 gene polymorphisms to Breast cancer susceptibility in Mizoram population, Northeast India. Biochemical Genetics, 54(1), 41-49. ***(Impact factor: 0.87)***

3. Sasmita Mohanty, Arabinda Mahanty, **Ravi Prakash Yadav**, Gopal Krishna Purohit, Biranchi Narayana Mohanty, Bimal Prasanna Mohanty (2014) The ATRI hot spring in Odisha - A natural ecosystem for global warming research. International Journal of Geology, Earth and Environmental Sciences. 4:85-90.

Communicated

4. **Ravi Prakash Yadav**, Souvik Ghatak, Freda Lalrohlui, Ravi Kannan, Rajeev Kumar, Zothan sanga, Jeremy L Pautu, John Zomingthanga, Rajendra Muthukumaran, Nachimuthu Senthil Kumar (2016) Association of SNPs in cell cycle regulatory and MMR genes with susceptibility to gastric cancer. Molecular Biotechnology (Under communication)

Book Chapter

1. Subhajit Mukherjee, Souvik Ghatak, **Ravi Prakash Yadav**, Zothansanga, G. Gurusubramanian and N. Senthil Kumar (2015) Advances in PCR based molecular markers and its application in biodiversity conservation. Biodiversity in Tropical Ecosystems (pp. 395-422). New Delhi: Today and Tomorrow's Printers and Publishers.

Workshops Attended

1. **Molecular Docking and Simulation for identification of novel molecules for Agricultural & Veterinary applications** in MBGE, College of Basic Sciences & Humanities GBPUAT Pantnagar, Uttarakhand during 13-17th Sept. 2013.

2. **Basics of Computer-Aided Drug Designing & Protein-Protein Interaction** in College of Veterinary Sciences, Assam Agriculture University, Assam during 18-20th Feb. 2014.

3. **Workshop on Capacity Building in Effective Management of Intellectual Property Right (IPRs)** from August 27 - 28, 2014 at Mizoram University, Aizawl, Mizoram.

4. **Molecular docking and virtual screening** from 2-4th October 2014 at DBT-Bioinformatics infrastructure facility, Department of Biotechnology, Mizoram University and Schrodinger Bangalore.

5. **Basic and Clinical Flow Cytometry course** jointly organized by the Cytometric Society of India, Indian Institute of Technology, Guwahati and Cachar Cancer Hospital and Research Center from November 10-11, 2014 at Cachar Cancer Hospital and Research Centre, Silchar, Assam.

6. **Cancer Mutations - Detection and Analysis** from April 17-18, 2015 at DBT-State Biotech HUB, Department of Biotechnology, Mizoram University.

7. **Browsing genome with Ensemble and UCSC genome browser** from 29-30th May, 2015 at DBT-State Biotech HUB, Department of Biotechnology, Mizoram University.

8. **Northeast autumn school on Human genetics technique and data analysis** from 8-11 Sept 2015 jointly organized by ISI Kolkata and Mizoram University.

ANALYSES OF MUTATION IN CELL CYCLE GENES ASSOCIATED WITH GASTRIC CANCER IN MIZO POPULATION

ABSTRACT

M. PHIL. THESIS

SUBMITTED FOR THE AWARD OF MASTER OF PHILOSOPHY
BIOTECHNOLOGY

Submitted by

Ravi Prakash Yadav
Regn. No. MZU/M. Phil./229 of 22.05.2015

Under the supervision of

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December, 2015

ANALYSES OF MUTATION IN CELL CYCLE GENES ASSOCIATED WITH GASTRIC CANCER IN MIZO POPULATION

Gastric cancer is the fifth most commonly diagnosed cancer (Ferlay *et al.,* 2013). Despite marked decreases in incidence over the last century, particularly in developed countries, Gastric cancer (GC) is still the third leading cause of cancer death worldwide, with highest estimated mortality rates are in Eastern Asia (Ferlay *et al.,* 2013). Incidence of gastric cancer varies among countries and even in different regions of the same country (Torres *et al.,* 2013). India is a pluralistic, multilingual, and multi-ethnic society. The Mizoram state has the highest rate of stomach cancer incidence in India (Rao *et al.,* 1998; Phukan *et al.,* 2004). Many studies revealed that different food habits tobacco, alcohol etc. promote the occurrence of most of the cancers including stomach cancer worldwide (Kim *et al.*, 2014). The people of Mizoram have unique set of dietary habits, consisting of heavy dosage of meat, dried and smoked fish and meat, Saum (fermented pork fat), dried and pickled vegetables and extra salt. The alcohol consumption and usage of tobacco products like khuva (leaf blended and mixed with fine lime paste), tuibur (tobacco smoke-infused water) etc. are also very high.

The cell cycle is the series of events that takes place in a cell leading to its division and duplication (replication), if it malfunction results into uncontrolled cell division and leads to cancer. Abnormal cell divisions can destabilize cancer genomes (Sintupisut and Yeang, 2013). Multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules and growth factor/receptor systems are involved over the course of the multi-step conversion of normal epithelial cells to gastric cancer (Tahara *et al.*, 2004). There is increasing interest in genetic polymorphisms and mutation studies as causes for gastric cancer, probably due to advances in DNA-analysis technologies (El-Omar *et al.,* 2004). Individual variations in cancer risk have been associated with specific genes polymorphisms and mutations. Polymorphisms and mutations in a wide variety of genes may modify the effect of environment (González *et al.,* 2002); these gene-environmental interactions may explain the high variation in the incidence of gastric cancer around the world (Ames *et al.,* 1993). Abnormalities in cell-cycle regulators are also involved in the development and progression of gastric cancers through unbridled cell proliferation (Yasui *et al.,* 2001). Specifically, genetic alterations and abnormal expression of various cyclins and cyclin-dependent kinases (CDKs), as well as CDK inhibitors, play a role in

gastric cancer pathogenesis. There are three main classes of genes involved in cancer development: oncogenes, tumor suppressor genes and stability genes.

P21 is a major regulator of the G1/S checkpoint, preventing inappropriate DNA replication in S phase. In response to various stimuli (oxidative stress, DNA damaging agents), P21 can become activated. Mouse double minute-2 (MDM2) is a negative regulator of tumour suppressor P53. MDM2 is one of the central nodes in the P53 pathway and can control P53 protein levels and activity. MLH1 gene normally function as mismatch repair genes, with the role of maintaining the microsatellite stability, because of their role in identifying and excising single-base mismatches and insertion-deletion loops that may arise during DNA replication. Aneuploidy is a good marker of a malignant cell population and an abnormal amount of DNA in solid tumours is a specific sign of neoplasia.

Very few studies has been done on prevalence of mutations and gastric cancer risk in Mizo population. To fill the lacunae in our knowledge, the present study was carried out to predict pathogenic mutations in the cell cycle regulator genes P21, MDM2, MLH1 (selected exon + intronic boundaries) using PCR and direct sequencing method and correlating identified mutations with demographic/clinicopathological characteristics of Gastric cancer patients from Mizoram and also to correlate with cell cycle progression study. Outcome of this study will provide better understanding of effect of demographic and genetic factors on gastric cancer risk and will help in better prognosis and diagnosis of gastric cancer.

The following techniques were employed in this study

- Sample collection and Processing.
- DNA isolation by FFPE tissues and Blood.
- PCR amplification of selected exons of P21, MDM2, MLH1 genes.
- Sequencing and Sequence Analysis to identify mutations and polymorphisms.
- Logistic regression analysis to correlate between demographic factors and gastric cancer risk.
- Multifactor dimensionality reduction (MDR) to predict the association between demographic factors and gastric cancer risk.
- Functional characterization of identified mutations by various in silico approach.
- Cell cycle estimation by Flow cytometry.

Objectives and Results of the proposed research work

1. To study the Somatic mutations and Germ line in p21, mdm2 and mlh1 genes in gastric cancer patients from Mizoram.
2. To study the correlation between gene mutations and cell cycle progression.

In the present study, we investigated the role of mutations in cell cycle (p21 and mdm2) and DNA damage repair (mlh1) genes in gastric cancer patients and correlated with various life style habits. In this population-based case control study, 40 gastric cancer patients and 40 healthy controls were included from Mizoram, Northeast India. The findings of this study are as follows:

1. Demographic study identified that smoked meat /vegetable (OR-16.214; 95% CI-2.746 – 95.749; P-0.002) and oral snuff (OR-10.496; 95% CI-2.410 - 45.710; P-0.002) are the major risk factor for GC for our study population.
2. The molecular analysis revealed total of five novel mutations in p21 gene. 36651738G>A and
3. In mlh1 gene two variants were identified one in exon 8 (37053568A>G; 219I > V) and other in intron 16 (37088831C>G) among which intronic variant is novel (37088831C>G). The exonic variant (37053568A>G; 219I > V) is of non-pathogenic type.
4. Flow cytometric analysis revealed high S-phase in gastric cancer samples. 17.5% of the samples were identified with aneuploidy with G2/M phase arrested.
5. The mutations in cell cycle regulatory genes is affecting the normal pathway of cell division which might lead to gastric cancer.
6. No mutations were identified in the MDM2 gene in this population.
7. The analysis revealed that 36653580C>T in 3'UTR of p21, branch point is changing with a CV variation of -1.53 due to which the motif might get abolished leading to

splicing defect. Similarly for mlh1 intron 16, 37088831C>G mutation the CV variation is -4.94 conferring to splicing defect.

8. The MDR analysis showed combination of smoked meat and oral snuff was found to be the best two-factor model which was also the best overall model with a CVC of 10/10 and TBA of 0.7389 ($p<0.0001$). In interaction entropy graph, smoked meat showed the highest independent effect (20.56 %) and also had moderate synergistic interaction with sa-um (0.43 %).
9. The oral snuff and mutations in p21 gene 3' UTR (OR, 9.256; 95% CI, 1.842-46.509; $P < 0.007$) were found associated with increased risk of GC. Similarly, smoked meat/vegetable and p21 intron 2 (OR, 4.149; 95% CI, 0.970-17.738; $P < 0.050$) were also found to be significantly associated with increased risk for GC.
10. Flow cytometric analysis predicted DNA aneuploid in 07 (17.5%) and diploid in 33 (82.5%) diploid tumor samples. Significantly higher S phase fraction (SPF) with arrested G2/M phase was observed in all the GC samples.

Conclusion

The findings of this study indicate that cell cycle regulatory gene (p21) and mismatch repair gene (mlh1) are more predisposed to variation in gastric cancer for Mizo population and may play an important role in tumorigenesis by the aberrant distribution of DNA content in the phases of cell cycle in tumor cells. Ethnicity and dietary habits are acting as crucial covariates, suggesting that the mutations have different penetrance according to ethnicity, dietary and life style habits. This study could afford early recognition of patients at risk of developing micro- or macroscopic, pathological lesions as well as the introduction of preventive measures. Due to the complexity with multiple genetic and environmental factors in the development of gastric cancer, large population studies are required that will be able to overcome the limitation of sample size and encompass all the variables including association with mutations in genes for DNA repair genes, cell cycle regulatory genes and cell cycle study, exposure to environmental factors, ethnic and demographic features.