Association of cyclooxygenase-2 genetic polymorphism with gastric cancer in eastern Indian population

Kanishka Uthansingh
Department of Gastroenterology, Institute of Medical Sciences and SUM Hospital, Siksha O Anusandhan deemed to be University, Bhubaneswar 751003

Debakanta Mishra
Department of Gastroenterology, Institute of Medical Sciences and SUM Hospital, Siksha O Anusandhan deemed to be University, Bhubaneswar 751003

Girish Kumar Pati
Department of Gastroenterology, Institute of Medical Sciences and SUM Hospital, Siksha O Anusandhan deemed to be University, Bhubaneswar 751003

Rabindra Nath Padhy (rnpadhy54@gmail.com)
Central Research Laboratory, Institute of Medical Sciences and SUM Hospital, Siksha O Anusandhan deemed to be University, Bhubaneswar 751003

Manoj Kumar Sahu
Department of Gastroenterology, Institute of Medical Sciences and SUM Hospital, Siksha O Anusandhan deemed to be University, Bhubaneswar 751003

Research Article

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Abstract

The Gastric cancer (GC) is the problem seen more frequently in tropical countries. The expressivity of cyclooxygenase-2 (COX-2) and inherent individual susceptibility to oncogenicity are due to over-expression of and single nucleotide polymorphisms (SNPs). The present experimental case-control study aims to evaluate the role of over-expression of COX-2 polymorphisms in the development of gastric cancer (GC) in the region at Eastern India. The genomic DNA was isolated from the peripheral blood samples of 124 GC patients and 176 controls attending the hospital, during February 2017 to December 2020. GC with or without infection of halophile, *Helicobacter pylori* was diagnosed by the histo-pathological assessment. DNA was isolated of GC-cases and controls by the salting-out method. Three SNPs of COX-2 gene such as, -1195GA, -765GC, and –1290AG were arbitrarily chosen to identify the genetic polymorphisms. Genotypes were analyzed by undertaking 'polymerase chain reaction restriction fragment length polymorphism' (PCR-RFLP). A total of 124 GC cases with 176 healthy individuals were enrolled in the study. A fraction of 10.5% cases had GG homozygous mutation alleles at -1195GA, while the 5% fraction had GG homozygous mutant alleles at -765GC, and an 8.9% fraction had GG homozygous mutant alleles at -1290AG, amongst all the GC cases. COX-2 polymorphisms were crucially associated with the risk of development of GC, compared to controls with $p = 0.003$, both in 1195 GA and 1290AG. COX-2 polymorphisms could play a crucial role in the development of GC in the coastal Eastern Indian region.

Introduction

Gastrointestinal cancer (GIC) is the most common occurrence, globally with an incidence of more than 1.22 million and nearly 865000 deaths [1]; despite its declining trend of mortality rates, gastric cancer (GC) is still the third most common cause of cancer-related decimation, nowadays [2]. GC is the 5th most commonly diagnosed cancer according to Global cancer statistics 2020. GC is the second most common cause of malignant deaths among the people aged, 15–44 years with7% incidences in India with 27% of 5-year survival rate after surgical resection [3, 4]. Risk factors associated with GC are *Helicobacter pylori (H. pylori)* infection, obesity, cigarette smoking, taking red meat, consumption of alcohol, low socioeconomic status and most importantly genetic mutation. *H. pylori* or the previously known *Campylobacter pylori* the gram - negative, microaerophilic bacteria has been observed in stomach. The infection of this bacterium causes chronic inflammation and lead to duodenal ulcer, gastric ulcer and GC. *H. pylori is* one of the vital risk factors for GC. Though, India has low incidence of GC, this stands the top five most common cancers among other cancers [3]. Indeed, in Odisha state the cancer incidence was 74,861 with the GC rate of 5%, during 2001-11. However, globally GC was the second most common cancer after oral cancer with a fraction of 13% incidence among males and it was the fourth most common cancer among women during 2012 [5, 6].

H. pylori pathogen colonizes approximately 50% of the global population across the globe. Generally, *H. pylori* generates the colonization in the gastric domain, the host immune response become ineffective in clearing this bacterium. The risk of *H. pylori* infection is directly related to overall use of water condition that is implicated as a possible mode of transmission in rural areas reliable supplies of potable water [6].

The mechanism of GC development is through two major pathways: one is a direct action on epithelial cells by inflammation, and the other is by the action through genetic mutation and protein modulation [10, 11].

Since COX-2 is an inducible enzyme, this catalyzes the conversion of arachidonic acid to prostaglandins, with an involvement in the modulation of potent lipid mediators during inflammation in cancer development [12]. The present study was aimed to explore its prevalence included with three single nucleotide polymorphisms (SNPs) namely, 1195GA, 765GC, and 1290AG, in GC cases. These genetic associations were reported in past by including single SNP of COX-2 individually or dually [13–15]. However, this is the first East Indian work to access the association of GC risk factors and three SNPs. It was observed in the study that the risk factors, smoking, chewing of tobacco and infection of *H. pylori* are associated with COX-2 mutation at regions 1195GA, 765GC and − 1290AG, for the development of GC.

Material And Methods

Study design and patients

Consecutive GC patients and healthy controls attending the Gastroenterology department, of this hospital, Bhubaneswar were enrolled in the study. Cases were identified by an endoscopy study and were confirmed by the histopathological (HP) examination. The study was carried out with good clinical practice (GCP) guidelines of Indian Council of Medical Research (ICMR, Government of India). Each participant of the study was agreed the written consent prior to the study. After getting the informed consent form the patients, blood samples were collected from both GC case group and healthy volunteer group, isolate the DNA, check the quality and quantity of the DNA, PCR procedure after designing and procuring the respective primers. PCR-RFLP procedure with the help of digestive enzyme, Analyze and identify the mutation band patterns for each individualsusceptible to GC. Finally compare the epidemiological, mutational results to observe the risk association by statistically analysis.

Inclusion and exclusion criteria

All consecutive biopsy-proven GC patients between February 2017 and December 2020 were included in this study. Healthy cases were taken as controls irrespective of individual family history of cancer, gender, and ethnicity. Participants those who have a family history of cancer, autoimmune disorders, and advanced stage of GC were excluded from the study.

Histopathological examination

To confirm the GC cases, the histopathological examination was done with tissue obtained during the endoscopy procedure. The staining procedure with hemotoxyline and eosin (H & E) involving dewaxing, dehydration, differentiation, bluing, eosin, dehydration again, clearing and cover-slipping were followed. Hematoxylin is dissolved in distilled water by a gentle heat. Dye and alum ratio to be observed for the selective stating; hence alum is added and dissolved followed by adding sodium iodate, citric acid and

chloral hydrate. After the completion of the H & E staining the slide was observed under the microscope and identified as the adenocarcinoma (Fig. 1).

Demographic data and collection of clinical samples

Two ml of venous blood were collected from each study participant. The demographic details such as, age, sex, food habits, laboratory parameters, infection of *H. pylori*, endoscopy and biopsy findings were entered on a pre-designed proforma. GC Cases were followed up at every 3 months for a duration of 1 year or, till death, whichever occurred earlier. The cause and date of death were also recorded in the designed proforma.

DNA extraction and polymerase chain reaction process

DNA was isolated from peripheral whole blood sample by the salting out method. The isolated DNA samples were heparinized and stored at -20 °C, for further work. The quantity and the quality of extracted DNA were routinely checked by a Multiskan Sky Microplate Spectrophotometer designed by Thermo Fisher Scientific appliances (Fig. 2). For mutational analysis, a primer was developed with the help of national Centre for Biotechnology Information (NCBI) and the Primer3 software. Three primers namely, 1195GA, -1290AG, and -765GC (Primers were procured from Thermo Fisher Scientific) were designed for PCR of both forward and reverse sequences (Table 1). The appropriate annealing temperature was standardized for the PCR process for each primer set. Thermo cycling conditions of PCR starting from denaturation to final extension time duration were recorded in the multiplex PCR machine (Table 1). The amplified products were recorded for further digestion process (Fig. 3). The PCR products were digested with restriction enzymes at 37°C for 3 hours. COX-2-1195GA products were digested by the Pvu II (procured from thermo scientific), COX-2-765GC PCR products were digested by the Bsh 1236I, and COX-2-1290AG PCR products digested by Rsa I restriction enzyme (RE) (procured from Thermo Fisher Scientific), respectively procured from thermo scientific appliances (Table 1). The digested products were separated using 2.5% agarose gel through Agarose gel electrophoresis, stained with ethidium bromide, and observed under Gel doc. The Bio-Rad application provides a comprehensive range of imaging systems for detecting, quantifying, and analyzing DNAs in gels and membranes.

Three pair-wise polymorphisms in COX-2 such as, GA, GC and AG base transitions were assessed at positions, -1195GA having 273 base pair (bp), -765GC having 153 bp, and -1290AG having 173bp from the transcriptional start site. The promoter region polymorphic variants of -1195GA, -765GC, and 1290 AG demonstrated to know the functional effect on COX-2 transcription [11, 12], which are the contributory factor for occurrence of GC in subjects.

Six samples were processed and submitted to Gene bank which is published in NCBI with Accession numbers MW592005.1, MW592004.1, MW592003.1, MW592002.1, MW592001.1, MW592000.1 (https://www.ncbi.nlm.nih.gov/nuccore?term=uthansingh).

Sequenced detail provided by Gene bank

1. Cyclooxygenase03-02-2021Seq1 MW592000- LOCUS MW592000 206 bp DNA linear BCT 19-MAR-2021 DEFINITION *Helicobacter pylori* clone KU1 cycloxygenase gene, promoter region.
2. Cyclooxygenase03-02-2021Seq2 MW592001- LOCUS MW592001 206 bp DNA linear BCT 19-MAR-2021 DEFINITION *Helicobacter pylori* clone KU2 cycloxygenase gene, promoter region.
3. Cyclooxygenase03-02-2021Seq3 MW592002- LOCUS MW592002 206 bp DNA linear BCT 19-MAR-2021DEFINITION *Helicobacter pylori* clone KU3 cycloxygenase gene, promoter region.
4. Cyclooxygenase_03-02-2021Seq4 MW592003- LOCUS MW592003 206 bp DNA linear BCT 19-MAR-2021DEFINITION *Helicobacter pylori* clone KU4 cycloxygenase gene, promoter region.
5. Cyclooxygenase_03-02-2021Seq5 MW592004- LOCUS MW592004 206 bp DNA linear BCT19-MAR-2021DEFINITION *Helicobacter pylori* clone KU5 cycloxygenase gene, promoter region
6. Cyclooxygenase_03-02-2021 Seq6 MW592005- LOCUS MW592005 206 bp DNA linear BCT 19-MAR-2021DEFINITION *Helicobacter pylori* clone KU6 cycloxygenase gene, promoter region.

Statistical analysis

Statistical analysis was performed by SPSS version 21(SPSS-V21). The frequency differences of various alleles and genotypes between GC cancer and healthy subjects were performed by the chi-square test (χ^2 test). The *p*-values obtained were further correlated by multiplying with the number of alleles tested. All *p*-values were two-sided and only values < 0.05 were considered statistically significant. The difference of survival rate of GC was determined by taking GC with any of the three mutations and GC without any mutation; which was evaluated by the Kaplan Meier survival assessment curve.

Results

Clinical characteristics of enrolled subjects

The consecutive 124 confirmed cases of GC and 176 healthy cases were enrolledrecording various demographic data in the study. Different SNPs, thermocycle temperatures, and repeat sessions with duration and further different characteristics of three primers with individual sizes and digestive enzymes were demonstrated (Table 1). The demographic characteristics such as, sex, clinical status, socioeconomic status,

food habits, cigarette smoking, alcohol consumption, chewing of tobacco and infection of *H. pylori* were recorded (Table 2). Although males were predominant in both groups (54% in GC cases and 57% in control group), statistically, there was no significant difference in the demographic factors such as gender, i.e., males and females had the same chance of GC occurrence. Likewise, spicy food consumption or pain in abdomen or vomiting, loss of appetite or loss of weight or alcohol consumption and financial status among both groups had no impact on the occurrence of GC, as the respective *p* values of each criterion was lesser than the tabulated values, as given respectively: $p > 0.05$ (0.077 for males/females; 0.661 spicy food consumptions; 0.264 occurrence of pain in abdomen; 0.702 irregular vomiting; 0.464 attended with loss of appetite; 0.342 concomitant loss of weight; 0.991 alcohol consumption regularly/ with 3 days gaps; and 0.848 financial status as rich/ comparatively more rich. Thus, it is concluded that the above cited parameters had insignificant effect on development of GC. Alternatively, the risk factors such as, smoking of cigarettes and chewing of tobacco have significant contribution in GC development, as evident from the χ^2 analysis. The 20.5% GC cases vs. 31.50% healthy controls, when tested with the χ^2 test, the tabulated value was lower than the obtained value as 0.03 for cigarette smoking; while the similar value was 0.001 (29% GC cases vs. 11.9% healthy controls in chewing tobacco). Moreover, the infection of *H. pylori* was significant GC patients for the test about 39% GC cases vs. 12.5% healthy controls; the computed *p*-value was 0.001and the tabulated value was <0.05 (Table 2).

Thus, the demographic variables result with χ^2 test revealed that the chewing of tobacco habit, cigarette smoking and infection of *H. pylori* were the causative agents for GC developments.

COX-2 polymorphism and occurrence of GC

The distributions of COX-2 genotype frequencies of -1195 GA, -765GC, and -1290AG in GC cases and control group are given (Table 3). At the -1195 location, a fraction of 10.5% homozygous mutation (-1195 GG-AA) was observed. Furthermore, a fraction of 12.9% of heterozygous mutation was observed; which were significantly higher compared to those of controls, where homozygous and heterozygous mutations were found in 6.7% and 8.3% subjects, respectively. Similarly, at the -765 location among GC cases, a fraction of 6% homozygous mutation and 11% heterozygous mutation were recorded in GC case group, which were significantly higher compared to those of control group. In -1290 location, a fraction of 7% homozygous mutation and 16.9% heterozygous mutation were observed, as significantly higher than those of the control group.

The prevalence of COX-2 mutations among GC cases along with their *H. pylori* infection status was represented. Among *H. pylori* infected GC cases at 1195 location, the homozygous mutation was in 18.8%, and heterozygous mutation was in 22.9%, which were significantly higher compared to GC cases without *H. pylori* infection. Similarly at the 765 locations, the homozygous mutation was in 10.4% patients, and heterozygous mutation was in 27.1% cases with *H. pylori* infection, but only in 2.6%; while 10.5% of cases were without *H. pylori* infection, respectively. At the 1290 location, both homozygous and heterozygous

mutations were found in a significantly higher proportion of cases with *H. pylori* infection. There was a significant co-relationship observed between the *H. pylori* infection and all the three mutations in the GC cases (p<0.005); which indicated that all these mutations along with *H. pylori* infection may contribute for the development of GC cases (Table 4). A one-year follow-up assessment of GC cases with and without *H. pylori* infection was undertaken with regular interval of three months. While a fraction of 6.3% patients with *H. pylori* infection died within a year; surprisingly, a higher number (14.5%) of GC cases without *H. pylori* infection died within 1 year (Table 5). The survival assessment by Kaplan-Meier curve analysis revealed that one-year survival of un-mutated GC cases was significantly less in comparison to the mutated GC patients (p=0.039) (Fig. 4). In univariate analysis, smoking, tobacco use, *H. pylori* infection, and COX-2 mutations at -1195, -765, and -1290 were significantly associated with the GC development; whereas *H. pylori* infection and tobacco abuse were independent predictors for GC inferred from the Multivariate analysis (Table 5).

Discussions

Various risk factors were seen associated with GC namely, lifestyle as aging, dietary intake, alcohol consumption, smoking habits, infection of *H. pylori,* along with SNPs of the COX-2 gene. The demographic characteristics and lifestyle habits between the GC cases and healthy control group were compared. Except smoking and tobacco abuse, there was not any statistically significant difference. It is known that unhealthy food habits namely, spicy foods, high cereal intake, soda, smoked, salted meat or fish have the putative risks for GC [16-18]. Smoking and tobacco-chewing habit were the significant factors for GC [16, 19]. The present study corroborated to an earlier study on association of alcohol-abuse and GC [17].

Infection from *H. pylori*was declared by WHO as a class-I carcinogen for GC, and the present study too corroborated it, along with an ample of in Indian studies have revealed the consistent results for *H. pylori* infection as a risk factor for GC [20-23]. Moreover, in the multivariate analysis, *H. pylori* was seen here as an independent predictor of GC development. Furthermore, SNPs of COX-2 gene at loci 1195GA, 765GC and 1290AG had a significant association in promoting GC. These results suggested that COX-2 gene polymorphism might be leading to inflammation followed by metastasis of gastrointestinal tumors as, COX-2 is the key mediator of inflammatory pathways [24]. Moreover, high level of COX-2 though over expression promotes tumor cell proliferation, which leads to inhibit tumor cell apoptosis to evade immune surveillance, and concomitantly accelerates the transformation of the precancerous lesion to cancer [25].

Both homozygous mutation (AA) and heterozygous mutation (AG) were at the 1195 location as significant among GC cases, compared to that of control group (p value 0.003). In the univariate regression analysis, both homozygous and heterozygous mutation status was significantly associated with the development of GC, as well. But, -1195GA was not the independent predictor for GC development, when adjusted for age, smoking, tobacco-chewing, and infection of *H. pylori*. The present study supported an increased association of GC with 1195 GA mutation, like reported [26]. Apart from GC, -1195 GA polymorphism might also be the risk factors for the development of other cancers such as, pancreatic cancer, and hepatocellular carcinoma (HCC) [27, 28). A few inconsistent of the present study was observed when corroborated with Asian and Caucasian population. The mechanism behind the inconsistency was hypothesized as the generation of a

new binding site for transcription factor C-MYB; which enhanced the transcription of COX-2 and ultimately leads to rise of over-expression [29].

Similarly, significant numbers of GC cases were observed when compared to healthy controls at -765 chromosomal location showing homozygous and heterozygous mutations. The present observation of SNPs mutation was corroborated with previous study revealing the association between 765GC mutation and the risk of GC [15, 30, 31]. Hence this particular 765G location of COX-2 has the positive increased susceptibility to GC when compared with other studies explored among Asian and Brazilian populations [14, 32, 33].

The present study also evaluated at -1290 locus of COX 2 gene and observed that the 1290 AG mutation in homozygous and heterozygous states revealing a significant number of GC cases compared to healthy controls. Very few studies have reported about the 1290 locus showing an increased risk of GC association with 1290 GA gene variant [34].

Although, the present study could not find a significant association of alcohol-abuse with GC risk; but, studies from different regions state a significant association of alcohol-abuse and smoking with -765GC genotype mutation with resultant increased risk of GC in their study subjects [33].

The predictors for the development of GCs by univariate analysis, the SNP variation were found to be significantly associated with GC. But, when adjusted for other factors like age, smoking, consumption tobacco, and *H. pylori* infection, those were not independent predictors for GC. Therefore, the relationship between *H. pylori* infection and the three SNPs observed in the present study significant among cases with *H. pylori* infection in comparison to that of cases without H. *pylori* infection. Although, a few previous studies suggested that COX -2 over expression induced by *H. pylori* infection and smoking; but till date, it was not fully established with available evidence [35]. Both *H. pylori* infection and smoking might be associated with an increased risk for GC in 765 C carriers, but not in 1195 A carriers revealed by a meta-analytical study elsewhere [29]. One year mortality was relatively higher in mutated case group compared to un-mutated case group. A study from North India revealed that a nine-fold increased risk of GC was significantly higher compared to all other addressed ethnical populations. This indicated that the variable effect of genetic polymorphism dependent on the targeted population, geographic variation, ethnicity, and environmental and dietary factors, which needs to be explored in the future by long term prospective studies.

Strength and limitations

To the best of our knowledge, this is the first study ever Indian study, which comprehensively and vividly evaluated the association of COX-2 polymorphisms at three different loci with the risk of development of GC in Eastern Indian population. Single-center evaluation and shorter duration of follow up might be the greatest limitation of this study. The exact patho-physiologic mechanism behind the effect of SNPs on the survival of GC could not be explored. Only 3 SNPs of COX 2 gene instead of assessing all the SNPs, which may variably affect the present result were evaluated.

Conclusions

It was observed that − 1195 GA, -765GC, and − 1290AG promoter polymorphisms (SNPs) of COX 2 gene were significantly associated with GC. These three SNPs might be taken as genetic markers for an early detection of GC in future. The present study findings supported the carcinogenic potentiality of chewing-tobacco and smoking abuse in GC; whereas it could not establish the carcinogenic potentiality of alcohol drinking in causation of GC. This study comprehensively evaluated the association of COX-2 polymorphisms at three loci and the risk of development of GC in the Eastern Indian population and evidenced a positive association. chewing-tobacco, smoking, and *H. pylori* infection were significantly associated with the development of GC.

Declarations

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Authors' contribution K.U. data collection, performed the experiment and data analysis, carried out statistical analysis and co-wrote the manuscript. D.M and G.K.P. consented patients for procurement of samples. R.N.P. Supervised the conduction of experiment, assisted in writing and revision of the manuscript. M.K.S. designed and directed the project, and co-wrote the manuscript.

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Conflicts of interest the authors have declare no conflict of interests to declare**.**

Ethical approval The study was conducted in compliance with all national and international ethical standards for research with humans. Patients provided informed consent for the sample and data acquisition and the study received full ethical approval from the Institutional ethics committee of the Institution bearing IEC Ref No. DMR/IMS-SU/SOA/160129, dated 2nd Dec 2016.

Consent to participate All patients provided informed consent for sample and data acquisition.

Availability of data and material The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Etemadi A, Safiri S, Sepanlou SG, et al. The global, regional, and national burden of stomach cancer in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease study 2017. Lancet Gastroenterol Hepatol. 2020;5(1):42–54.
2. Pourhoseingholi MA, Vahedi M, Baghestani AR. Burden of gastrointestinal cancer in Asia; an overview. Gastroenterol Hepatol Bed Bench. 2015;8(1):19–27.
3. Murugesan CS, Manickavasagam K, Chandramohan A, et al. Gastric cancer in India: epidemiology and standard of treatment. Updates in surgery. 2018;70(2):233–39.
4. Shrikhande SV, Sirohi B, Barreto SG, et al. Indian Council of Medical Research consensus document for the management of gastric cancer. Indian J Med Paediatr Oncol. 2014;35(04):239–43.
5. Hussain MA, Pati S, Swain S, et al. Pattern and trends of cancer in Odisha, India: a retrospective study. Asian Pac J Cancer Prev. 2012;13(12):6333–336.
6. Chatterjee S, Levine PH, Senapati SN, et al. Cancer patterns in Odisha: An important mining state in India. Int J Cancer Clin Res. 2019;6(5):126.
7. Uthansingh K, Sahu MK, Narayan J, *et al*. COX-2 and CYP2C9 polymorphism and the risk of gastric cancer in the population of Odisha. J Gastroenterol Hepatol. 2019;34: 258.
8. Du J, Xu Y, Dai J, et al. Genetic variants at 5p15 are associated with risk and early onset of gastric cancer in Chinese populations. Carcinog. 2013;34(11):2539–42.
9. Rawla P, Barsouk A. Epidemiology of gastric cancer: global trends, risk factors and prevention. Prz Gastroenterol. 2019;14(1):26–38.
10. Chiba T, Marusawa H, Seno H, et al. Mechanism for gastric cancer development by Helicobacter pylori infection. J Gastroenterol Hepatol. 2008;23(8pt1):1175–81.
11. Ke-Xiang Z, Yu-Min L, Xun L, et al. Study on the association of COX-2 genetic polymorphisms with risk of gastric cancer in high incidence Hexi area of Gansu province in China. Mol Biol Rep. 2011;38(1):649–55.
12. Akkız H, Bayram S, Bekar A, et al. Functional polymorphisms of cyclooxygenase-2 gene and risk for hepatocellular carcinoma. Mol Cell Biochem. 2011;347(1):201–08.
13. Tian J, Liu G, Zuo C, et al. Genetic polymorphisms and gastric cancer risk: a comprehensive review synopsis from meta-analysis and genome-wide association studies. Cancer Biol Med. 2019;16(2):361–89.
14. Liu JL, Liang Y, Wang ZN, et al. Cyclooxygenase-2 polymorphisms and susceptibility to gastric carcinoma: a meta-analysis. World J Gastroenterol. 2010;16(43):5510–17.
15. Hou L, Grillo P, Zhu ZZ, et al. COX1 and COX2 polymorphisms and gastric cancer risk in a Polish population. Anticancer Res. 2007;27(6C):4243–47.
16. Mathew A, Gangadharan P, Varghese C, et al. Diet and stomach cancer: A case-control study in South India. Eur J Cancer Prev. 2000;9(2):89–97.
17. Rao DN, Ganesh B, Dinshaw KA, et al. A case-control study of stomach cancer in Mumbai, India. Int J Cancer. 2002;99(5):727–31.

18. Siddiqi M, Kumar R, Fazili Z, et al. Increased exposure to dietary amines and nitrates in a population at high risk of esophageal and gastric cancer in Kashmir (India). Carcinog. 1992;13(8):1331–35.
19. Phukan RK, Zomawia E, Narain K, Tobacco use and stomach cancer in Mizoram, India. Cancer Epidemiol Biomarker Prev. 2005;14(8):1892–96.
20. Saxena A, Nath Prasad K, Chand Ghoshal U, et al. Association of Helicobacter pylori and Epstein-Barr virus with gastric cancer and peptic ulcer disease. Scand J Gastroenterol. 2008;43(6):669–74.
21. Ghoshal UC, Tiwari S, Dhingra S, et al. Frequency of Helicobacter pylori and CagA antibody in patients with gastric neoplasms and controls: The Indian enigma. Dig Dis Sci. 2008; 53(5):1215–22.
22. Misra V, Misra SP, Singh MK, et al. Prevalence of H. pylori in patients with gastric cancer. Indian J Pathol Microbiol. 2007;50(4):702–07.
23. Khanna AK, Seth P, Nath G et al. Correlation of *Helicobacter pylori* and gastric carcinoma. J Postgrad Med. 2002;48(1):27–28.
24. Liu Y, Sun H, Hu M, et al. The role of cyclooxygenase-2 in colorectal carcinogenesis. Clin Colorectal Canc. 2017;16(3):165–72.
25. Misra S, Sharma K. COX-2 signalling and cancer: new players in old arena. Curr Drug Targets. 2014;15(3):347–59.
26. Li Y, Dai L, Zhang J, et al. Cyclooxygenase-2 polymorphisms and the risk of gastric cancer in various degrees of relationship in the Chinese Han population. Oncol Lett. 2012;3(1):107–12.
27. Wang Y, Jiang H, et al. Cyclooxygenase-2-1195G > A (rs689466) polymorphism and cancer susceptibility: an updated meta-analysis involving 50,672 subjects. Int J Clin Exp Med. 2015;8(8):12448–462.
28. Zhang XW, Li J, Jiang YX, et al. Association between COX-2 -1195G > A polymorphism and gastrointestinal cancer risk: A meta-analysis. World J Gastroenterol. 2017;23(12):2234–45.
29. Luo MX, Long BB, Li F, et al. Roles of cyclooxygenase-2 gene – 765G > C (rs20417) and – 1195G > A (rs689466) polymorphisms in gastric cancer: A systematic review and meta-analysis. Gene. 2019;685(2019):125–35.
30. Sitarz R, Leguit RJ, De Leng WW, et al. The COX-2 promoter polymorphism–765 G > C is associated with early-onset, conventional and stump gastric cancers. Mod Pathol. 2008;21(6):685–90.
31. Saxena A, Prasad KN, Ghoshal UC, et al. Polymorphism of-765G > C COX-2 is a risk factor for gastric adenocarcinoma and peptic ulcer disease in addition to *H. pylori* infection: a study from northern India. World J Gastroenterol. 2008;14(10):1498–03.
32. Wang XF, Huang MZ, Zhang XW, et al. COX-2-765G > C polymorphism increases the risk of cancer: a meta-analysis. PloS one. 2013;8(9):e73213.
33. Campanholo VM, Felipe AV, Lima JM, et al. -765 G > C Polymorphism of the COX-2 gene and gastric cancer risk in Brazilian population. Arq. Gastroenterol. 2014;51(2):79–83.
34. Zhang XM, Miao XP, Tan W, et al. Genetic polymorphisms in the promoter region of cyclooxygenase-2 and their association with risk of gastric cancer. Zhongguo yi xue ke xue Yuan xue bao. Acta Acad Med Sin. 2006;28(2):119–23.

35. Zhang XM, Zhong R, Liu L, et al. Smoking and COX-2 functional polymorphisms interact to increase the risk of gastric cardia adenocarcinoma in Chinese population. PLoS. One. 2011;6(7):e21894.

Abbreviations

GC	Gastric cancer
H. pylori	*Helicobacter pylori*
COX-2	Cycloxygenase-2
SNP	Single nucleotide polymorphism
PCR-RFLP	Polymerase chain reaction- restriction fragment length polymorphism
GCP	Good clinical practice
WHO	World Health Organization
ICMR	Indian Council of Medical Research
HCC	Hepatocellular carcinoma

Tables

e 1 Three primers with their sizes, digestive enzymes and PCR program

ıo.	SNPs	Nucleotides	Base pair	Annealing temp. (In °C)	Digestive enzyme
	- 1195 GA	Forward: 5’-CCCTGAGCACTACCCATGAT-3' Reverse: 5’-GCCCTTCATAGGAGATACTGG-3'	273	54	Pvu II
	-765 GC	Forward: 5’-ATTCTGGCCATCGCCGCTTC-3’ Reverse: 5’-CTCCTTCTTTCTTGGAAAGAGCG-3’	157	56	Bsh1236I (Bst UI)
	- 1290 AG	Forward: 5’-CAGGTTATGCTGTCATTTTCC-3’ Reverse: 5’-TAGTGCTCAGGGAGGAGCAT-3’	173	62	Rsa I

{ program

.e ne	Step-1 Denaturation	Step-2 Annealing	Step-3 Elongation	Step-4 Extension	Step-5 Final Extension
ζ-2 5GA	95 ° for 5 mins	35 cycles of 95°for 30 sec	54°for 30 seconds	70° for 30 sec	72 ° for 5 mins
ζ-2 G>C	95°C for 10 min	35 cycles of 95°C for 30sec	56°C for 30sec	72°C for 30sec	72°C for 7 min
ζ-2- 0A>G	95°C for 5 min	35 cycles of 95°C for 30sec	62°C for 30 sec	72°C for 30 sec	72°C for 5 min

The enzyme Pvu II recognizes CAG^CTG sites, Bsh1236I (BstUI) recognizes CG^CG sites and Rsa I recognize GT^AC site.

Table 2 Distribution of selected demographic variables and risk factors of cases and control groups

	Control N_1=174 (58%)		Cases N_0=124 (42%)		*P* value
Variables	n_1	%	n_0	%	
Male	98	55.7	67	54	0.77
Female	78	44.3	57	46	
Spicy food consumers	102	58	75	60.50	0.661
Pain Abdomen	131	74.4	85	68.5	0.264
Vomiting	72	40.9	48	38.7	0.702
Appetite loss	77	43.8	49	39.5	0.464
Weight loss	85	48.3	53	42.7	0.342
Alcohol	37	21	26	21.00	0.991
Smoking	36	20.5	39	31.50	0.03
Tobacco chewing	21	11.9	36	29	0.001
Socio-economic Status					
Poor (<67.64$ per month)	90	51.1	60	48.4	
Middle (>67.64<676.42$ per month)	84	47.7	63	50.8	
Higher >676.42$	2	1.1	1	0.8	0.848
Infection of *H. pylori*					
H. pylori +ve	22	12.5	48	38.7	<0.001
H. pylori -ve	154	87.5	76	61.3	

N1= Total number of samples in case group

N0= Total number of samples in control group

n_1 (Column 2) = Individual frequency distribution of different factors for control group

% (Column 3) = Fraction distribution of individual factors for the control group

n_0 (Column 4) = Individual frequency distribution of different factors for case group

% (Column 5) = Fraction distribution of individual factors for the case group

Gender (Male and Female), Intake of spicy food yes or no, pain abdomen yes or no, vomiting yes or no, appetite loss yes or no, weight loss yes or no, alcohol drinking yes or no, smoking habit yes or no, Chewing of tobacco yes or no.

a Two-sided χ^2 test; the tabulated χ^2 value at df= 298 (as sample size is 300); the tabulated values of all individual criteria were in the SPSS demi table was less the tabulated value, for df=298, the *p* value = 0.05; for the comparison between cases and control using χ^2 tests.

‡χ2 test; $\chi^2 = \sum (O_i - E_i)^2/E_i$

χ2 =Chi square

Oi= observed value

Ei = Expected value

‡ *p*-value < 0.05 is significance

Factors like smoking, tobacco chewing, and *H. pylori* infection the *p* values < 0.05, hence rejected the null hypothesis. Factors like gender, spicy food intake, abdominal pain, vomiting, loss of appetite, weight loss and socio-economic status the *p* values > 0.05, hence retain the null hypothesis

Table 3 COX- 2 genetic mutations in gastric cancer cases and the control group

SNP Mutations	Control (N_1=176)		GC case (N_0=124)		Total (N=300)		'*p*' value
- 1195GA	n_1	%	n_0	%	n = (n_1+n_0)	%	
Wild type (AA)	160	90.9	95	76.6	255	85	0.003
Heterozygous (AG)	9	5.1	16	12.9	25	8.3	
Homozygous mutants (GG)	7	4	13	10.5	20	6.7	
-765GC							
Wild type (CC)	159	90.3	96	77.4	255	85	0.007
Heterozygous (GC)	11	6.3	21	16.9	32	10.7	
Homozygous mutants (GG)	6	3.4	7	5.7	13	4.3	
-1290AG							
Wild type (AA)	157	89.2	92	74.2	249	83	0.003
Heterozygous (AG)	12	6.8	21	16.9	33	11	
Homozygous mutants (GG)	7	4	11	8.9	18	6	

N1= Total number of samples in case group
N0= Total number of samples in control group
n1 (Column 2) = individual frequency distribution of different factors for control group
% (Column 3) = Fraction distribution of individual factors for the control group
n_0 (Column 4) = individual frequency distribution of different factors for case group
% (Column 5) = Fraction distribution of individual factors for the case group
Statistical analysis irrespective of *H. pylori* infection among the case and control group
aTwo-sided $\chi 2$ test; the tabulated $\chi 2$ value at df= 298 (as sample size is 300)
† Student t test.
‡$\chi 2$ test.
‡ *p*-value < 0.05 is significance

SNP mutation among the case and control group, the significant p value < 0.05, hence rejected the null hypothesis

Table 4 Co-relation between COX-2 (-1195GA, -765 GC, -1290 AG) mutations with *H. pylori* and without *H. pylori* GC*

		COX21195GA			p-value
		AA	AG	GG	
H. pylori	+ve	28(58.3%)	11(22.9%)	9(18.8%)	0.001
	-ve	67(88.2%)	5(6.6%)	4(5.3%)	
		COX2765GC			
		CC	GC	GG	
H. pylori	+Ve	30(62.5%)	13(27.1%)	5(10.4%)	
	-Ve	66(86.8%)	8(10.5%)	2(2.6%)	0.006
	COX21290AG				
		AA	AG	GG	
H. pylori	+Ve	30(62.5%)	13(27.1%)	5(10.4%)	0.040
	-Ve	62(81.6%)	8(10.5%)	6(7.9%)	

H. pylori: *Helicobacterpylori*

*Two-sided χ^2 test; the tabulated χ^2 value at df= 122 (as sample size is 124)

‡ p-value ≤ 0.05 is significance

Compared different SNPs among GC with *H. pylori* infection and without *H. pylori* infection, the significant p value < 0.05, hence rejected the null hypothesis in all SNPs of three locations of COX-2 (-1195, -765 and -1290)

Table 5 Univariate and multivariate analysis for various risk factors

Univariate analysis				Multivariate analysis			
Different risk factors	OR	95% C.I. for OR		'p' Value	AOR	95% C.I. for AOR		'p' Value
Lower	Upper	Lower	Upper					
Age	1.032	1.013	1.050	0.001	1.019	0.998	1.039	0.071
Sex	0.936	0.590	1.485	0.777	0.811	0.483	1.362	0.429
BMI	0.988	0.924	1.056	0.724				
Rice based	1.434	0.478	4.303	0.521				
Wheat based	1.396	0.869	2.242	0.167				
Spicy food	1.110	0.695	1.773	0.661				
Alcohol	0.997	0.567	1.752	0.991				
Smoking	1.784	1.053	3.023	0.031	1.395	0.766	2.539	0.276
Tobacco chewing	3.019	1.660	5.493	0.000	2.499	1.297	4.818	0.006
H. pylori	4.421	2.489	7.853	0.000	2.922	1.560	5.471	0.001
COX-2 1195AG	3.128	1.206	8.114	0.019	2.376	0.841	6.717	0.103
COX-2 1195GG	2.994	1.273	7.042	0.012	1.720	0.663	4.463	0.265
COX-2 765GC	1.932	0.631	5.919	0.249	1.273	0.357	4.547	0.710
COX-2 765GG	3.162	1.461	6.844	0.003	1.958	0.671	5.711	0.219
COX-2 1290AG	2.682	1.005	7.159	0.049	1.839	0.611	5.540	0.279
COX-2 1290GG	2.986	1.404	6.351	0.004	1.451	0.507	4.152	0.488

OR and 95% CI for log-additive model for demographic and each allele estimated by unconditional logistic regression analysis

OR>1 Associated with disease (Null hypothesis is rejected)

OR<1 Protective

OR=1 No association

$$Odds\ ratio(OR) = \frac{Odds\ of\ being\ exposed - case}{Odds\ of\ being\ exposed - control}$$

If the 95% confidence interval for an OR includes 1, it means the results are not statistically significant

AOR: Adjusted odds ratio

AOR is a conditional odds ratio

p value significant ≤ 0.05

The degrees of freedom for the chi-square df = (r-1) (c-1)

when chi-square test statistic is greater than the critical value, the null hypothesis is rejected.

Chewing tobacco and smoking is the only factors showed the significance in multivariate analysis having *p* value < 0.05, all other factors *p* value > 0.05

Figures

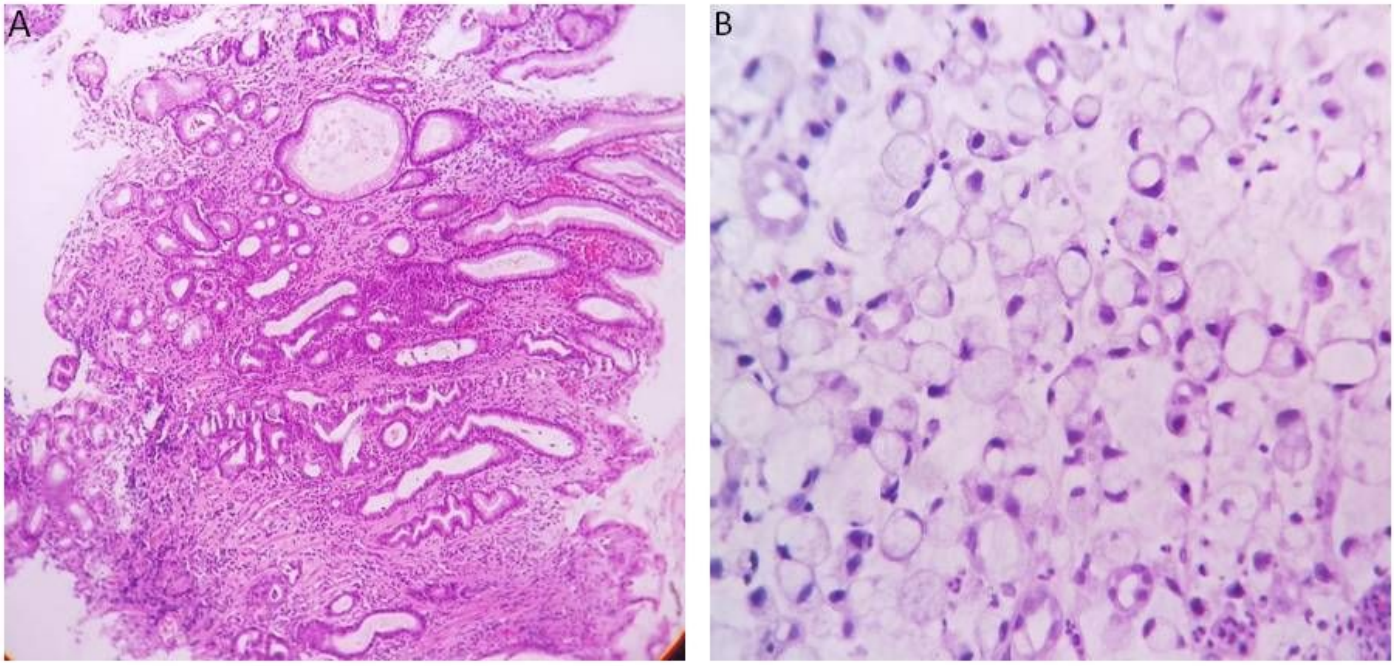

Figure 1

Microscopic examination of tissue, morphological and cellular illustrations of suspicious gastric growth that was diagnosed as GC on biopsy and resected. A: Mucinous adenocarcinomas tumors originated from epithelial tissues, B: Signet ring cell adeno carcinomas are the highly malignant adenocarcinomas producing mucin

Figure 2

Confirmation of DNA Quality and its quantity by spectrophotometer, Nucleic acid based analysis A,B,C,D, E,F, G and H denotes different DNA samples

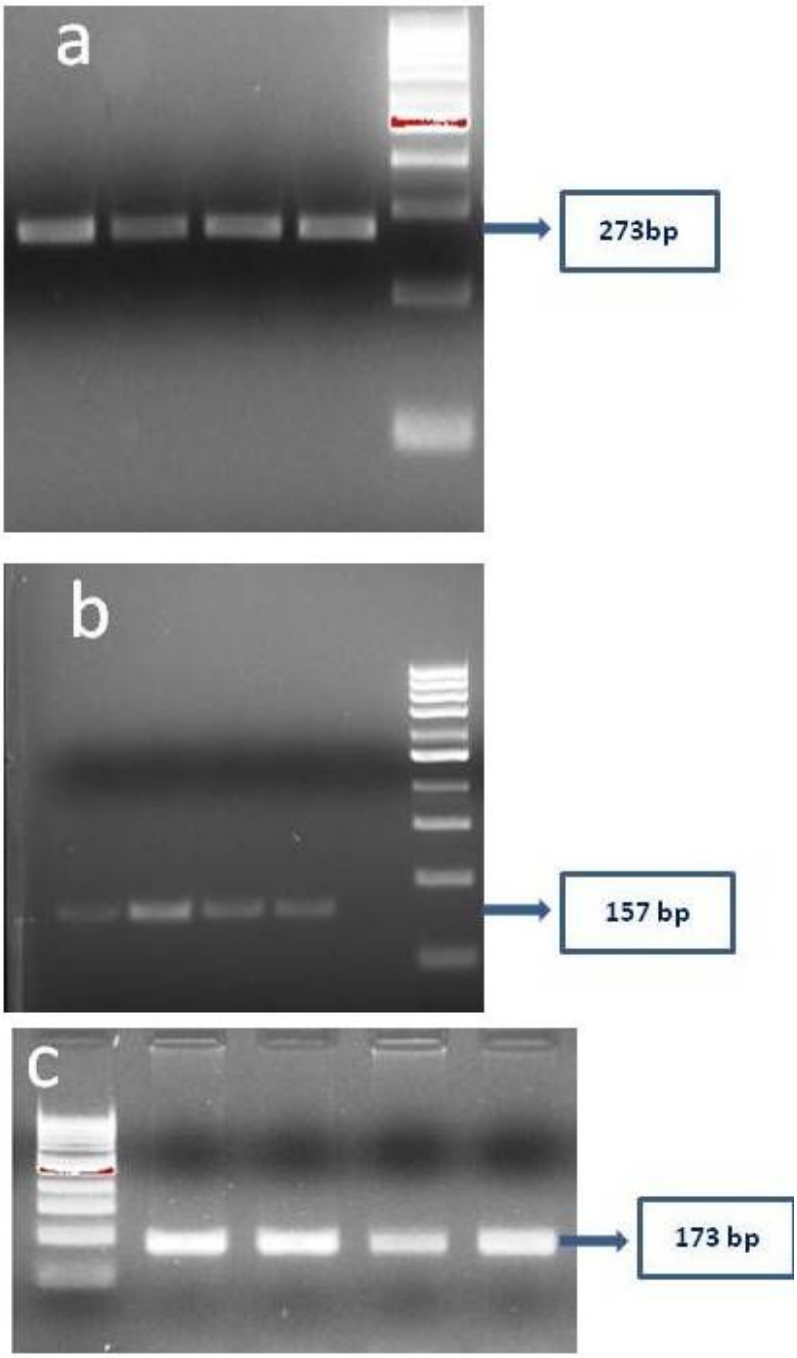

Figure 3

PCR for three SNPs (-1195GA, 765GC, and 1290AG); PCR agarose gels, PCR products amplified with primers a; COX-2 1195GA having size of273 bp, b; 765GC having size of 157bp, c;1290AG having size of 173 bp

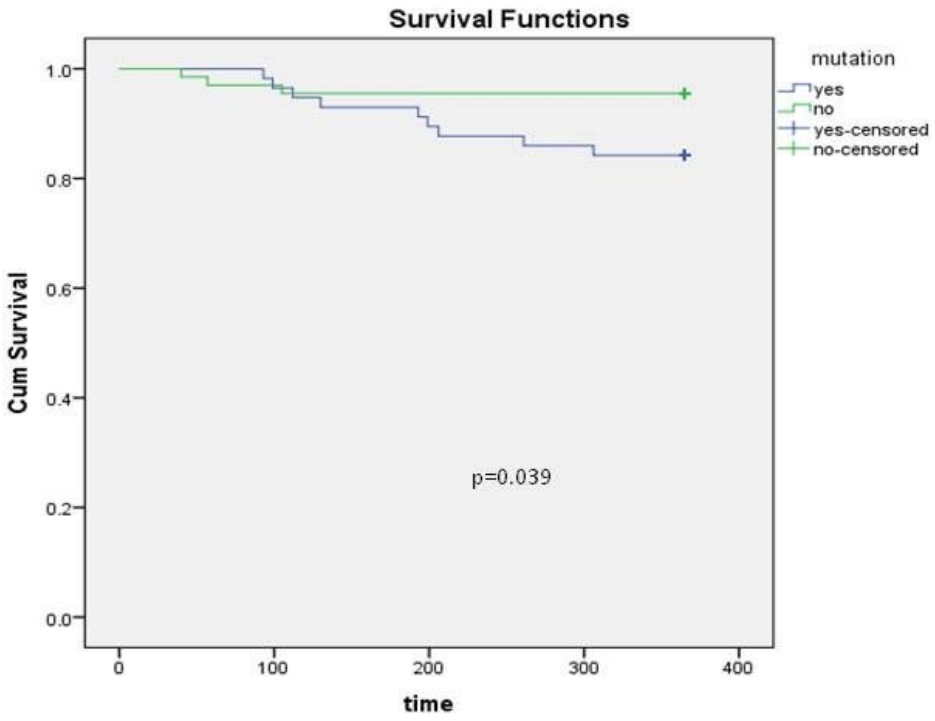

Figure 4

Kaplan Meier survival analysis of GC with respect to overall SNP mutation; Kaplan Meir plot for two conditions associated with patient survival