Variants of SMAD1 gene increase the risk of colorectal cancer in the Bangladeshi population

Priyanka Florina Karmokar1*, Samia Shabnaz1*, Md. Abdul Aziz2, Md. Asaduzzaman1, Mohammad Shahrriar1, Mohiuddin Ahmed Bhuiyan1, Abu Syed Md Mosaddek3 and Mohammad Safiqul Islam2

Abstract
Colorectal cancer is the fourth most common type of malignancy worldwide that may develop due to the accumulation of several genetic variations. Different single nucleotide polymorphisms of SMAD1 gene are assumed to be linked with increased colorectal cancer risk. The current case-control study was conducted to verify the association of genetic polymorphisms of SMAD1 (rs11100883 and rs7661162) with colorectal cancer in the Bangladeshi population. This study was performed on 275 colorectal cancer patients and 300 healthy volunteers using polymerase chain reaction–restriction fragment length polymorphism method. The odds ratios were adjusted for age and sex with logistic regression analysis. In case of SMAD1 rs11100883 polymorphism, GA heterozygous genotype, GA + AA (dominant model), and minor allele “A” were significantly associated with colorectal cancer (adjusted odds ratio = 1.55, 95% confidence interval = 1.09–2.20, p = 0.014; adjusted odds ratio = 1.59, 95% confidence interval = 1.13–2.23, p = 0.008; and odds ratio = 1.35, 95% confidence interval = 1.06–1.73, p = 0.015, respectively) and the significance exists after the Bonferroni correction. Again, single nucleotide polymorphism rs7661162 showed significant association with an elevated colorectal cancer risk for AG heterozygous genotype, AG + GG (dominant model), AG versus AA + GG (overdominant model), and minor allele “G” (adjusted odds ratio = 1.78, 95% confidence interval = 1.24–2.56, p = 0.002; adjusted odds ratio = 1.68, 95% confidence interval = 1.18–2.39, p = 0.004; adjusted odds ratio = 1.76, 95% confidence interval = 1.23–2.53, p = 0.002; and odds ratio = 1.47, 95% confidence interval = 1.08–2.00, p = 0.014, respectively) and significance withstands after the Bonferroni correction. No significant age and gender differences between cases and controls were observed. In silico, gene expression analysis showed that the SMAD1 mRNA level was downregulated in the colon and rectal cancer tissues compared to healthy tissues. In conclusion, our findings indicate that SMAD1 rs11100883 and rs7661162 polymorphisms are responsible for increasing the susceptibility of colorectal cancer development in the Bangladeshi population.

Keywords
Colorectal cancer, genetic polymorphism, polymerase chain reaction–restriction fragment length polymorphism, SMAD1, Bangladesh

Introduction
Colorectal cancer (CRC) is the fourth leading cause of cancer-associated deaths worldwide. It is estimated that around 2.2 million people will be affected by CRC leading to almost 1.1 million deaths by 2030.1 CRC is the third most common cancer of men (746,000 cases,
which are 10.0% of the total) and the second in case of women (614,000 cases, which are 9.2% of the total) globally.² CRC typically initiates as a polyp, a noncancerous development that grows slowly and progresses on the internal coating of the rectum or colon over a period of 10–20 years.³ ⁴ Apart from deskbound lifestyle, age and race, Western diet, and smoking are behaviors habitually allied with high-income countries are the most identified risk factors for CRC. Besides, an individual or family history of CRC or adenomatous polyps and individual history of chronic inflammatory bowel disease over an extended period are non-variable factors that are likely to increase the risk of CRC.⁵–⁷

Despite an effective treatment present for both early and localized diseases involving surgery, a considerable number of patients having advanced localized colon carcinomas (CCs) will eventually build up liver metastases, which remain largely inoperable.⁸ Hence, reassessment of existing ideas and identification of novel ways to fight CCs and metastatic disease is essential.

A better understanding of the genetics of the CRC, whether of the familial origin or sporadic, has been attained after the discovery of human homologues of the Drosophila Mad gene called SMAD (small mothers against decapentaplegic) genes.⁹ They are equivalent to the gene yields of the Drosophila gene named “mothers against decapentaplegic” (Mad) and the Caenorhabditis elegans gene called Sma.⁸ ¹⁰ Till now, eight mammalian SMADs are described, which are classified into three groups according to their functions in signal transduction.¹¹ SMAD proteins have been found to play a key role in the pathogenesis of inflammatory bowel disease (IBD), that have exceptional characteristics compared to hereditary and sporadic cancer.¹² One of the SMAD proteins that are recently reported to be linked with CRC is SMAD1 (SMAD family member 1), which falls into the receptor-activated SMAD’s group. It is encoded by the SMAD1 gene and mediates the signals of the bone morphogenetic proteins (BMPs), which are implicated in a variety of biological activities as well as cell growth, apoptosis, morphogenesis, development, and immune responses. This protein can be phosphorylated and triggered by the bone morphogenetic protein (BMP) receptor kinase in response to BMP ligands. The phosphorylated form of this protein further generates a complex with SMAD4, which is important for its task in the transcription regulation.⁸⁻¹³

Anomalies in SMAD1 functions are associated with various types of developmental defects and diseases. For instance, SMAD1 plays an imperative role in cell invasion and metastasis because it can be induced by many tumors stimulating cytokines such as the bone morphogenetic protein 2 (BMP2) and tumor necrosis factor alpha (TNFα).¹⁴ ¹⁵ Moreover, SMAD1 promotes the epithelial–mesenchymal transition (EMT) process in the CRC cells since it is upregulated by B7-H3 via the PI3K-Akt pathway.¹⁶ Even though SMAD1 has been found to be involved in the metastatic progression of many cancer types, the thorough molecular signaling pathway underlying the regulatory link between SMAD1 and metastasis remains obscure.¹⁷ It has recently been exposed that the BMP-SMAD1 pathway functions as a tumor suppressor and stabilizes the distinguished p53 tumor suppressor. Consequently, it is suspected that the disruption of this interaction plays a role in tumorigenesis and the development of juvenile polyposis and cancer.¹⁸ Analysis of >500 colorectal tumors and comparison of the rectal tumor against paired paraneoplastic subjects revealed that SMAD1 was upregulated in tumor samples, which was due to p53 defects and DNA damage.¹⁵ Another study that sheds light on the pivotal role of SMAD1 in CRC reported that Snail and Ajuba are upregulated by SMAD1, which in turn promotes cell migration for the progression of CRC. Furthermore, the expression of Ajuba and SMAD1 is firmly correlated, signifying that SMAD1 and Ajuba may be prospective therapeutic targets and prognostic factors for CRC.¹⁷ The genetic variation of SMAD1 may mislead the transcriptional regulation and causes cancer according to the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/dbget-bin/www_bget?pathway:hsa05202).

Although no genetic association study has been performed particularly with SMAD1 gene variants in the Bangladeshi population, we hypothesized that genetic variants of this gene might be associated with the risk of CRC found from the network-based (http://UALCAN. path.uab.edu/analysis.html) SMAD1 mRNA analysis. A significant downregulation of SMAD1 mRNA expression was found in the colon and rectal cancer tissues compared to healthy tissues. We also analyzed the BEB (Bengali from Bangladesh) population data from the 1000 genome database and found these rs11100883 and rs7661162 single nucleotide polymorphisms (SNPs) are suitable for analysis based on the minor allele frequency and heterozygosity. In the present case-control study, two SNPs of SMAD1 such as rs11100883 and rs7661162 were chosen to assess the association between these variants and the susceptibility of CRC. Our study might help to establish the role of the SMAD1 gene for the progression of CRC risk in the Bangladeshi ethnic group.

Materials and methods

Study subjects

This study incorporated 275 CRC patients and 300 cancer-free controls. All patients were histologically detected with CRC who reported in the National Institute of Cancer Research and Hospital (NICRH), Dhaka, Bangladesh. The cancer-free controls were
genetically unconnected to the cases, and had no individual or family history of cancer. Informed consent was taken from each of the qualified subjects before recruitment. An approved questionnaire from NICRH was used to obtain demographic and risk factor information about the study subjects. The clinicopathological variables, including tumor site, age, sex, metastasized cancer, and family history of cancer, were collected from the medical records of patients with a validated questionnaire. The patients who had a family history of cancer in their first relatives were excluded from the study. The study protocol and questionnaire were also approved by the ethical committee of NICRH. This research was conducted following the guidelines of the International Conference of Harmonization for Good Clinical Practice and in compliance with the Declaration of Helsinki and its further amendments (adopted by the 18th WMA general assembly, Helsinki, Finland, on June 1964 and last amendment in Fortaleza, Brazil, on October 2013). The genotyping analysis was performed in the Laboratory of Biotechnology of the Department of Pharmacy, University of Asia Pacific, Bangladesh.

Gene and SNPs selection

We selected this gene as we found it is significantly downregulated in the colon and rectal cancer compared to healthy tissue from network-based tissue expression analysis (http://ualcan.path.uab.edu/analysis.html). We selected these two SNPs from the 1000 genome database based on the (1) minor allele frequency will be higher than 10% in the Bengali in Bangladesh (BEB), (2) heterozygosity more than 15% in the BEB population, and (3) SNPs that are not enlisted in the published genome-wide association studies (GWAS) to identify some new findings for CRC. We analyzed the genotype-specific expression data for rs11100883 from GTEx (https://www.gtexportal.org/home.snp/rs11100883).

Genotyping

Genomic DNA from the whole blood was extracted using a commercial kit (FavorPrep, Favorgen, Taiwan) and stored at −80°C until use. DNA extraction was done by strictly following the protocol book supplied with the kit. Amplification of genomic DNA was performed using the newly designed forward and reverse primers. The primers for SMAD1 rs11100883 and rs7661162 were designed by primer blast. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping both the SMAD1 alleles. PCR products of 471 and 237 bp were obtained for SMAD1 rs11100883 and rs7661162 SNPs, respectively. Digestion of 471 bp PCR product of SMAD1 rs11100883 was done with Mph1103I (NsiI) (Thermo Fisher Scientific, USA) by incubating at 37°C overnight. After cutting the PCR product of 471 bp with Mph1103I (NsiI), two fragments were obtained, including 206 and 265 bp for A allele (Figure 1). Restriction enzyme HpyF3I (DdeI) (Thermo Fisher Scientific, USA) was applied to cleave the A allele of rs7661162 at 37°C overnight, giving three fragments of 25, 88, and 124 bp (Figure 2). The digested fragments were stained with Ethidium Bromide (EtBr) and visualized on 2% agarose gel electrophoresis. More than 25% of the samples were randomly re-evaluated using both positive and negative controls for confirmation. The sequences of primers, required PCR conditions with fragmentation patterns, and restriction enzymes used in the study are listed in Table 1.

Statistical analysis

Statistical analysis was carried out with the SPSS software package 17.0 (SPSS Inc., Chicago, IL). The corresponding 95% confidence intervals (CIs) and odds
ratios (ORs) were calculated by the MedCalc software program. Hardy–Weinberg equilibrium (HWE) was tested with a goodness of fit chi-square test ($\chi^2$) to compare the observed genotype frequencies among the subjects. Adjusted odds ratios (adjusted ORs) and 95% CIs were calculated with multivariate logistic regression to assess the effect age and sex on the association between SMAD1 polymorphism and CRC. p-value and OR of clinicopathological characteristics were also determined for both variant carriers and non-carriers. p < 0.05 is considered statistically significant for all analyses. Bonferroni correction was made to avoid the false positive by considering p < 0.025 statistically significant for two SNPs.

**Results**

**Characteristics of the study population**

The study included 147 (53.45%) males and 128 (46.55%) females CRC patients with a mean age of 44.20 (range 14–82) years and the control group comprised of 139 (46.33%) of males and 161 (53.67%) of females with a mean age of 33.39 (range 14–70) years. The male-to-female ratio for the patient group is 1.15, and that for the control group is 0.86. There were no significant differences for both age and sex between cases and controls (p > 0.05). The most prevalent primary tumor site is colon 185 (67.27%), and the majority of the patients had stage II disease 125 (45.45%) (Table 2). In the case of rs11100883 polymorphism, parameter like primary tumor location in rectum (OR = 12.16, 95% CI = 5.06–29.24, p = 0.001) was found to have a statistically significant association with CRC (Table 3). Not any other parameter was considerably associated with SMAD1 rs11100883 and rs7661162.

**Smad1 rs11100883 polymorphism**

Table 4 displays the genotype frequencies in cases and controls for SNP rs11100883. The GG homozygous genotype was statistically lower in patients than in controls (33.45% vs 44.33%). The frequency of GA heterozygous genotype was found to have 1.55 times more chance of developing CRC, and it was statistically significant (p = 0.014, aOR = 1.55, 95% CI = 1.09–2.20). The AA genotype was found to have 1.82 times more chance of developing CRC even though it was statistically insignificant (p = 0.060, OR = 1.82, 95% CI = 0.97–3.40). Keeping resemblance, the dominant model and allele model were found statistically significant (GA + AA vs GG: p = 0.008, adjusted OR = 1.59, 95% CI = 1.13–2.23 and A vs G: p = 0.015, OR = 1.35, 95% CI = 1.06–1.73, respectively), whereas the risk factor of developing CRC found to be statistically not significant in both recessive model and overdominant model (AA vs GG + GA: p = 0.242, adjusted OR = 1.42, 95% CI = 0.79–2.55 and GA vs GG + AA: p = 0.055, adjusted OR = 1.38, 95% CI = 0.99–1.92, respectively).

**Smad1 rs7661162 polymorphism**

The genotype frequencies of rs7661162 for both cases and controls are presented in Table 4. In this case, the AA homozygous genotype was statistically lower in patients than in controls (61.09% vs 72.67%). The frequency of AG heterozygous genotype was found to
have 1.78 times more chance of developing CRC, and it was statistically significant (p = 0.002, adjusted OR = 1.78, 95% CI = 1.24–2.56). The GG genotype was found to have 0.88 times lower chance of developing CRC while it was statistically insignificant (p = 0.834, OR = 0.88, 95% CI = 0.27–2.85). The distribution of AG and GG together in dominant model was found statistically significant (AG + GG vs AA: p = 0.004, adjusted OR = 1.68, 95% CI = 1.18–2.39). The allele model and dominant model showed statistically strong significance in developing CRC as that of the dominant model (G vs A: p = 0.014, OR = 1.47, 95% CI = 1.08 to 2.00 and AG vs AA + GG: p = 0.002, adjusted OR = 1.76, 95% CI = 1.23–2.53, respectively), whereas the recessive model found to be statistically not significant (GG vs AA + AG: p = 0.600, adjusted OR = 0.73, 95% CI = 0.23–2.35).

**In silico gene expression analysis**

We found significantly low expression of SMAD1 mRNA in the colon cancer tissues ($1.50 \times 10^{-10}$) and rectal cancer tissues ($6.80 \times 10^{-4}$) compared to healthy tissues from UALCAN database (http://ualcan-path.uab.edu/analysis.html) (Figure 3). We also found low tissue expression for rs11100883AA genotype compared to the GG genotype in cultured fibroblast and testis from the GTEx database (https://www.gtexportal.org/home/) (Figure 4). No data were found for polymorphism rs7661162 in GTEx, and no data for CRC tissue were available for any of these SNPs.

**Discussion**

CRC is associated with more or less than 10% of all incidence of mortality due to cancers or cancer-related diseases worldwide. Both family history and environmental factors play a key part in the outgrowth of CRC. Studies showed that positive family history might predispose about 10%–20% of CRC in all patients. Besides, the risk may vary depending on the age of diagnosis, and the number and status of exposure of relatives. Moreover, the inheritance of CRC accounts for approximately 10%–35% in twins. The gradual growth of genetic and epigenetic alterations in colon epithelium is the main culprit behind the commencement and progression of CRC development. Genome-wide association studies (GWAS) revealed that a cluster of genes is thought to be contributing to the initiation of colorectal carcinogenesis. In this study, we have found a statistically significant association of CRC risk with SMAD1 rs11100883 and rs7661162 genetic polymorphisms in the Bangladeshi population.

While various GWAS have successfully identified genes or their variants for CRC susceptibility, most of
the risk factors responsible for heritability are yet elusive. However, previous studies provide properly recorded evidence for the association of SMAD1 with advanced cancer stage and migration. The upregulation of SMAD1 in human CRC patients, both in Caucasian and Asian populations, has been reported by several research groups. Korchynskyi et al. conducted methodical research where they showed the expression of all the groups of SMAD proteins in the indifferent variety of human CRC of the Ukrainian population. Another study validated the upregulation of SMAD1 in 542 CRC patients (from stages I to IV) in the Chinese Han population, where SMAD1 elevation may act as compensatory for TP53 mutation/loss. Strong evidence showed that SMAD1 leads to the maximal induction of regulatory TP53 protein that repairs DNA damage and initiates apoptosis. Other genetic studies on human and mouse reported that the BMP-SMAD pathway possesses a tumor suppressor function and found the downregulation of BMP-SMAD1 signaling in human colon cancer samples. Another research conducted by Yang et al. provides evidence of the positive influence of SMAD1 gene via induced expression of Ajuba and Snail in CRC.

Although scientists are shedding light on this area, there are still considerable challenges in elucidating the influence of genetic variation on the expression of SMAD1, such as resolving the different functional consequences of perhaps quite delicate perturbations of the pathway at many points. A case-control study conducted on the Caucasian population by Slattery et al. reported no association between SMAD1 (rs714195, rs653735, rs2118438, rs1016792, and rs12505085) and risk of colon and rectal cancer. Several studies revealed that inactivating mutations in other SMAD genes interrupt the signal transduction pathway between the cell membrane receptors and the cell nucleus. For instances, mutations in SMAD2 are particularly associated with...
early-stage sporadic colorectal carcinoma, whereas SMAD4 mutations arise in advanced stage cancers (lymph node involvement and metastasis). Animal studies revealed that SMAD3 is another influential tumor suppressor for colonic epithelium since it demonstrated that SMAD3 homozygous mutants build up colon adenocarcinoma in various stages. Numerous GWAS have been conducted to recognize the genetic variation in the SMAD7 gene on 8q21 as being associated with CRC.

To ascertain a more accurate assessment of the relationship between the SMAD1 gene and CRC, we undertook this case-control study in the Bangladeshi population, which included a total of 575 subjects (275 cases and 300 controls). In the case of SMAD1 rs11100883 polymorphisms, the study revealed that individuals carrying GA heterozygous and AA mutant homozygous genotypes possess 1.55- and 1.82-fold increased risk of developing CRC compared to control group, respectively, where GA heterozygous is statistically significant (p = 0.014). Besides, the dominant model and allele model increased the risk of CRC development (adjusted OR = 1.59 and 1.35, respectively), and the findings are statistically significant (p = 0.008 and 0.015, respectively). However, the over-dominant model and recessive model showed an increased risk that was not statistically significant.

This study also found a significant association between the SMAD1 rs7661162 polymorphism and CRC risk in the Bangladeshi population. It showed that AG genotype increased the risk of CRC by 1.78-fold (adjusted = 1.78, p = 0.002) in comparison with AA genotype. Along with this, the dominant model, overdominant model, and allele model (adjusted OR = 1.68, p = 0.004; adjusted OR = 1.76, p = 0.002;
OR = 1.47, p = 0.014) also presented a significant risk association with CRC except recessive model (p = 0.600) in the Bangladeshi population. All the significant values withstand after the Bonferroni correction. There was no family history of cancer in the first relatives for both the cases and controls.

Our study also tried to establish a correlation of SMAD1 rs11100883 and rs7661162 polymorphisms with different clinicopathological characteristics of the patients. For the rs11100883 variant, primary tumor location in the rectum (OR = 12.16, p = 0.0001) was found in association with CRC that was statistically significant. However, we did not observe any significant difference in the case of age and sex between CRC patients and healthy controls in this study.

However, besides the strengths, there are a few limitations in our study that need to be addressed. First, the inclusion of some other related genes and SNPs would help to observe a wider and more precise correlation between genetic polymorphism and CRC. Second, the sample size was relatively small; therefore, more extensive investigations with larger samples and other SNPs of SMAD1, including additional detailed environmental exposure data, are defensible for validating these results.

Conclusion

In summary, this study indicates that both SNPs (rs11100883 and rs7661162) of the SMAD1 gene are associated with an augmented risk of CRC in the Bangladeshi population.

Author contributions

S.S. and P.F.K. carried out the study and drafted the manuscript. M.S.I., M.A.A., and S.S. conceived the study design and performed the statistical analysis. M.S.I., M.A., and M.S. guided the study conducted in the laboratory. M.A.B. and A.S.M.M. checked the manuscript and suggested some improvisations. All the authors finally discussed and gave conceptual comments and approved the final manuscript.

Declaration of conflicting interests

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ORCID iD

Mohammad Safiqul Islam https://orcid.org/0000-0003-4924-5319

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