RESEARCH ARTICLE

Intragenic DNA Methylation Concomitant with Repression of ATP4B and ATP4A Gene Expression in Gastric Cancer is a Potential Serum Biomarker

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Abstract

Based on our previous report on gastric cancer which documented ATP4A and ATP4B mRNA down-regulation in gastric tumors relative to normal gastric tissues, we hypothesized that epigenetic mechanisms could be responsible. ATP4A and ATP4B mRNA expression in gastric cancer cell lines AGS, SNU638 and NUGC-3 was examined using reverse transcriptase PCR (RT-PCR). AGS cells were treated with TSA or 5'-AzaDC and methylation specific PCR (MSP) and bisulfite sequencing PCR (BSP) analysis were performed. MSP analysis was on DNA from paraffin embedded tissues sections and plasma. Expression analysis revealed downregulation of ATP4A and ATP4B genes in gastric cancer cell lines relative to normal gastric tissue, while treatment with 5'-AzaDC re-activated expression of both. Search for CpG islands in their putative promoter regions did not indicate CpG islands (CGI) but only further downstream in the bodies of the genes. Methylation specific PCR (MSP) in the exon1 of the ATP4B gene and exon7 in ATP4A indicated methylation in all the gastric cancer cell lines tested. MSP analysis in tumor tissue samples revealed methylation in the majority of tumor samples, 15/19, for ATP4B and 8/8 for ATP4A. There was concordance between ATP4B and ATP4A down-regulation and methylation status in the tumour samples tested. ATP4B methylation was detectable in cell free DNA from gastric cancer patient's plasma samples. Thus ATP4A and ATP4B down-regulation involves DNA methylation and methylated ATP4B DNA in plasma is a potential biomarker for gastric cancer.

Keywords: ATP4A - ATP4B - gastric cancer - DNA methylation

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Introduction

Gastric cancer is one of the leading sites of cancer seen in the South Indian population and one of the leading causes of death from cancer. High incidence rate of gastric cancer in the region combined with majority of lesions (more than 90%) presenting in advanced stages of the disease, presents an urgent need for population based screening which can identify early lesions using bio-markers, preferably serum based. In our previous study, where we compared gene expression profile of gastric tumor with the corresponding paired normal (PN) and apparently normal (AN), we identified several genes including ATP4A and ATP4B to be repressed in gastric tumor tissues compared to PN and AN tissues (Rajkumar et al., 2010).

The H,K- ATPase, a heterodimeric, P-type ATPase, secretes H+ in exchange for K+ at the expense of ATP. The H,K- ATPase is composed of two subunits, an α (ATP4A) and a β (ATP4B) subunit (Chow and Forte, 1995). ATP4A, a 114-KDa subunit with an ATP and a cation binding site, is the catalytic unit of the membrane protein, while ATP4B, a 35-KDa heavily glycosylated subunit is involved in the proper folding and membrane localization of protein (Morley et al., 1992). The H,K-ATPase is the most critical component of the ion transport system in the parietal cell that mediates acid secretion in the stomach. Being a major portion of the apical and basolaternal sides of the plasma membranes it is thought to play an important role in maintaining membrane integrity. ATP4B knockout mice show defects in the membrane integrity of the parietal cells consistent with this hypothesis (Scarff et al., 1999). In the instance of ATP4A knockout, adult mice exhibited achlorhydria, and hypergastrinemia, changes in the parietal cell membrane, and metaplsia of gastric mucosa, with no changes in parietal cell viability or chief cell differentiation (Judd et al., 2005). Inhibition of H,K-ATPase activity by drugs like omiprazole in mice is reported to cause epithelial cell proliferation and suppression of its differentiation (Kakei et al., 1995). Therefore H,K-ATPase is essential for normal functioning and homeostasis of gastric tissue.

Epigenetic mechanisms such as DNA methylation, histone modifications and micro-RNA are well known for regulating gene expression at transcriptional and post transcriptional levels (Berdasco and Esteller, 2010). Our

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present report indicates that intragenic DNA methylation seen concomitantly with repressed expression levels of ATP4A and ATP4B genes is involved in the down regulation of expression in gastric cancer tumor tissues.

Materials and Methods

Patient samples

10cc of blood was collected from patients in the presence of 200μl of 10% ethylene diamine tetra acetic acid (EDTA). All the patients and normal donors provided their informed consent for the study, which was approved by the Institutional Ethical committee. The paraffin embedded tissue sections from the identified paraffin blocks were obtained from the Department of Pathology, Cancer Institute (WIA). The criteria for choosing apparently normal (AN), and paired normal (PN) was based on our previous report on gastric cancer (Rajkumar et al., 2010). Briefly, AN gastric tissues were obtained from patients who were admitted for treatment for other malignancies and underwent stomach resection as part of their primary surgery and did not exhibit any lesions in the stomach. PN gastric tissue samples were obtained away from the resected margins of the tumor. The study was approved by the Institutional ethical committee and an informed consent was obtained. All normal samples were HE stained and confirmed for presence of cytologically normal cells and absence of tumor cells.

Cell culture and drug treatment.

Gastric adenocarcinaoma cell lines AGS was cultured in HAMS-F12 (HiMedia Laboratories, India) supplemented by 10% fetal bovine serum (Invitrogen), NUGC3 and SNU638 were cultured in RPMI (HiMedia Laboratories, India) supplemented by 10% fetal bovine serum (Invitrogen). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For treatment with 5-Aza-2'-deoxycytidine (5-AzaDC) cells were seeded at a density of 1 X 10^6 in a 100 mm^{-1} dish. After 24 h, cells were treated with 1 and 5 μm 5-Aza-2'-deoxycytidine (Sigma Chemical Co., USA). The same concentration of Dimethyl sulfoxide (DMSO) was used as control for nonspecific solvent effect on cells. AGS cells were also treated with Trichostatin A (TSA) (Sigma Chemical Co., USA) at a concentration of 200 nM supplemented in HAMS-F12 containing 10% FBS in a 100mm dish for 6 hours.

RNA isolation, reverse transcriptase PCR (RT-PCR) and real-time PCR

Total RNA from DMSO, 5-Aza-2'-deoxycytidine and TSA treated AGS cells were isolated using RNeasy kit (Qiagen) as per manufacturer's instructions. RNA was quantitated using NanoDrop™ ND1000 (NanoDrop Technologies) spectrophotometer. 2μg of Total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 2μl of the cDNA was used for the subsequent PCR reaction for ATP4A gene mRNA using primer set (sense): 5' - GCAATCGCTCTCATTGCTGTGGTT-3', (antisense):5'-TCTGGAATTTGTCTCCATCGCGGA - 3 and for the ATP4B gene mRNA using primer set (sense): 5' GCAATCGCTCTCATTGCTGTGGTT 3', (antisense) : 5' TCTGGAATTTGTCTCCATCGCGGA 3'; and GAPDH gene primer set (sense) 5'GTGAAGGTCGGAGTCAACG 3', (antisense) 5'TGAGGTCAATGAAGGGGTC 3'. After electrophoresis the image of the ethidium bromide stained agarose gel was obtained using Typhoon Trio™ (GE life sciences) variable mode image scanner. The bands in the respective lanes were quantitated using Imagequant™ (Molecular Dynamics Inc., USA). The maximum intensity of each band was plotted as histograms.

Bisulfite treatment, methylation specific PCR analysis (MSP) and sequencing.

The DNA isolated from all samples was quantitated using NanoDrop™ ND1000 (NanoDrop Technologies) spectrophotometer. Bisulfite treatment was performed

Table 1. List of MSP Primers and BSP Primers Used in the Study

Gene Methylation Primers	Primer Sequence
ATP4B MSP set-1	
Methylated Forward (5'-3')	TTTTATGTATTTAGTCGGTTCGTTTTTCGTAC
Methylated Reverse (5'-3')	CGAACACGAAACAAATACTAAACCG
Un Methylated Forward (5'-3')	TTTTTTATGTATTTAGTTGGTTTGTTTTTTGTATG
Un Methylated reverse (5'-3')	CTAAAACCCAAACACAAAACAAATACTAAACCA
ATP4B MSP set-2	
Methylated Forward (5'-3')	ATAATTCGGTGGTTTTGAGC
Methylated Reverse (5'-3')	AACGTAACCGATAATACGACG
Un Methylated Forward (5'-3')	TATATAATTTGGTGGTTTTGAGT
Un Methylated reverse (5'-3')	AAACATAACCAATAATACAACACT
ATP4B BSP set-1	
Forward (5'-3')	GGTAGGTTTGGTTTATTTTTTATTTT
Reverse (5'-3')	TAATTCCTTAATCACACAAATTCAC
ATP4B BSP set-2	
Forward (5'-3')	GTATTTTATTTGGATAGGGTG
Reverse (5'-3')	AAAAATCTCAAACCAAAAAC
ATP4A MSP set-1	
Methylated Forward (5'-3')	CGATGGGTGTTTTTTCGTTTTTTATTTTCGAC
Methylated Reverse (5'-3')	ACACCGTACAAAACCTAATAATAAACACGAACG
Un Methylated Forward (5'-3')	AGTGATGGGTGTTTTTTTGTTTTTTATTTTTGATG
Un Methylated reverse (5'-3')	AACACCATACAAAACCTAATAATAAACACAAACA

modifications. For genomic DNA isolated from paraffin sections, 1μg was used for the bisulfite treatment. In the case of plasma samples the total DNA was concentrated using vacuum concentrator Speed Vac™ (Savant) and subjected to bisulfite treatment in the presence of 2μg yeast tRNA. Sequences upstream and downstream of the transcription start site of ATP4A and ATP4B genes were searched for CpG islands using cpgplot tool (http://www.ebi.ac.uk/Tools/emboss/cpgplot). The list of MSP primers and BSP primers used for analysis of ATP4B and ATP4A genes are listed in Table I. The primer sets for both genes were designed using MethPrimer program (http://www.urogene.org/methprimer/index.html). The PCR products from the MSP, and BSP analysis were purified from agarose gels using Qiaquick™ gel extraction kit (Qiagen)

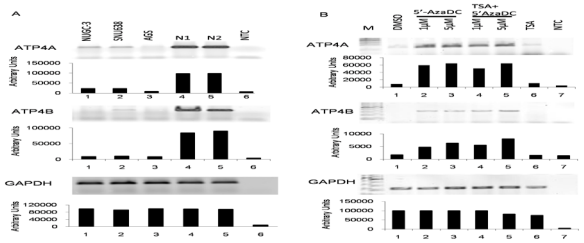

Figure 1. A,B: RT-PCR Analysis for ATP4A and ATP4B Expression in Gastric Cancer Cell lines. 1a: RT-PCR analysis of ATP4A and ATP4B expression in the three gastric cancer cell lines and two normal gastric mucosa, GAPDH expression was used as a control. Below each of the gels showing RT-PCR data for ATP4A, ATP4B and GAPDH gene expression is the corresponding intensity plots of the bands. 1b: RT-PCR for ATP4A and ATP4B in AGS cells treated with 5'Aza Deoxycytidine(5'-AzaDC), or TSA. GAPDH was analysed as a control. Below each of the gels showing RT-PCR data for ATP4A, ATP4B and GAPDH gene expression after drug treatment is the corresponding intensity plots of the bands. Ethidium bromide stained gels were quantitated as described in the materials and methods section.

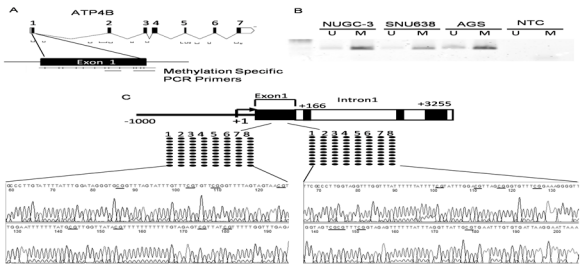

Figure 2. A-C: DNA Methylation Analysis of ATP4B Gene. 2a: Diagrammatic representation of the organization of Exons and Introns in ATP4B with the CpG islands locations depicted below. The ATP4B premessenger comprises of 7 exons and complete mRNA is 1497 base pairs. 2b: MSP analysis of three gastric cancer cell lines (NUGC3, SNU638 and AGS). 2c: BSP analysis of CpG islands in the Exon 1 of ATP4B gene in AGS cells. The PCR product from BSP analysis was cloned as described in the materials and methods section. Ten individual clones were picked and recombinant plasmids sequenced, methylation on individual CpG's is represented as solid circles. The sequencing electrophorogram of a representative recombinant plasmids is presented.

on genomic DNA isolated from formalin fixed paraffin embedded tissue sections and DNA isolated from plasma samples using EZ DNA Methylation-GoldTM kit (Zymo Research) as per the manufacturer's instructions with some and cloned into pCR™ 2.1 vector using Topo TA cloning™ kit. Ten colonies containing the insert was sequenced using Big dye terminator V3.1 cycle sequencing kit (Applied Biosystems) and sequencing reaction was resolved using ABI PRISM™ 310 genetic analyzer (Applied Biosystems) and the sequence analysed using Sequence Analysis 3.7 (Applied Biosystems) software.

Statistics

OpenEpi version 2.3.1 (www.OpenEpi.com) statistics software package was used to calculate significance. Significance for differences in the methylation pattern between the "normals" (AN and PN) and tumors was estimated using Fisher exact test, p-value less than 0.05 was considered as significant.

Results

Expression levels of ATP4A and ATP4B genes were evaluated in gastric cancer cell lines NUGC-3, SNU638, AGS and AN gastric tissue samples. The results showed that ATP4A and ATP4B are highly expressed in AN gastric tissues and repressed in NUGC-3, SNU-638 and AGS cells. GAPDH gene expression was used as control for estimating loading of equal amounts of c-DNA in the respective PCR reactions (Figure 1a). AGS cells were treated with DNMT inhibitor 5'-AzaDC or TSA or combination of both the drugs. Treatment with 5'-AzaDC induced the expression of both ATP4A and ATP4B expression in AGS cells relative to DMSO control at both 1μM and 5μM concentrations. No significant increase in expression levels with TSA treatment in combination with 1μM and 5μM concentrations of 5'-AzaDC was observed and treatment with the TSA alone failed to induce the expression of ATP4A and ATP4B under the conditions tested (Figure 1b).

Next we analysed for the presence of DNA methylation in the ATP4B gene. Search for CpG islands (CGI) in the promoter region of the ATP4B gene did not identify significant CpG rich regions. When the intra-genic sequences downstream of transcription start site were analysed the region encompassing the body of the gene indicated the presence of several CpG islands (Figure 2a). MSP analysis of the CpG's in exon 1- intron 1 boundary revealed the presence of methylation in all the 3 gastric cancer lines tested (Figure 2b). Subsequently BSP analysis in AGS cell line DNA encompassing the CpG island revealed hypermethylation in 16 CpG's confirming the presence of dense methylation in the region (Figure 2c). MSP analysis was performed using two sets of primers interrogating different CpG's for methylation in the same region in gastric tissue samples. The 19 gastric tumors taken up for analysis comprised of 18 adenocarcinomas, and 1 poorly differentiated neuro-endocrine carcinoma. Among the 18 adenocarcinomas 12- intestinal, 4-diffuse, and 2-mixed subtypes were present. All the tumor samples showed down regulation of ATP4B expression. In the list of 19 tumors 12 cases were mucin positive and 7 were mucin negative. The mucin positive phenotype comprised of 9 intestinal, and 3 diffuse and the mucin negative phenotype comprised of 3 intestinal, 1 diffuse, and 2

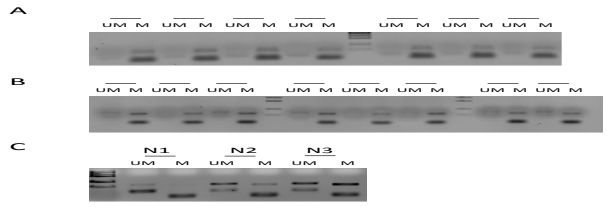

Figure 3. A, B: MSP Analysis of ATP4B Gene in Gastric Cancer, Paired Normal and Apparent Normal Tissue Samples. 3a: A representative ethidium bromide stained gel of a set of samples from MSP analysis using primer set 1. 3b: A representative ethidium bromide stained gel of a set of samples from MSP analysis using primer set 2

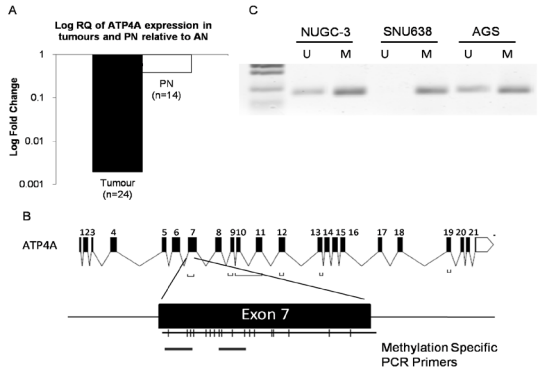

Figure 4. `A-C: DNA Methylation Analysis of ATP4A Gene. 4a: ATP4A Expression Levels in Tumours, Paired Normals Relative to Absolute Normal Samples. Realtime PCR analysis for ATP4A and GAPDH gene expression analysis was performed using primer sets mentioned in the materials and methods section. 4b: Diagrammatic representation of the exon intron organization in ATP4A with the positions of CpG islands depicted below. The ATP4A pre-messenger comprises of 22 exons and complete mRNA is 3709 base pairs. 4c MSPCR analysis of the exon 7 of ATP4A in the gastric cell lines (NUGC-3, SNU638, AGS)

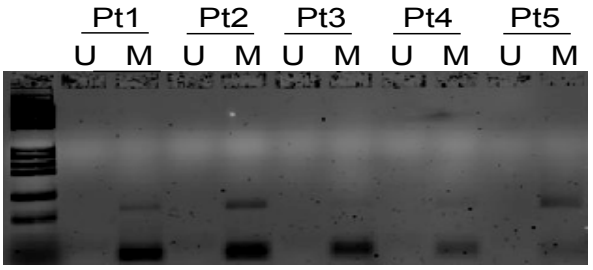

Figure 5. DNA Methylation Analyses of ATP4B Gene in Cell-free DNA. Data presented is a representative gel of the MSP analysis performed on cell free DNA isolated from plasma of gastric cancer patient samples

mixed types. One poorly differentiated neuro endocrine carcinoma was mucin negative. Analysis in 19 tumors showed the presence of exclusive methylation in 15/19 (78%) samples, 4/19 samples showed a mixed pattern indicated by the presence of unmethylated DNA besides methylation. In 12 PN samples evaluated using MSP1 set majority 10/12 (83%) showed mixed pattern in 4 AN samples evaluated all showed mixed pattern (Figure 3a). The differences in the methylation patterns between the tumors and "normals" were found to be significant (Fisher exact p-value = 0.00004). Similar results were obtained from MSP analysis using another primer set (Figure 3b) in the same region indicating a concordance in the detection of methylation.

We then compared the mRNA expression levels and DNA methylation status of samples. The fold changes in mRNA expression of individual samples were retrieved from our previous study (Rajkumar et al., 2010). 18/19 tumor samples showed down regulation and one sample did not show a change in the mRNA expression level. In majority 83% (15/18) of tumor samples showing mRNA down regulation, DNA methylation was exclusively indicated. On the other hand DNA methylation pattern showed unmethylated DNA in all AN samples tested. In the case of PN higher expression levels of mRNA was indicated in 6/15, no change in 3/15 and lower levels of expression in 6/15 samples relative to AN. It is important to note that expression levels in PN did not reach the lower levels seen in the case of tumor. MSP analysis in PN samples indicated a mixed pattern with presence of un-methylated DNA. Methylation analysis showed an inverse correlation with expression levels of ATP4B gene in the gastric tumor samples analysed. When compared to subtype and mucin status of the samples there was no relation with the methylation status.

Our previous gene expression analysis using micro-array showed that ATP4A was down regulated in tumours (Rajkumar et al., 2010). In the present study we validated the micro-array result using real-time PCR analysis for ATP4A expression in gastric tumors, PN and AN samples. In all, 24 tumors, 14 PN and 4 AN samples were taken up for analysis. The fold change in tumors and PN were calculated using AN as calibrator. The results indicate a striking down regulation in tumors relative to AN and PN samples validating our previous finding (Figure 4a). Similar to the case of ATP4B gene, CGI search in the intragenic sequences revealed the presence of interspersed CpG rich regions starting from exon 7 near the 5' end of the gene to exon 18 in the 3' end (Figure 4b). MSP analysis of the intragenic CpG's located in the region exon 7 was performed in the 3 gastric cancer cell lines NUGC-3, SNU636 and AGS revealed DNA methylation indicated by the presence of MSP product for methylation primers (Figure 4c). We could not include all the samples analysed for ATP4B gene methylation as the DNA isolated from the paraffin sections was limiting. The 8 gastric tumors taken up for analysis comprised of 7 adenocarcinomas, and 1 poorly differentiated neuro endocrine carcinoma. In the list of 8 tumors 6 cases were mucin positive and 2 were mucin negative. Among the 7 adenocarcinomas 4-intestinal, 2-diffuse, and 1-mixed subtypes were present. The results from the analysis showed the predominant presence of methylation in tumors (8/8) where as in the normals the DNA was unmethylated, further mRNA expression of ATP4A in these eight tumors also showed down regulation. AN gastric DNA which indicated presence of unmethylated DNA was concordant with elevated levels of mRNA expression in these samples. As in the case of ATP4B gene, methylation was present irrespective of the subtype and mucin status of the tumor samples. Our study in patient tumor samples mirrors the

results from gastric cancer cell lines in as much as the hypermethylation in intragenic region of ATP4B and ATP4A genes is concomitant with downregulation of mRNA expression levels.

We then tested for the presence of methylated DNA from ATP4B gene in cell free DNA isolated from normal and gastric cancer patient plasma samples (Figure 5). 25 plasma samples from gastric cancer patients, and 9 "normal" samples were taken up for analysis. Our results from MSP analysis indentified the presence of methylated DNA from gastric cancer patients in 16/25 (64%) and negative for unmethylated DNA, 9 samples were negative for methylated and unmethylated DNA (Table II). In the 9 normal samples tested none showed positivity for DNA methylation and were negative for unmethylated DNA also. Our results indicate that DNA methylation in the ATP4B gene is detectable in plasma samples of gastric cancer patients.

Discussion

DNA methylation is a well known epigenetic modification involved in the stable and heritable down regulation of genes. We analysed for DNA methylation in the respective intragenic regions of ATP4A and ATP4B genes which revealed DNA methylation in the exon 1 of ATP4B and exon7 of ATP4A genes. The methylation pattern was also observed in the tumor tissue samples and was consistent with the repressed levels of mRNA expression of the genes which indicated that DNA methylation in the intragenic regions is involved in the down regulation of expression. The linkage between gene promoter methylation and consequent down regulation of gene expression is well recognized. However the role of intragenic methylation in the repression of gene expression in mammalian cells is poorly understood. Recent studies have indicated that intragenic methylation is more common especially in the exonic regions than previously estimated (Edwards et al., 2010, Feng et al., 2010, Maunakea et al., 2010, Brenet et al., 2011). In a recent report by Brenet et al., it was revealed that methylation in the intragenic 5' end of the gene was associated with transcriptional silencing. The same study identified that densely methylated regions are more common in the intragenic regions with a bias for exonic regions and DNA methylation downstream of the TSS especially in exon1 is vital for transcriptional silencing (Brenet et al., 2011). The presence of DNA methylation in the exonic regions at 5' end of ATP4B and ATP4A is consistent with current observations in as much as the presence of hypermethylation coincides with down regulation of gene expression. A previous study analysed tissue specific methylation levels in 10 non- island –CpG sites located in upstream sequences of ATP4A and ATP4B genes however methylation levels in the intragenic regions of ATP4A and ATP4B were not analysed (Hong et al., 2009). Considering the role of ATP4A and ATP4B in the differentiation, membrane integrity of gastric cell types it is plausible that progressive loss of expression in gastric glands may coincide with the emergence of a neoplastic phenotype.

Cell free DNA circulating in the blood has been widely used to study putative biomarkers for various cancers (Lo, 2001). Some of the applications include oncogene mutations, oncogene amplification, and tumor related viral DNA. Several studies have used circulating DNA to study aberrant methylation in genes (RUNX3 (Kim et al., 2004), DAP-kinase, E-cadherin, GSTP1, p15, and p16 (Lee et al., 2002) specific to tumor tissue and correlate it with clinical outcome. In the present study we have demonstrated the detection of DNA methylation of ATP4B gene in circulating cell free DNA in plasma samples of gastric cancer patients. Since the sample size studied is small, future studies comprising of a larger number of samples will need to be performed to assess the role of aberrantly methylated ATP4A or ATP4B gene in cell free DNA from plasma as a viable biomarker for detection of gastric cancer.

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