The epigenetic study of Fgfr2 as a potential biomarker in the differential diagnosis of intestinal metaplasia and gastric cancer

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Abstract

Background Epigenetic alterations represent the potential role in the pathological process of cancer development. Epigenetic modulators affect some key gene methylation that can be applied as cancer biomarkers. Gastric cancer is faced with some limitations in diagnosis and differential diagnosis with intestinal metaplasia (IM). Intestinal metaplasia (IM) is generally considered a precancerous lesion in the carcinogenesis of gastric cancer cascade that is a major health burden worldwide. The fibroblast growth factor receptor 2 (*FGFR2*) gene is a receptor tyrosine kinase in which aberrant expression has a direct connection to GC. The purpose of the present study was to evaluate the prognostic relevance of *FGFR2* methylation in the whole blood specimens obtained from patients with GC and IM and normal individual controls to examine the possible implication of epigenetic biomarker for differential diagnosis GC from IM.

Material and Methods Appropriate epigenetic control regions in *FGFR2* CpGs Island were specified by bioinformatic and differentially methylated regions (DMRs) enrichment analysis. The methylation aberration of *FGFR2* selected CPG region was determined using MSRE-PCR and Real-time PCR on DNA extracted from blood samples of 125 participants, including 30 IM cases, 60 GC cases, and 35 normal controls individuals.

Results A significant *FGFR2* hypomethylation has been obtained in IM ($p = 0.01$) and GC ($p < 0.001$) versus the normal control samples. ROC statistical analysis revealed sensitivity (96.67 %) and specificity (100 %) for *FGFR2* as DNA epigenetic biomarker diagnostic of gastric cancer test with $p < 0.001$. These results suggest that the change in the methylation of *FGFR2* (AUC = 0.97) is a promising epi-biomarker.

Conclusions This study is the first study to show that blood-based biomarkers *FGFR2* gene may be a powerful epigenetic biomarker for diagnosing GC and IM and providing insights into gastric cancer pathogenesis and diagnosis.

Background

Malignant Gastric adenocarcinoma (GAC), the most common type of Gastric cancer (GC), is one of the major causes of cancer-related deaths worldwide [1]. The GC at early stages is usually asymptomatic or manifests with vague symptoms. Advanced stages may be accompanied by persistent abdominal pain, anorexia, and irrational weight loss [2]. Although GC's morbidity and mortality rates have been reduced over the past decades, it remains the third most common cancer-related death worldwide [3]. In most cases, GC is developed from a multistep progression according to Correa's cascade [4] and is often initiated by H. *pylori* infection. Subsequent processes have been led to atrophic gastritis and intestinal metaplasia (IM). The IM is considered gastric cancers' precursors, and it is defined as replacing the normal gastric mucosa with mucin-secreting intestinal mucosa [5].

Despite the advances in diagnosis and treatment of cancers but in regards to GC, the clinical outcome is still facing limitations because of its late diagnosis. Hence, a non-invasive effective method is required to

be employed in the early diagnose of GC in patients. [6]. Since tumorigenesis is a stepwise accumulation of genetic and epigenetic alterations in oncogenes and tumor suppressor genes [7]. Recent advances in cancer epigenetic offer a better understanding of the underlying mechanism(s) of carcinogenesis and provide insight into the discovery of putative cancer biomarkers for early detection, disease monitoring, prognosis, and risk assessment [8]. Epigenetic modifications are heritable changes that do not alter the DNA sequences. Aberrated epigenetic processes can lead to altered gene expression and malignant cellular transformation.

The fibroblast growth factor (FGF) receptor (FGFR) family is involved in various significantly affect tumorigenesis. Activation of these receptors can lead to activation of the RAS-MAPK pathway and the PI3K-AKT pathway, among others. The mechanisms by which FGFR can be misregulated vary between cancers. The FGFR1-4 are a family of receptor tyrosine kinases regulating fundamental processes, including cell proliferation, differentiation, migration, and survival [9, 10].

The isoform IIIb of *FGFR2* (known as keratinocyte growth factor receptor) is recognized as the regulator of epidermal differentiation and homeostasis in normal human tissues. Also, it is considered as an important tumor-suppressor role in-Vitro and -Vivo. The FGFR-2 IIIc isoform is expressed in epithelial and mesenchymal cells. The altered *FGFR2* splicing and the aberrant expression of the mesenchymal *FGFR2* IIIc isoform induce changes in the specificity for FGFs, leading to impairment in cellular differentiation, epithelial-mesenchymal transition (EMT), and tumor creation characteristics [11].

In this study, methylation aberration of *FGFR2* promoter region was assessed in DNA extracted from blood samples WBC of patients with IM and GC and normal controls as a potential epigenetic biomarker.

Material And Methods

Data Acquisition

The DMRs of gastric tumors and non-tumoral tissue checked by downloaded microarray datasets (GSE25869 and GSE30601), including non-tumoral and gastric cancerous tissues, from the "NCBI GEO database" (Home - GEO - NCBI) and previous studies. A Venn diagram was plotted for hypomethylated ($-1 < logFC < 0$) genes using the online Venn diagram plotter tool (Draw Venn Diagram - Bioinformatics and Systems Biology) to identify the intersections between two datasets.

Protein-Protein Interaction (PPI) Network Construction

Obtained genes uploaded to the String database (STRING: functional protein association networks) to construct a PPI network. Afterward, the constructed PPI network was analyzed using Cytoscape (Cytoscape: An Open-Source Platform for Complex Network, version: 3.8.2). Topological parameters of the PPI network, such as betweenness centrality, closeness centrality, and degree, were analyzed with the Network Analyzer.

Enrichment Analyses (Gene Ontology)

The selected gene was explored by the gene ontology (GO) analysis website (GOnet - DICE Tools) to understand the genes' function. The GO analysis was also undertaken from the biological process and molecular function categories.

Differentially Methylated Regions (DMRs) of chosen genes and designing primers

We investigated the regulator regions from the "GeneCards database" (GeneCards - Human Genes | Gene Database | Gene Search) to identify the promoter regions. The "SMART APP" (SMART - bioinfo-zs.com) is used to recognize the significant distinguished methylated region's accurate location. The SMART App explored and interpreted the DNA methylation data across 33 cancer types from the TCGA database [12]. Afterward, the restriction endonuclease recognition sites are determined by the "NEBcutter analysis tool" (NEBcutter V2.0), and the specific methylation-sensitive restriction enzymes have been chosen. Then, primer sequences are designed by "Primer3Plus" (https://primer3plus.com/) on either side of the selected restriction enzyme recognition sites. (Fig. 4)

Study design and participants

In this study, 125 samples were analyzed, including 30 patients with gastric cancer and 30 patients with intestinal metaplasia, and 35 normal control samples by healthy individuals. All patients sign the consent form to use their clinical samples under the supervision of their physician.

This study has been confirmed by the National Institute of Genetic Engineering and Biotechnology's ethical committee (Code number: IR.NIGEB.EC1398.12.3.A).
About 4 mL of whole blood samples were collected in EDTA tubes from participants. The participant's clinicopathological features are presented in Table 1, including age, sex, and disease type.

Table 1
Demographic and pathologic data for studied participants

Factors			Patients (Total = 125)	Methylation Status		
				MIN	MAX	Mean
Gender	Female		58 (46.4 %)			
	Male		67 (53.6 %)			
Diagnosis	Intestinal Metaplasia		30	0	100	72.5
	Cancer	Intestinal Type	30	5	100	25.2
		Diffuse Type	30	5	100	28.3
	Normal		35	85	100	94.8

Genomic DNA isolation

DNA samples were extracted using the GeneAll Biotechnology DNA extraction Kit (GeneAll Biotechnology, Korea) and stored at -20°C. The quality and quantity of DNA were determined by The NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Also, extracted DNAs run on the 1% agarose gel to evaluate the quality of their extraction.

Analysis of promoter methylation status with MSRE-PCR and MSRE-qPCR

Promoter's CpG islands methylation changes carried out using the MSRE-PCR method. According to the manufacturer's instruction, extracted DNA was digested using the appropriate methylation-sensitive restriction enzyme (RE), NaeI (Takara, Japan). 50 ng of DNA treated with 0.5 µL of RE, one µL 10× universal buffer. Sterile distilled water was added to reach the solutions' total volume to 10 µL. For undigested samples (as controls), 0.5 µL sterile distilled water was added instead of restriction enzyme. All sample tubes were incubated overnight at 37°C.

Each PCR reaction consisted of 1 µL of the DNA solution, 0.5 µL of each forward and reverse primer (with the concentration of 10 pmol/µL, FGFR2-Forward: 5′ AGTCCTTTCTTCCTCTCGTTCC 3′, FGFR2-Reverse: 5′ ACGCAGAAGAGTGGTCCTTG 3′), and ten µL of Taq 2× master mix, sterile distilled water to achieve a 20 µL reaction mixture for each sample. Treated DNA amplified by PCR using the Taq PCR Master Mix Kit (Ampliqon, Denmark).

PCR condition was performed in 35 cycles, including strings denaturation (95°C for 30"), primer annealing (60°C for 30"), extension (72°C for 30"). Also, initial denaturation set at 95°C for 5' and a final extension set at 72°C for 7' were considered. PCR products run on the 1.5% agarose gel, stained with ethidium bromide, and bands visualized under UV radiation.

Each treated sample with RE was compared with its undigested one as 100 % methylated due to no RE were used in these tubes to identify the accurate samples methylation. The intensity of the treated samples amplified products has a direct relationship with the methylation level. The methylation intensity was calculated by gel analyzer software (GelAnalyzer 19.1).

Digested and undigested DNA samples were also amplified using real-time PCR. All PCRs were performed in a Rotor-Gene 6000 thermal cycler (Corbett Life Science, Australia). Real-time PCR performed with the following constituents: 1 µL of DNA solution was added to 9 µL of a PCR mixture made up of 5 µL of 2× SYBR Green PCR Master Mix (Takara, Japan), 0.2 µL of forward and reverse primers, and 4.6 µL of water. PCR condition was performed in 40 cycles, including strings denaturation (95°C for 20"), primer annealing (61°C for 40"), extension (72°C for 60"). Also, initial denaturation and final extension were set at 95°C for 2' and 72°C for 7'. ΔCt values determined as the difference between the obtained Ct values of the non-treated DNA and treated DNA ($\Delta\Delta C_T = \Delta C_{T\,(UN)} - \Delta C_{T\,(D)}$).

Methylation fraction (%) = 100($e^{-0.7(\Delta\Delta CT)}$).

Statistical analysis

Results expressed as means ± standard deviation (SD), and all statistical analyses were performed using IBM SPSS version 26 (SPSS, Inc., Chicago, IL, USA) and Prism (GraphPad Software, San Diego, CA) version 9.0.0. The statistical significance of the difference between groups was determined using one-way ANOVA to determine the three groups' differences. $P < 0.05$ were considered statistically significant.

Results

Identification of DMRs in the GEO datasets

Raw data were downloaded from two independent GEO datasets (GSE25869 and GSE30601); DMRs were identified using GEO2R. The top 250 hypomethylated regions have been selected from each dataset. Volcano plots displayed the differently methylated regions between tumoral samples and normal controls. Venn diagram indicated the intersections containing 68 DMRs were commonly hypomethylated in two datasets. As shown in Fig. 1, a regulatory network of obtained genes was constructed. The top 10 hub genes were *EGFR, ERBB2, TJP1, YAP1, FOXA1, APP, GATA6, FGFR2, KLF4, and CXCL1*. (Fig. 1)

GO enrichment analyses

The DMRs analysis was conducted using GO. The GO enrichment analyses indicated that DMRs were significantly related to the molecular function of "protein kinase binding" (p = 1.53E-06) and "transmembrane receptor protein tyrosine kinase activity" (p = 1.53E-06) and "transmembrane receptor protein tyrosine kinase activity" (p = 2.51E-06). Biological pathway results also showed that DMRs were mainly enriched in "positive regulation of protein kinase B signaling" (p = 8.00E-10) and "cellular response to growth factor stimulus" (p = 1.60E-09). (Fig. 2)

Validation of FGFR2 with TCGA database

The *FGFR2* was chosen for validation according to the differentially methylated regions and the protein-protein interaction result. The data of TCGA and GTEx (SMART - bioinfo-zs.com and New tab (cancer-pku.cn)) revealed the methylation status of *FGFR2* was significantly (p = 0.032) lower at the region of probe cg12835048 (chr10: 121598092, 121598093), and the expression was upregulated in GC compared to the normal gastric tissue ($p < 0.05$). (Fig. 3)

Clinicopathological parameters

The present study analyzed the association between the promoter methylation status of *FGFR2* and clinicopathological characteristics. Participants consisted of 46.4% female and 53.6% male. The cohorts' age range consisted of 39–82 years old, 37–65 years old, and 34–83 for normal, IM, and GC cohorts, respectively. (Table 1).

Methylation analysis by MSRE-PCR method

Implication of MSRE-PCR assessments of *FGFR2* promoter methylation indicated a hypermethylation in normal samples (mean = 94.8 %) compared with that in IM (mean = 72.5 %) and in GC intestinal type (mean = 25.2 %) and diffuse (mean = 28.3 %) patients. Intriguingly, there is no significant difference

between patients' methylation status with Intestinal type of GC versus diffuse type (Table 1, Fig. 5, Fig. 6-A).

A significant difference among *FGFR2* promoter hypomethylation were detected among normal controls versus metaplasia ($p < 0.01$) and gastric cancer ($p < 0.001$) cases. The Receiver operating characteristic (ROC) curve assay of *FGFR2* methylation levels in GC patients and normal controls ($p < 0.001$) revealed high sensitivity, specificity 96.67, 100%, respectively, with a cut-off < 77.50% and area under the ROC curve (AUC) of 0.9700. (Fig. 6- B)

Conclusion

This is the first study based on bioinformatics and experimental assays to examine the methylations alteration of the *FGFR2* promoter region in GC and IM compared to normal controls. *FGFR2* has been studied as a potential epigenetic biomarker in peripheral blood WBCs as a non-invasive, reliable, cost-effective tool to be employed in GC and IM diagnosis.

Significant methylation changes were observed among studied GC and IM cases compared to normal controls. The level of significances was $p < 0.001$ for GC, and $p = 0.02$ for IM. Also, significant differences were observed between the methylation status of GC and IM cases themselves ($p < 0.001$). Our results conveyed that the mean of methylation changes in the *FGFR2* promoter was equal to 26.75 % in total GC cases, whereas it was 72.5 % in IM cases and 94.8 % in normal individuals in DNA samples extracted from WBCs.

This study's bioinformatics analysis is based on the TCGA database and represented methylation aberrations and mRNA expression profiling data of GC samples. The obtained data were used as the training dataset to screen for significant DMRs. Interestingly, based on the TCGA data and NCBI GEO database, the *FGFR2* in patients with GC has significant demethylation of the chosen *FGFR2* promoter region ($p = 0.032$). As a result, the expression becomes upregulated consequently.

The high expressed levels of *FGFR2* in colorectal [13], lung[14], gastric [15–17] cancer, coding mutations[18], and more recently, FGFR fusions that lead to pathway activation [19] have been demonstrated that the *FGFR2* has a significant oncogenic potential across multiple cancer types. ROC analysis was employed to compare the predictive accuracy of the methylation status of patients with gastric cancer. Our findings demonstrated the methylation status of *FGFR2* (with a cut-off at < 77.50 %, $p < 0.001$) could reflect the presence of malignant gastric lesions.

The MSRE-PCR has some advantages in assessing the methylation of genes promoter over another common method that used bisulfite DNA treatments. For instance, Methylation-specific PCR (MS-PCR) uses bisulfite conversion to detect methylated DNA sequences. In the ideal state, this conversion deaminates unmethylated cytosines to uracil but does not affect methylated cytosines. In the experimental condition, DNA manipulation and treatments may result in incomplete conversion, false-positive extensive, and DNA degradation (of up to 90%) [20]. Because the WBCs extracted DNA's amount

and quality are high, studies are shifting to assay WBCs DNA as a potential biomarker [21]. One study demonstrated a positive relationship between estrogen receptor alpha methylation in leukocytes and colonic tissue of patients with colorectal cancer (CRC) [22].

Gastric cancer is accompanying a high mortality rate and remaining an important cause of cancer death worldwide. The prognosis of GC is poor because cases are diagnosed at an advanced stage; hence, the treatment options are limited. Our findings indicated that *FGFR2* hypomethylation status in WBC DNA of patients with IM and GC significantly relates to lesions' malignancy. Also, the cornerstone of the study's approach is the non-invasiveness and preciseness of the utilized method. Hence, MSRE-PCR is used to evaluate the methylation status. In conclusion, we can deduce the methylation status of *FGFR2* as an important prognostic biomarker of gastric cancer.

Declarations

Ethics approval and consent to participation

The Ethical Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB) approved the study (Ethical code #: IR. NIGEB.EC1398.12.3.A). The informed consent form has been prepared and signed by all participants and the minors' parents under eighteen years old to use their clinical samples and personal data under their physician's supervision. All methods were carried out following relevant guidelines and regulations.

Availability of data and materials

The data generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author who was an organizer of the study.

Consent for publication

Not applicable.

Competing interests

All authors have read the manuscript and declared that they have no competing interest.

Authors' contributions

SAA designed the concepts and methodology. AM, NA, and MA carried out the sampling and experimental laboratory work. AM performed the data acquisition, analyzing and interpretation. AM and SAA performed the manuscript writing and revisions. Administrative, technical, and material were supported by SAA. SAA supervised this study. All authors read and approved the final manuscript.

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Figures

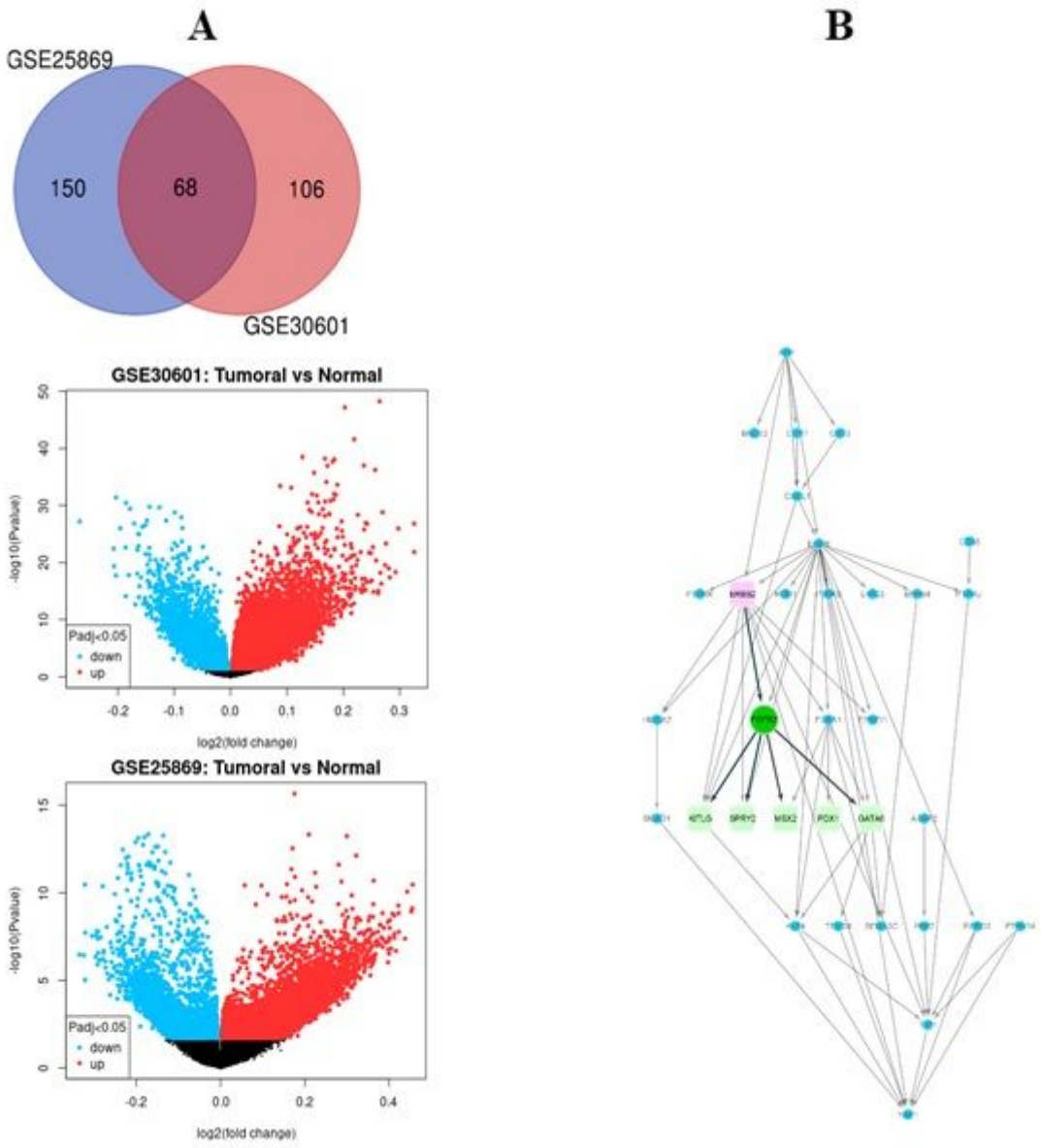


Figure 1

Statistical comparison of the hypomethylated genes and volcano plots presenting for differentially methylated regions. (A) Venn diagram analysis of shared genes between GSE25869 and GSE30601. Sixty-eight shared genes were obtained from the analysis and volcano plots of aberrantly methylated genes for each dataset ($p < 0.05$). (B) The PPI network of intersections genes obtained from two datasets.

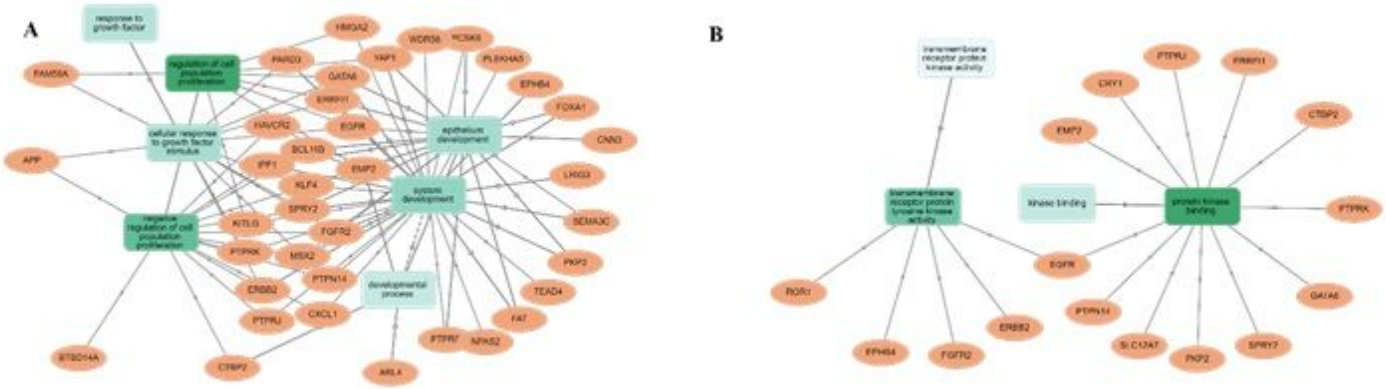


Figure 2

Gene Ontology (GO) analysis. (A) GO analysis of biological process: GOnet analyzed the Top 68 hypomethylated genes to generate gene clusters (q-value threshold ≤ 0.001). P-value ≤ 2.277e-7 was considered to obtain pivotal pathways. (B) Molecular function analysis. The top 68 hypomethylated genes list was analyzed by GOnet functional annotation to produce gene clusters (q-value threshold ≤ 0.01). P-value ≤ 8.43e-6 was set to gain more significant associated enrichment, and the protein kinase binding (GO:0019901) molecular function was the most highlighted annotated term (p = 1.53E-06). The networks are constructed by (GOnet - DICE Tools).

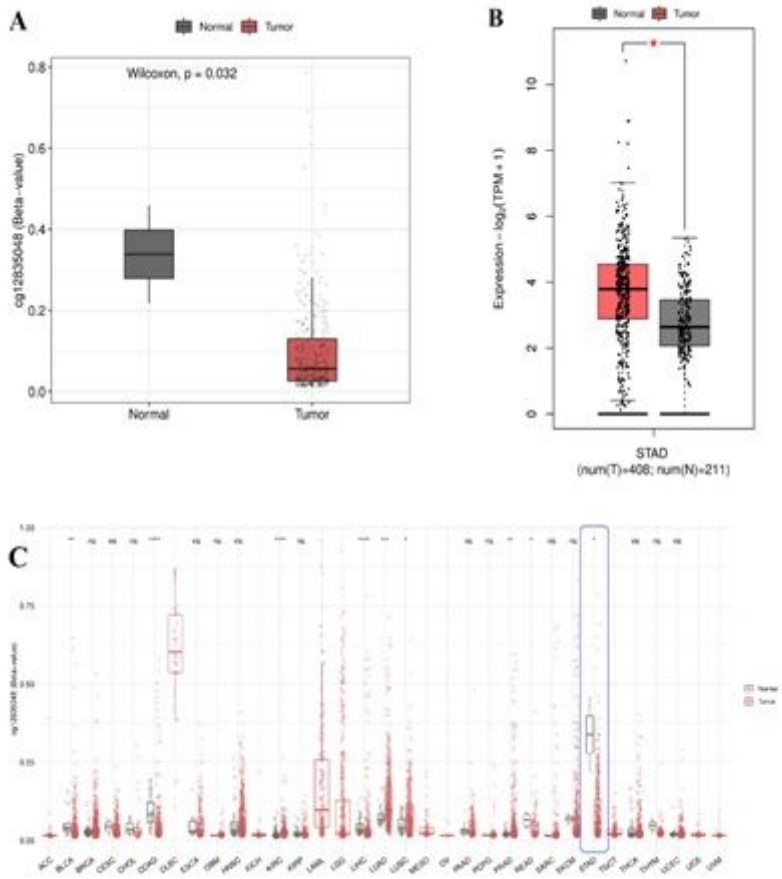
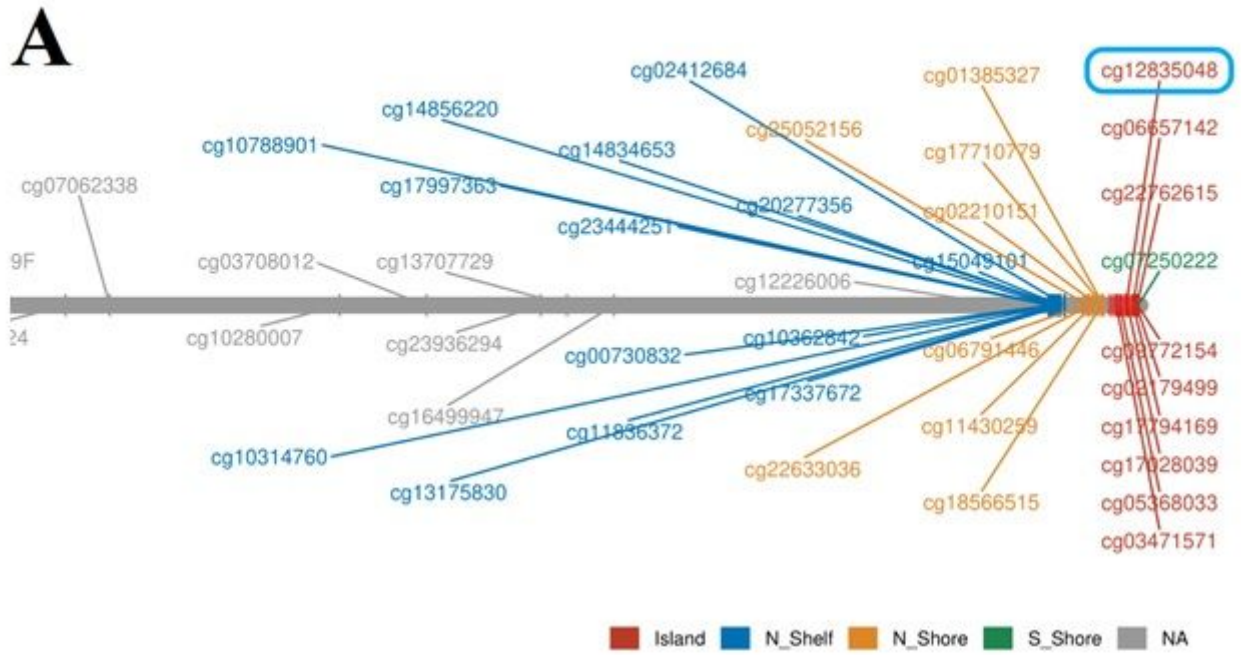


Figure 3

Methylation level and gene expression analysis. (A) Methylation level of FGFR2 in the region chr10: 121598479, 121598093, The analysis conveyed a significant difference between tumoral and normal samples (p = 0.032). (B) Expression analysis of FGFR2 also showed a significant upregulation (p < 0.05). (C) The methylation status of chr10: 121598092, 121598093 between the cancer types depicted and revealed significant differences (p < 0.05) in gastric cancer (STAD). STAD: Stomach Adenocarcinoma The pictures are adopted from (SMART - bioinfo-zs.com and GEPIA 2)



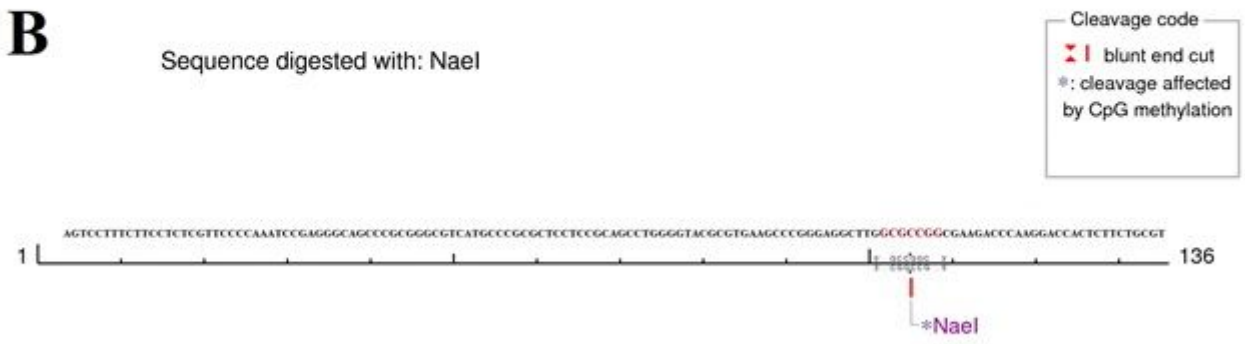


Figure 4

Information of the selected region. (A) Infinium 450k methylation array (Illumina) probes sites. The picture is adopted from (SMART - bioinfo-zs.com) (B) The sequence of PCR product and the selected

restriction enzyme. The picture is adopted from (NEBcutter V2.0)

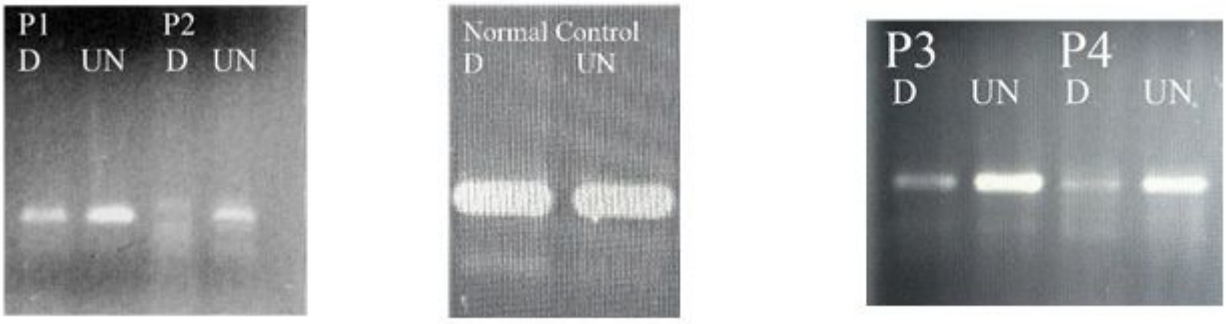


Figure 5

Methylation status of FGFR2 after digestion and MS-PCR of DNA samples. The samples derive from the same experiment, and that gels were processed in parallel. P1 and P2 are patients with IM. The sample of normal control did not digest. P3 and P4 are patients with GC. D: Treated samples, UN: Untreated control of the same sample, IM: intestinal metaplasia, and GC: Gastric Cancer.

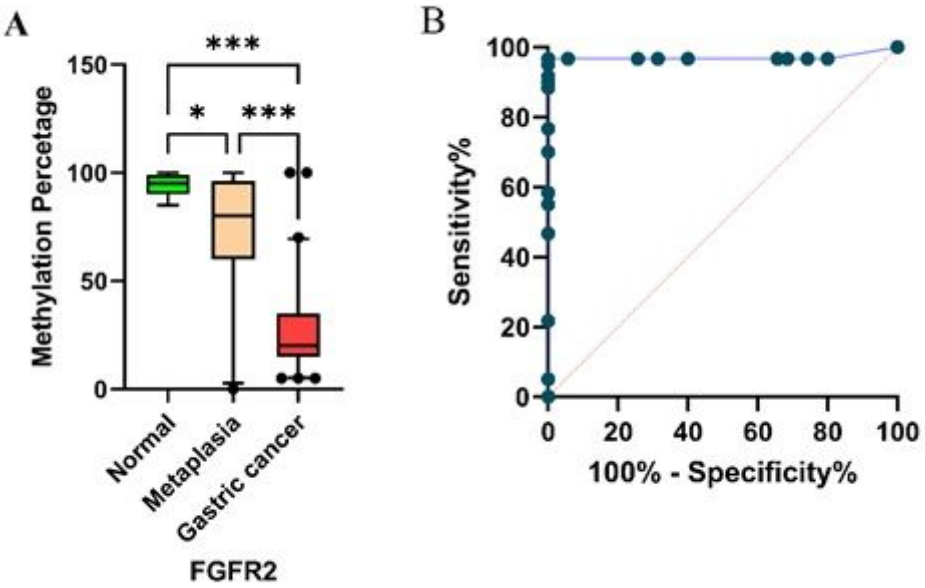


Figure 6

FGFR2 methylation status in the DNA obtained from whole blood. (A) Decreased methylation of FGFR2 promoters correlated with the presence of various gastric lesions. Each bar represents the mean ± SD. (p-value = 0.05 considered, *: $p = 0.01$, *** $p < 0.001$ vs. normal control), (B) ROC curve of FGFR2 methylation ($p < 0.001$) exhibiting a well-validated gene with high sensitivity (96.67 %) and high specificity (100 %).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GSE25869Supplement1.tsv
- GSE30601Supplement2.txt
- VennSupplement3.txt
- BiologicalpathwaySupplement4.csv
- MolecularFunctionSupplement5.csv