Deficiency in vitamin B12 and ferritin is associated with epigenetic alterations in cancer patients

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Research Article

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Abstract

Background: Cancer is the second-leading cause of death worldwide, caused by several mutations in DNA within the cells including epigenetic alteration. The epigenetic changes are external modifications to the DNA that switch “on” or “off” gene expression. The present study was conducted to investigate the epigenetic modifications and its correlation with the levels of vitamin B12 and ferritin in cancer patients with hepatocellular carcinoma (HCC), breast cancer (BC), lung cancer (LC), or colon cancer (CC).

Methods and Results: A total of 200 blood samples were obtained from cancer patients and healthy individuals. The relative expression of DNA methyltransferases (DNMTs), Ten-Eleven translocation (TET), and methionine synthase (MS) was evaluated in patients with the normal level of vitamin B12/ferritin and patients with the deficient levels of them. DNA methylation within the promoter regions was investigated of each indicated genes using the methylation-sensitive restriction enzyme HpaII and bisulfite PCR. Interestingly, the expression of DNMT1, DNMT3a, and DNMT3b was increased in patients with low levels of vitamin B12 and ferritin, while the expression of MS, TET1, and TET3 was significantly decreased. DNA methylation analysis in patients with deficient levels of vitamin B12/ferritin showed a methylated-cytosine within the location 318/CG and 385/CG in the promoter region of TET1 and TET3, respectively. Moreover, the bisulfite PCR assay further confirmed the methylation changes in the promoter region of TET1 and TET3 at the indicated locations.

Conclusion: These data indicate that the deficiency in vitamin B12 and ferritin in cancer patients plays a key role in the epigenetic exchanges during cancer development.

Introduction

Cancer is a kind of disease in which cells are characterized by unlimited growth and rapid proliferation. These cancerous cells can invade and destroy tissues, or migrate to distant tissues during a metastasis process. Unlikely, the benign tumor is characterized by specific growth and the inability to invade or transmit. However, the benign tumor can sometimes develop into a malignant tumor. Cancer is the second leading cause of death worldwide and is responsible for an estimated 9.6 million deaths in 2018 [1]. The most common types of cancer in males are lung cancer, colorectal cancer, prostate cancer, and stomach cancer. In females, the most common types are breast cancer, colorectal cancer, lung cancer, and cervical cancer [2]. Noteworthy, about 10% of all newly diagnosed cancers are due to inherited genetic traits [3]. Another 10% is due to obesity, malnutrition, lack of physical activity, or excessive drinking of alcohol. In addition to 30% caused by other factors include smoking, microbial infections, radiation exposure, and environmental pollutants [4].

Vitamin B12 (cobalamin) is a water-soluble vitamin that is derived from animal source and well known with a crucial role in the brain function and nervous system through the formation of myelin and the maturation of red blood cells. Vitamin B12 participates in the progress of the representation of each cell including the cells contributed to the synthesis of DNA, the metabolism of fatty acid, and myelin [5]. Vitamin B12 was recognized due to its association with pernicious anemia, an autoimmune disease in which the parietal cells of the stomach are destroyed. The parietal cells are responsible for producing the intrinsic factor and secreting the gastric fluid. The deficiency of vitamin B12 causes extensive dysplastic changes such as pancytopenia, macrocytosis, and hypersegmentation of neutrophils that contribute to the progress of acute leukemia [6]. Therefore, vitamin B12 deficiency contributes to various medical disorders and diseases including anemia, malabsorptive GI diseases such as sprue, celiac disease, partial gastrectomy, short bowel syndrome, and inflammatory bowel disease. The

deficiency of vitamin B12 also implicates in neurologic, psychiatric disorders, and increases the risk of cardiovascular [5, 7]. Meanwhile, the deficiency of iron is quite common in cancer patients due to multiple compatible mechanisms including medications, bleeding, nutrient starvation, and hepcidin- driven iron to macrophages leading to restricted formation of erythropoiesis [8].

In cancer, epigenetic alterations can affect the long-term expression of tumor suppressor genes and implicate in cancer initiation, development, and progression [9]. These epigenetic modifications can causally affect chromatin structure, gene silencing, and cell division and proliferation [10]. Several studies in genome and epigenome analysis reported the potential hypermethylation or hypomethylation of tumor suppressor genes and aberrant histone modification in cancer. On the other hand, DNA alteration refers to methylation activity that mainly occurs on cytosine at position 5 in CpG dinucleotides context and is activated by DNA methyl-transferase (DNMT) [11]. Methylation of the CpG islands promoter suppresses targeted genes due to poor recognition by transcription factors [12]. Consequently, DNA methylation pattern modifies a certain protein expression profile which is ultimately associated with various human disorders such as Alzheimer's and carcinogenicity [13, 14]. Several studies have been implicated the biological role and molecular interaction of DNMTs in cancer and medical disorders. For instance, The expression of DNMT1 is increased in breast cancer and highly induces DNA methylation of various tumor suppressor genes such as ataxia telangiectasia mutated (ATM) and adenomatous polyposis coli (APC) [15].

Noteworthy, vitamin B12 implicates in epigenetic modifications as a cofactor of the methionine synthase (MS) which is located in the cytoplasm and required vitamin B12 to form methylcobalamin and to catalyze the conversion of homocysteine to methionine. Through this process, a methyl group is transferred from methyltetrahydrofolate to methionine, an essential amino acid. Overall, vitamin B12 in association with methionine synthase is involved in the DNA methylation pathway through the synthesis of the methyl donor S-adenosylmethionine (SAMe), and synthesis of purine and pyrimidine groups via generation of tetrahydrofolate [16].

Accordingly, we sought to investigate the expression profile of different mediators of epigenetic process in blood samples obtained from patients with different types of cancer including lung cancer, breast cancer, colon cancer, and liver cancer. Furthermore, we demonstrated a concurrent association between the low levels of vitamin B12/ferritin in patients and hypermethylation of methionine synthase, the essential co-factor of DNMT and ten-eleven translocation (TET) 1 and 2, the methycytosine dioxygenases.

Materials And Methods

Ethical issues

This work was conducted at the faculty of medicine (Mansora University, Egypt) and Genetic Engineering and Biotechnology Research Institute (University of Sadat City, Egypt) from May 2018 to March 2020. The present study has been approved by the medical ethics and scientific ethics committees of the Faculty of Medicine and Genetic Engineering and Biotechnology Research Institute, respectively. The aim of this work was explained to patients who were agreed and understood all the rules and regulations. The most important criteria to be stated are that all samples were obtained from patients with a mean age of 40±10 years and before exposure to any treatment. Exclusion criteria were including age (<50 or >30 years), patients diagnosed with diabetes mellitus, patients with renal failure, patients treated with drugs affected plasma ferritin, vitamin B_{12}, folate, or zinc, and patients who were underweight or suffered from cachexia, or with a history of an eating disorder.

Samples conditions

A total of 160 blood samples were obtained equally from patients with hepatocellular carcinoma (HCC), breast cancer (BC), lung cancer (LC), and colon cancer (CC), in addition to 40 blood samples obtained from healthy individuals investigated as control. All patients were under health care, medical supervision, and accurate diagnosis of the cancer development and they were all in the advanced stage of cancer. Collected blood samples were further analyzed for the signs of cancer including tumor markers, the presence of cancer cells, and proteins or other substances made by cancer.

Biochemical analysis

The levels of serum ferritin, vitamin B12, and folate were measured using Elecsys Ferritin (REF 04491785190), Elecsys B12 II (REF 07212771190), and Elecsys Folate III (REF 07559992190), respectively via the electrochemiluminescence immunoassay (ECLIA) (Roche) [17]. For serum ferritin measurement, the blood samples collected in a trace-element free 8 mL tube (Becton Dickinson) were allowed to clot and centrifuged. 10 µL of each sample was incubated with a biotinylated monoclonal ferritin-specific antibody and a monoclonal ferritin-specific antibody labeled with a ruthenium complex for 9 min. Then the streptavidin-coated microparticles were added to the mixture which incubated for 9 min. The reaction was then transferred into the measuring cell and the chemiluminescent emission was measured by a photomultiplier [18].

To measure vitamin B12, 15 µL of the collected serum was incubated with vitamin B12 pretreatment 1 and pretreatment 2 reagents for 9 min. Then the pretreated samples were incubated with the ruthenium-labeled vitamin B12 binding factor for 9 min followed by incubation with streptavidin-coated microparticles and vitamin B12 labeled with biotin for 9 min. The reaction was then transferred into measuring cells and the induced chemiluminescent emission was measured by a photomultiplier. The final values were determined using the master curve provided via reagents barcode. Similarly, the level of folate in prepared serum was measured using 25 µL serum incubated with folate pretreated 1 and 2 reagents, ruthenium-labeled folate binding protein, and folate labeled with biotin.

DNA isolation and epigenetic analysis

The genomic DNA was isolated from obtained samples by using a DNA purification kit (Qiagen, USA) according to the manufacturer's protocol. Genomic DNA was digested with the methylation-dependent restriction enzyme HpaII (Thermo Scientific, USA) that unable to cleave the 5-methyle cytosine in its own restriction site 5-CCGG-3 by using five units of the enzyme to digest 200 ng of DNA during the incubation period of 4 hrs at 37 $^{\circ}$C. The digested products were loaded in 1% agarose gel and electrophoresed using 1X-TBE buffer. The electrophoresed fragments were monitored using the UV trans-illuminator with a long-wave (320 nm) UV and gel documentation system [19].

DNA elution and sequences

The HpaII-cleaved genomic DNA of cancer patients were eluted form the agarose gel using GeneJET gel extraction kit (Thermo Scientific, USA). Briefly, the gel slice was excised using a clean scalper and was weighted in 1.5ml-eppendorf tube. The gel slices (100mg) was then incubated with 100µL of binding buffer for 15min at 60 $^{\circ}$C. The dissolved gel was mixed very well by inversion and vortex, and then 100µL isopropanol was added to the mixture before loaded on the purification columns. The columns were centrifuged at 12000 rpm for 1 min and finally the DNA fragment was dissolved and collected from the columns using 50µL elusion buffer. Sequencing analysis of

eluted fragments we performed using Illumina NovaSeq 6000 sequencing system [20]. The DNA alignment between sequences data and the 3`-UTR of Atg7 and LC3B was done by using online tool [21].

Bisulfite PCR assay

Bisulphite analysis of *TET1 and TET3* promoter methylation was performed using the spin columns of EpiTect Bisulphite kit (Qiagen, USA). According to the manufacturer protocol, the purified genomic DNA was thermal denaturized and treated with sodium bisulfite using DNA protect buffer and incubated for 7 hrs for complete conversion. The converted DNA was applied to an EpiTect spin column using optimized buffers, then washed to remove all traces of sodium bisulfite and eluted from spin columns. Firstly designed oligoniclutids have been used to detect the methylated or unmethylated fragments into the promoter region of TET1 and TET3 at the location 318/CG and 385/CG, respectively. These specific oligonucleotides contain the forward primer specific for each gene location and two reverse primers. The first reverse primer contains the original or wild type sequences indicating the amplified unmethylated fragments. While the second reverse primer or the methylation-insensitive primer (MIP) has degenerated bases (G/A) to cope with the uncertain C/U conversion indicates the amplified methylated fragments. Therefore, the oligonucleotides contain triple primers specific for TET1; TET1-F-C-5'-GGCTCGGGCCTTGACTGTGCTG-3, TET1-R-W-5'-AGGTTTTGGTCGCTGGCCGGGT-3' and TET1-R-M-5'-CACTGGCCAGGTCACATTCCCA -3'. The following triple primers have been used for amplification of TET3 fragment, TET3-F-C-5'-GAGGCGGCGGGCAGGCAGCACT-3', TET3-F-W-5'-AAAGGGATCATCTTGGCCCGGT-3', and TET3-R-M-5'-GCCCAGTGGGTTCTTCACCCTC-3'[22]. The conventional PCR was used to amplify the methylated and unmethylated fragments using the following parameters; 95 $^{\circ}$C for 5 min, and 40 cycles (95 $^{\circ}$C for 30 sec, 62 $^{\circ}$C for 30sec, and 72 $^{\circ}$C for 30 sec) [23, 24]. The PCR products were loaded in 1% agarose gel and electrophoresed using 1X-TBE buffer and the amplified fragments were monitored using the UV trans-illuminator with a long-wave (320 nm) UV and gel documentation system.

RNA isolation and qRT-PCR

Total RNA were isolated from obtained samples by using TriZol (Invitrogen, USA), chloroform and isopropanol. Then the total RNA was purified by using RNA purification kit (Invitrogen, USA). The complementary DNA (cDNA) was synthesized from total RNA using QuantiTech Reverse Transcriptase Kit (Qiagen, USA) according to the manufacturer's protocol. The relative gene expression of DNMT1, DNMT2, DNMT3a, DNMT3b, MS, TET1, TET2 and TET3 was assessed in samples derived from patients compared with their expression in samples obtained from healthy individuals using specific oligonucleotides listed in table 1. The steady state mRNA of the indicated genes was quantified using the QuantiTect SYBR Green PCR Kit (Qiagen, USA). Expression level of the housekeeping gene, GAPDH, was used for normalization. The following PCR parameters were used to detect fold changes in genes expression, 94°C for 5 min, 40 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec). Delta-delta Ct analysis has been used to determine the relative gene expression indicated by fold changes in steady state mRNA [14, 25].

Statistical analysis and prediction tools

The data obtained by q-RT-PCR that reveal the cycle threshold values (Ct) were analyzed using the previously described ΔΔCt equations. Thus, the relative gene expression of targeted genes at RNA level was indicated by fold

changes that equaled to $2^{-\Delta\Delta ct}$ [26]. Student's two tails *t*-test was used to determine the differences in analyzed data. *P<0.05 was considered statistically significant and **P<0.01 as highly significant.

Results

Deficiency of serum vitamin B12 and ferritin is recognized in cancer patients

Out of 200 patients screened and offered baseline hemoglobin, serum ferritin, serum folate, and vitamin B12 estimation, 40 patients in each type of cancer were finally enrolled in the study. As shown in table 2, the prevalence of anemia was almost 80% in patients with HCC, since the persons who showed deficient levels of hemoglobin were 32 out of 40. These patients also showed deficient levels of ferritin, vitamin B12, transferrin saturation, and increasing levels of homocysteine. In patients with BC, 75% of patients have severe anemia with deficient levels of ferritin, vitamin B12, transferrin saturation, and increasing levels of homocysteine. The prevalence of anemia in patients with LC was 70% of patients accompanied by deficient levels of ferritin, vitamin B12, and transferrin saturation, and high levels of homocysteine. Likewise, 85% of patients with CC were anemic with deficient levels of ferritin, vitamin B12, and transferrin saturation, and high levels of homocysteine. Unlikely, more than 80% of cancer patients showed normal levels of serum folate, while a maximum of 20% of cancer patients showed low levels of serum folate. These data indicate that the cancer patients with HCC, BC, LC, or CC represented deficient levels of hemoglobin, serum ferritin, vitamin B12, and subsequently low saturation levels and accumulated levels of homocysteine.

The relative expression of DNMTs is associated with deficiency of vitamin B12/ferritin in cancer patients

To assess the correlation between DNA methylation and vitamin B12/ferritin deficiency in cancer, the relative gene expression of DNMT1, DNMT2, DNMT3a, and DNMT3b in obtained blood samples from patients with HCC, BC, LC, and CC were compared to blood samples derived from healthy individuals using qRT-PCR. Our results revealed a significantly increased expression level of DNMT1, DNMT3a, and DNMT3b, but not DNMT2 that connected with the low level of vitamin B12 and ferritin, while their expression significantly reduced in patients represented normal levels of vitamin B12 and ferritin (Figure 1A, B, C, and D). Notably, the calculated *P* values for the expression level of DNMT1 and DNMT3b were lower than 0.01 in samples derived from patients with HCC, BC, LC, and CC. Whereas the calculated *P* values for the expression level of DNMT3b were about 0.05 (Tables 3-6). These findings indicate the possible connection between DNMTs expression, particularly DNMT1, DNMT3a, and DNMT3b, and levels of vitamin B12/ferritin in patients with the indicated cancer diseases.

Deficiency of vitamin B12 and ferritin associated with low expression level of MS, TET1, and TET3 genes in cancer patients

To achieve the correlation between low levels of vitamin B12/ferritin and gene expression of other essential cofactors involved in DNA methylation in cancer patients, the relative gene expression of MS, TET1, TET2, and TET3 was evaluated by qRT-PCR in obtained blood samples. Interestingly, the results showed a significant reduction in the expression level of indicated genes; expect TET2 expression, associated with the deficiency of vitamin B12/ferritin in patients with HCC, BC, LC, and CC (Figure 2A, B, C, and D). Importantly, the calculated *P* values for MS, TET1, and TET3 expression level were lower than 0.01 in obtained samples from patients with low level of vitamin B12/ferritin compared with samples derived from patients with normal level of vitamin B12/ferritin and healthy individuals (Tables 7, 8, and 10). Whereas the calculated *P* values for TET2 expression level showed insignificant differences between patients and healthy individuals (Table 10). These data reveal that the deficiency

of serum vitamin B12 and ferritin in patients with cancer may contribute in the regulation of MS, TET1, and TET3 genes expression and subsequently controlling the DNA methylation process.

Patients with deficient vitamin B12 and ferritin revealed a methylation activity particularly in TET1 and TET3 promoter region

DNA methylation at CpG dinucleotides is a major epigenetic marker leading to gene silencing when located in the promoter region of a certain gene [27]. Noteworthy, the identified restriction site for HapII enzyme is (CCGG) with an independent sensitivity to cleave the methylated CpG [28, 29]. Based on this, we incubated the purified genomic DNA with HapII to compare and analyze the differences in cleaved fragments in cancer patients that are associated with the level of vitamin B12 and ferritin. Interestingly, agarose gel electrophoresis of HapII-cleaved fragments showed an alteration in the number of cleaved fragments in patients with a normal level of vitamin B12/ferritin and healthy on one side and patients with a low level of vitamin B12/ferritin on the other side. As shown in figures 3A and B, almost 7 cleaved fragments were found in patients with the normal level of vitamin B12/ferritin, while only 2 cleaved fragments were detected in patients with the low level of vitamin B12/ferritin. These findings clearly indicate that the HapII resection enzyme has failed to digest the genomic DNA isolated from cancer patients with a deficient level of vitamin B12/ferritin due to the potential DNA methylation activity on its binding site. Furthermore, to identify the potential methylated region, the low-intensity band cleaved from genomic DNA purified from patients with the normal level of vitamin B12/ferritin (approximately 100bp) was sequenced and aligned with deferent sequences of various related genes. Fortunately, the sequence and alignment analysis showed a consensus sequence of about 80 nucleotides within the promoter region of TET1 and TET3, started at the location 318/CG and 385/CG, respectively (Figure 3C and D). These findings strongly suggest that TET1 and TET3 were downregulated in cancer patients in a vitamin B12/ferritin dependent manner due to the methylation potential in their promoter regions.

Bisulfite-converted DNA revealed a significant CpG methylated motif in TET1 and TET3 genes associated with vitamin B12/ferritin level in cancer patients

Bisulfite DNA sequencing was recognized as an accurate method to detect DNA methylation-based on the conversion of genomic DNA using sodium bisulfite [30]. In this way, the methylated cytosine is converted into uracil and recognized in PCR amplification as thymine [31]. Therefore, we designed specific oligoniclutids for both TET1 and TET3 methylated regions including common forward primers and two reverse primers for each TET gene. The first reverse primer contains the complementary sequences of the normal motif CG named wild-type reverse primer (R-W) at the 3^- end of the primer. While the second reverse primer contains the complementary sequences of converted cytosine in CpG motif and named methylated reverse primer (R-M) at the 5^- end of the primer, to make the amplified fragment shorter than the amplified wild-type fragment, and contained adenine nucleotide instead of guanidine nucleotide. These triple primers were used to amplify the specific fragments from bisulfate-converted DNA isolated from cancer patients with a vary level of vitamin B12 and ferritin. Interestingly, the agarose gel electrophoresis of PCR products showed only one specific band with a molecular size of about 175bp when used the triple specific primers for TET1 and genomic DNA isolated from cancer patients with the normal level of vitamin B12/ferritin (Figure 4A). Whereas, two specific bands with a molecular size of 175bp and 165bp were detected using the genomic DNA isolated from cancer patient with a low level of vitamin B12 and ferritin (Figure 4B). Likewise, the specific triple primers for TET3 amplified only the wild-type fragment when using the genomic DNA isolated from cancer patients with a low level of vitamin B12/ferritin. Meanwhile, the same primers amplified both of the fragments with the molecular size of 175bp and 165bp using the obtained DNA from cancer patients with a

low level of vitamin B12 and ferritin (Figure 4C and D). These findings reveal the methylation potential in the promoter region of TET1 and TET3 associated with deficiency of vitamin B12 and ferritin in cancer patients like HCC, BC, LC, and CC.

Discussion

In the current work, we sought to investigate the DNA methylation process and the expression profile of its related cofactors in cancer patients to address the correlation between epigenetic changes and the levels of vitamin B12 and ferritin in patients' blood. Interestingly, the biochemical analysis of the patients and their collected samples demonstrated that more than 75% of the cancer patients showed low levels of vitamin B12 and ferritin, while more than 90% of the patients showed normal levels of serum folate. Therefore, the blood samples obtained from patients with HCC, BC, LC, or CC were divided into two main groups according to the levels of vitamin B12 and ferritin, patients with normal levels and patients with deficient levels. Our findings showed that the expression profile of DNMT1, DNMT3a, and DNMT3b was increased in patients with low levels of vitamin B12 and ferritin, while the expression of MS, TET1, and TET3 was significantly decreased. The methylation analysis of purified DNA using the HapII restriction enzyme and sequences analysis of cleaved fragments showed a potential methylated-cytosine at the location 318/CG and 385/CG in the promoter region of TET1 and TET3, respectively. These methylation changes were also achieved in patients with a low level of vitamin B12 and ferritin. Moreover, the bisulfite PCR using triple specific primers further confirmed the methylation changes in the promoter region of TET1 and TET3 at same indicated sites. Accordingly, the current data demonstrated that the low levels of vitamin B12 and ferritin in cancer patients plays a crucial role in hypermethylation and gene silencing of TET1 and TET3 and subsequently the balance between hyper and hypo-methylation processes in cancer cells.

The methylation activity of DNA is maintained by DNMTs that contain three major types, DNMT1, DNMT2, and DNMT3. DNMT2 mainly regulates the methylation activity of transfer RNA molecules (tRNA), although very recent evidence indicating the ability of DNMT2 to make methylation changes in the genomic DNA of aged macrophages [14, 32]. DNMT3 includes two different subtypes, DNMT3a and DNMT3b, which can make methylation changes in the genomic DNA [33]. Several studies demonstrated the variation of mRNA levels of DNMT1 and DNMT3 in association with human diseases such as atopic dermatitis and systemic lupus erythematosus in which their mRNA levels were decreased [34, 35]. While the overexpression of DNMT1 and DNMT3 has been connected with cancer development and autoimmune diseases indicated by the hypermethylation of certain genes expression [36]. In this way, our findings revealed the overexpression of DNMT1, DNMT3a, and DNMT3b in blood samples obtained from cancer patients with low levels of vitamin B12 and ferritin when compared with the blood samples obtained from cancer patients with normal levels of vitamin B12 and ferritin. This conclusion supports the hypothesis suggesting the role of vitamin B12 and ferritin in hypermethylation activity of genomic DNA during cancer development such as HCC, BC, LC, and CC. The current data also indicated the depletion of MS expression in patients with a deficient level of vitamin B12/ferritin. Noteworthy, MS is a crucial cofactor of DNMTs which responsible for the metabolism of SAMe, the donor of methyl groups required for DNA methylation, nucleic acids metabolism, and membrane structure and function [37].

Consequently, we addressed how DNA hypermethylation of certain genes can occur during cancer development; however, the methyl donor is interrupted due to deficient levels of vitamin B12 and ferritin. We hypothesize that the downregulation of TET1 and TET3 gene expression is indirectly responsible for the DNA hypermethylation of cancerous cells since TET1 and TET3 are oxidize 5-methylcytosines (5mCs) and promote locus-specific reversal of DNA methylation [38]. Most likely, the downregulation of TET1 and TET3 is accumulated due to epigenetic

exchanges in their promoter regions, as suggested by the current data, by which the expression profile and the molecular function of these effectors are prevented in cancer patients. *Numerous studies reported that TET proteins contribute to preventing the onset and transformation of malignancies; however, the exact mechanism is still unclear [38, 39]. Other evidences indicated that TET genes are often mutated in several cancers including* myeloid malignancies and solid tumors such as colorectal cancer, breast cancer, lung and liver cancer [40–42]. In normal cells, the hemimethylated DNA strands are normally converted to symmetrical methylation condition by the contribution of DNMT1 and ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) protein complex which recognizes hemimethylated CpGs and remains DNA methylation progress [43].

Recently, the TET proteins family were identified as dioxygenases that can converts 5-methylcytosine in DNA to 5-hydroxymethylcytosine, which plays a critical role in restoring and decreasing the symmetrical methylation progress [44]. Thereby, we provide evidence for the mechanism by which *TET* genes are mutated in cancer patients with deficient levels of vitamin B12 and ferritin via methylation of CpG islands in the promoter region of *TET1* and *TET3* genes. Interestingly, we developed an accurate method to efficiently determine single-base pair DNA methylation patterns on TET1 and TET 3 promoter regions via exploiting triple specific primers for each region that contain a forward primer and two reverse primers recognized the methylated CpG island with either wild-type sequences or complementary sequences for the methylated islands. Based on the conversion of 5-methyl cytosine at CpG islands to uracil in bisulfite-converted DNA, the triple primers were designed to recognize a certain fragment with CpG frequencies. In this method, the forward primer recognizes the upper sequences of the targeted fragment, while the wild-type reverse primer recognizes the end of targeted fragment that contains unmethylated CpG islands. The methylated reverse primer recognized the methylated CpG islands and amplifies a shorter fragment in comparison with the amplified one using the wild-type reverse primer. Accordingly, the agarose gel electrophoresis of bisulfite-PCR products using the indicated tripe primers can be used to distinguish between the two amplified fragments and confirm the potential methylation activity in the targeted region.

Declarations

Ethics statement

The current study has been approved by the medical ethics committee and scientific ethics committee of the Faculty of Medicine, Mansora University and Genetic Engineering and Biotechnology Research Institute, University of Sadat City, respectively.

Author's contributions

HK, SF, and SA established the study design, provided the research strategy and supervised overall the research plan. HK and KE performed the experiments, interpreted the data and provided the scientific and statistical analysis. HK prepared and wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

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Tables

Table 1: Oligonucleotides sequences used for quantification analysis of the indicated genes using qRT-PCR

Description	Primer sequences 5'-3'
DNMT1-sense	CCCATGCATAGGTTCACTTCCTTC
DNMT1-antisense	TGGCTTCGTCGTAACTCTCTACCT
DNMT2-sense	CATACAATGCCCGTGTGAGTTCTTAAGG
DNMT2-antisense	CGTGTGTCTAAATGGCTTGAGTACAGT
DNMT3-sense	TGCAATGACCTCTCCATTGTCAAC
DNMT3-antsense	GGTAGAACTCAAAGAAGAGGCGG
MS- sense	GAGATGCCTGAGACACCCA
MS- antisense	GTGCACCAGTTTTCGTTCCT
TET1- sense	GCACGATGCACCTGTACGAT
TET1- antisense	CACCAAGCTTTTTTGCTGTGAGT
TET2- sense	GCTCCTGGTGATGGCTACTG
TET2- antisense	TGTTTGCAGAGGTGAGTGGT
TET3- sense	GAAGAGCTCCCAAATTGCCT
TET3- antisense	GCTACAACATGAGCTACTGGC
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT

Factor / Cancer		Hemoglobin ≤1mg/dL	Ferritin >300ug/L	Transferrin saturation ≤20%	Vitamin B12 ≤200ug/L	Serum folate ≤140ng/ml	Homocysteine >15mcmol/L
HCC (n=40)	N	8 (20%)	8 (20%)	8 (20%)	6 (15%)	32 (80%)	6 (15%)
	D	32 (80%)	32 (80%)	32 (80%)	34 (85%)	8 (20%)	34 (85%)
BC (n=40)	N	10 (25%)	10 (25%)	10 (25%)	10 (25%)	34 (85%)	8 (20%)
	D	30 (75%)	30 (75%)	30 (75%)	30 (75%)	6 (15%)	32 (80%)
LC (n=40)	N	12 (30%)	12 (30%)	12 (30%)	10 (25%)	32 (80%)	10 (25%)
	D	28 (70%)	28 (70%)	28 (70%)	30 (75%)	8 (20%)	30 (75%)
CC (n=40)	N	6 (15%)	6 (15%)	6 (15%)	8 (20%)	36 (90%)	6 (15%)
	D	34 (85%)	34 (85%)	34 (85%)	32 (80%)	4 (10%)	34 (85%)

Table 2: Demographic variables of cancer patients in serum vitamin B12, ferritin, and the other related factors

Table 3: Mean Ct values of the DNMT1 and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		DNMT1		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	20.5	21.21	22.1	23.44	1.62	2.2	0.00	0.00	1.00	1.00	1.00	0.00	
	D	18.4	20.81	22.6	23.90	4.21	3.1	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	18.2	18.63	25.0	23.10	6.82	4.5	5.92	2.24	0.18	0.28	0.23	0.21	**0.001****
	D	19.3	20.86	20.8	22.18	1.45	0.8	-2.76	-2.24	7.17	4.87	6.02	2.30	**0.004***
BC	N	18.9	19.66	21.0	20.89	2.13	1.2	2.08	1.01	0.29	0.54	0.41	0.26	**0.04***
	D	19.6	19.85	21.3	19.68	1.64	-0.1	-2.02	-1.63	4.24	3.18	3.71	0.78	**0.01****
LC	N	20.0	20.88	23.8	24.58	3.84	3.7	2.22	1.46	0.22	0.48	0.35	0.24	**0.04***
	D	19.9	20.20	22.1	20.60	2.21	0.4	-1.45	-1.18	2.79	2.41	2.60	0.27	**0.01****
CC	N	18.9	19.19	22.1	22.39	3.21	3.2	0.89	0.97	0.56	0.63	0.59	0.34	**0.02***
	D	19.7	20.08	21.8	20.36	2.14	0.3	-1.52	-1.30	2.95	3.06	3.00	1.55	**0.001****

Table 4: Mean Ct values of the DNMT2 and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		DNMT2		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	18.5	19.21	27.1	26.44	8.62	7.2	0.00	0.00	1.00	1.00	1.00	0.00	
	D	17.7	18.28	26.5	25.56	8.85	7.3	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	18.2	18.63	25.8	25.58	7.62	7	-1.00	-0.28	2.08	1.31	1.70	0.66	**0.11**
	D	17.8	18.86	26.1	25.11	8.32	6.3	-0.53	-1.03	1.67	2.27	1.97	0.96	**0.08**
BC	N	17.6	18.16	25.7	25.70	8.06	7.5	-0.57	0.31	1.67	1.17	1.42	0.58	**0.24**
	D	19.1	19.18	26.4	26.33	7.25	7.2	-1.60	-0.13	3.29	1.41	2.35	1.33	**0.28**
LC	N	19.0	18.88	27.5	26.68	8.48	7.8	-0.14	0.56	1.22	0.68	0.95	0.38	**0.87**
	D	17.4	16.70	26.6	25.10	9.21	8.4	0.36	1.12	0.80	0.49	0.64	0.22	**0.14**
CC	N	16.9	17.19	26.1	25.73	9.21	8.5	0.59	1.30	0.78	0.45	0.61	0.30	**0.14**
	D	16.7	18.08	26.2	25.03	9.48	6.9	0.63	-0.34	0.65	1.49	1.07	0.59	**0.88**

Table 5: Mean Ct values of the DNMT3a and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		DNMT3a		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	16.5	17.21	25.1	24.44	8.62	7.2	0.00	0.00	1.00	1.00	1.00	0.00	
	D	17.9	17.21	26.5	25.56	8.64	8.4	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	17	17.23	26.0	25.27	9.02	8.0	0.40	0.81	1.02	0.87	0.95	0.71	**0.13**
	D	17.8	18.86	24.5	24.71	6.70	5.9	-1.94	-2.50	3.98	5.88	4.93	1.57	**0.05***
BC	N	17.6	18.16	25.5	25.70	7.83	7.5	-0.79	0.31	1.98	1.17	1.58	0.58	**0.29**
	D	19.1	19.18	26.2	26.16	7.09	7	-1.55	-1.37	3.03	3.28	3.15	0.92	**0.001****
LC	N	19.0	18.88	27.1	26.41	8.14	7.5	-0.48	0.30	1.47	0.86	1.16	0.44	**0.65**
	D	17.4	16.70	24.1	23.60	6.71	6.9	-1.93	-1.45	3.85	2.74	3.29	0.79	**0.05***
CC	N	16.9	17.19	25.5	25.06	8.54	7.9	-0.08	0.64	1.08	0.65	0.87	0.30	**0.60**
	D	16.7	18.08	23.5	24.03	6.81	5.9	-1.83	-2.41	3.66	5.32	4.49	1.17	**0.05***

Table 6: Mean Ct values of the DNMT3b and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		DNMT3b		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	16.4	17.22	24.3	24.84	7.85	7.6	0.00	0.00	1.00	1.00	1.00	0.00	
	D	17.9	17.21	26.5	25.56	8.64	8.4	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	17.8	17.23	26	25.43	8.20	8.2	0.35	0.58	0.86	1.02	0.94	0.68	**0.69**
	D	17.8	18.86	24.1	24.84	6.29	6	-2.35	-2.38	5.19	5.54	5.36	1.01	**0.001****
BC	N	17.6	18.16	25.5	25.77	7.85	7.6	0.00	-0.01	1.15	1.52	1.34	0.50	**0.29**
	D	19.1	19.18	26.0	26.16	6.87	7	-1.77	-1.37	3.49	3.28	3.38	0.75	**0.001****
LC	N	19.0	18.88	26.1	26.34	7.14	7.5	-0.71	-0.16	1.92	1.21	1.56	0.50	**0.25**
	D	17.4	16.70	23.6	22.46	6.21	5.8	-2.43	-2.60	5.51	6.09	5.80	0.41	**0.003****
CC	N	16.9	17.19	24.8	25.73	7.87	8.5	0.02	0.91	1.57	0.59	1.08	0.70	**0.60**
	D	16.7	18.08	22.6	24.03	5.93	5.9	-2.71	-2.41	6.78	5.32	6.05	1.48	**0.02***

Table 7: Mean Ct values of the MS and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in

comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		MS		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	17.2	18.29	20.4	20.74	3.19	2.4	0.00	0.00	1.00	1.00	1.00	0.00	
	D	19.9	19.81	20.31	21.18	0.4	1.37	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	20.6	21.52	21.0	21.50	0.42	-0.0	-1.67	-2.47	3.26	5.77	4.52	1.78	**0.002****
	D	19.1	19.64	21.3	22.22	2.25	2.3	2.74	1.95	0.16	0.27	0.21	0.08	**0.004****
BC	N	20.2	20.14	20.3	20.43	0.09	0.3	0.16	0.37	1.03	0.79	0.91	0.36	**0.5**
	D	19.2	18.95	22.2	21.20	2.92	2.1	2.11	2.16	0.27	0.25	0.26	0.09	**0.001****
LC	N	19.6	19.74	20.9	22.44	1.30	2.7	-1.20	-1.31	2.51	2.55	2.53	0.89	**0.001****
	D	19.2	19.20	21.3	22.44	2.06	3.2	1.30	2.20	0.41	0.23	0.32	0.13	**0.001****
CC	N	20.6	19.80	21.7	22.55	1.09	2.7	-0.40	-0.39	1.37	1.37	1.37	0.50	**0.1**
	D	19.5	19.86	19.9	20.15	0.40	0.3	3.37	3.39	0.27	0.10	0.18	0.17	**0.01****

Table 8: Mean Ct values of the TET1 and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		TET1		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	18.3	20.21	19.10	20.14	0.8	-0.1	0.00	0.00	1.00	1.00	1.00	0.00	
	D	19.9	19.81	20.31	21.18	0.4	1.37	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	20.6	21.72	20.84	21.50	0.2	-0.2	0.51	-0.15	0.73	1.20	0.97	0.33	**0.5**
	D	19.0	19.68	21.53	21.34	2.5	1.45	1.72	2.23	0.32	0.22	0.27	0.09	**0.004****
BC	N	20.2	20.14	20.27	20.43	0.1	0.30	0.16	0.37	1.03	0.79	0.91	0.36	**0.5**
	D	19.6	19.11	20.95	20.88	1.4	1.77	1.57	1.85	0.38	0.36	0.37	0.10	**0.008****
LC	N	19.3	19.18	20.6	22.24	1.27	3.0	-1.23	-0.95	2.37	2.15	2.26	0.34	**0.001****
	D	19.3	19.20	22.3	21.76	3.1	2.6	3.03	1.66	0.12	0.32	0.22	0.14	**0.013***
CC	N	19.9	19.50	21.6	22.55	1.9	3.1	0.36	-0.09	0.86	1.06	0.96	0.30	**0.7**
	D	20.2	20.53	19.9	20.15	-0.3	-0.4	3.23	2.72	0.37	0.16	0.27	0.26	**0.019***

Table 9: Mean Ct values of the TET2 and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		TET2		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	18.0	19.21	21.1	20.44	3.09	1.2	0.00	0.00	1.00	1.00	1.00	0.00	
	D	17.9	18.81	21.6	20.39	3.66	1.6	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	19	18.63	19.6	20.50	0.62	1.9	-0.47	0.64	1.43	0.75	1.09	0.52	**0.6**
	D	19.0	20.86	21.7	22.53	2.72	1.8	-0.94	0.23	2.10	1.03	1.57	0.76	**0.4**
BC	N	19.4	19.66	21.6	20.64	2.22	1	-0.83	-0.24	2.86	1.29	2.08	1.60	**0.3**
	D	18.3	19.18	22.2	21.18	3.97	1.7	0.31	0.21	1.01	1.05	1.03	0.39	**0.3**
LC	N	19.0	20.88	22.8	22.24	3.84	1.4	0.05	0.13	1.12	1.02	1.07	0.08	**0.2**
	D	18.9	20.20	21.6	21.60	2.71	1.4	-0.95	-0.18	1.95	1.20	1.58	0.53	**0.2**
CC	N	18.6	19.19	22	21.22	3.43	2.0	-0.28	0.45	1.24	0.84	1.04	0.28	**0.9**
	D	18.7	19.08	21.2	22.36	2.48	3.3	-0.25	0.27	1.35	1.12	1.23	0.48	**0.1**

Table 10: Mean Ct values of the TET3 and GAPDH genes expression in blood samples derived from patients with the indicated cancer diseases connected with the levels of vitamin B12 and ferritin in comparison with healthy individuals using qRT-PCR

Samples	B12-F	GAPDH		TET3		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
		MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Co	N	16.9	18.71	19.1	20.14	2.20	1.4	0.00	0.00	1.00	1.00	1.00	0.00	
	D	18.9	19.81	20.3	21.18	1.41	1.4	0.00	0.00	1.00	1.00	1.00	0.00	
HCC	N	18.8	19.06	19.8	20.49	1.01	1.4	-0.31	0.00	1.39	1.15	1.27	0.55	**0.07**
	D	18.6	18.7	22.9	22.70	4.30	3.6	2.89	2.21	0.17	0.22	0.20	0.11	**0.001****
BC	N	18.3	18.89	20.0	20.24	1.69	1.4	0.37	-0.07	0.82	1.12	0.97	0.49	**0.8**
	D	18.8	18.95	23.3	23.79	4.53	4.5	3.12	3.14	0.13	0.14	0.13	0.07	**0.001****
LC	N	18.7	18.67	20.8	20.43	2.18	1.8	-0.02	0.33	1.20	0.89	1.04	0.22	**0.81**
	D	18.3	18.20	23.3	22.95	5.06	4.7	3.65	3.37	0.10	0.14	0.12	0.03	**0.001****
CC	N	18.6	18.17	20.4	19.89	1.86	1.7	-0.34	0.29	1.53	0.98	1.25	0.71	**0.45**
	D	18.5	18.86	24.3	23.72	5.73	4.9	4.32	3.49	0.06	0.15	0.11	0.10	**0.002****

Figures

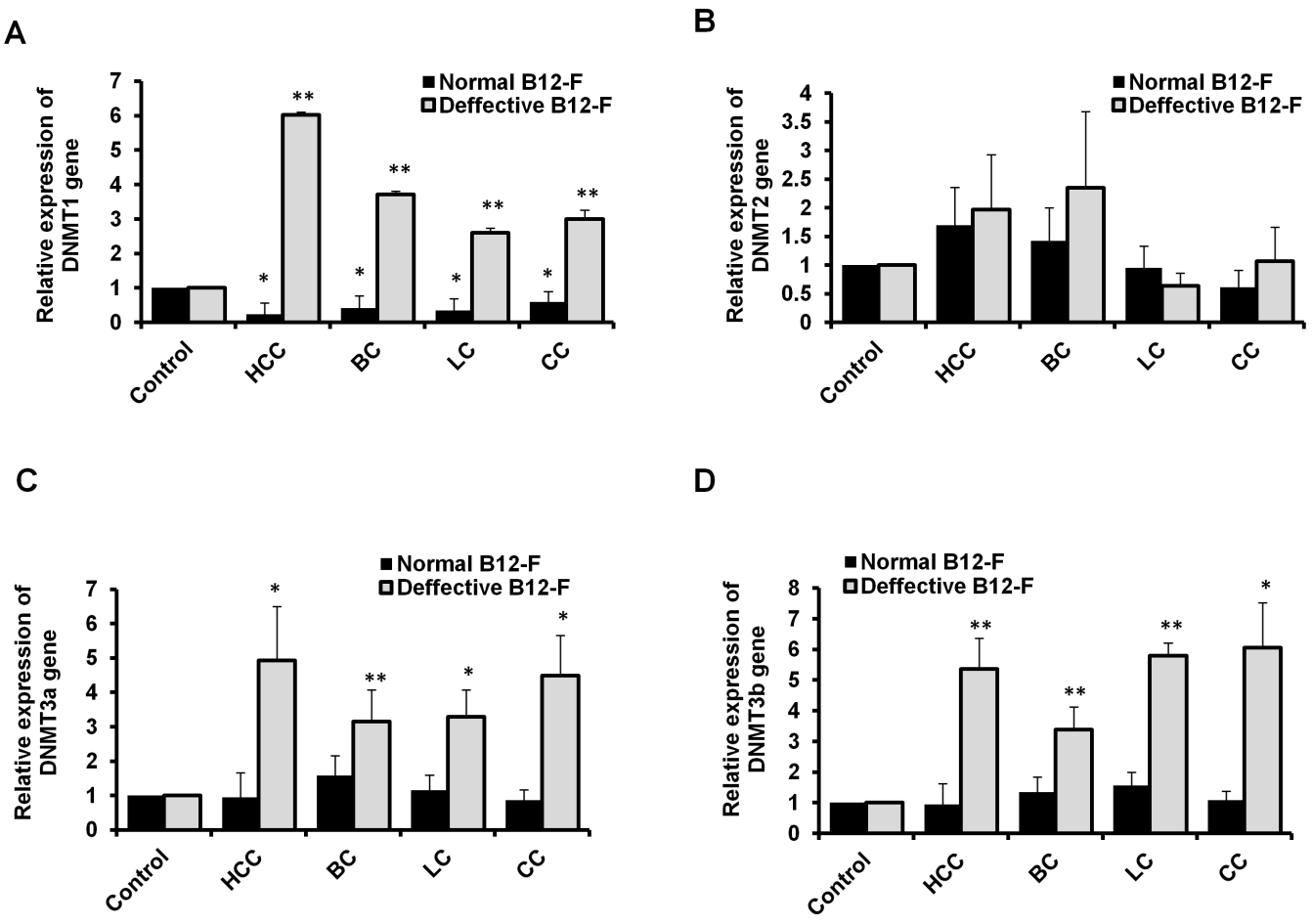

Figure 1

Quantification analysis of DNMTs in blood samples derived from cancer patients based on level of vitamin B12/ferritin (A) The relative gene expression of DNMT1 in samples derived from cancer patients with a deficiency of vitamin B12/ferritin in comparison with patients with a normal level of vitamin B12/ferritin and control-obtained samples using qRT-PCR assay. (B) The relative gene expression indicated by fold change in the expression of DNMT2 in obtained samples from cancer patients with either a normal level or low level of vitamin B12/ferritin using qRT-PCR. (C) The relative gene expression indicated by fold change in the expression of DNMT3a in samples derived from cancer patients with a normal level or low level of vitamin B12/ferritin using qRT-PCR. (D) The relative gene expression indicated by fold change in the expression of DNMT3b in samples derived from cancer patients with a normal level or low level of vitamin B12/ferritin using qRT-PCR. Error bars indicate the standard deviation (SD) of 3 independent experiments. Student two-tailed t-test was used for statistical differences of Ct values in different groups. (*) indicates P-values ≤0.05 and (**) indicates the P ≤0.01

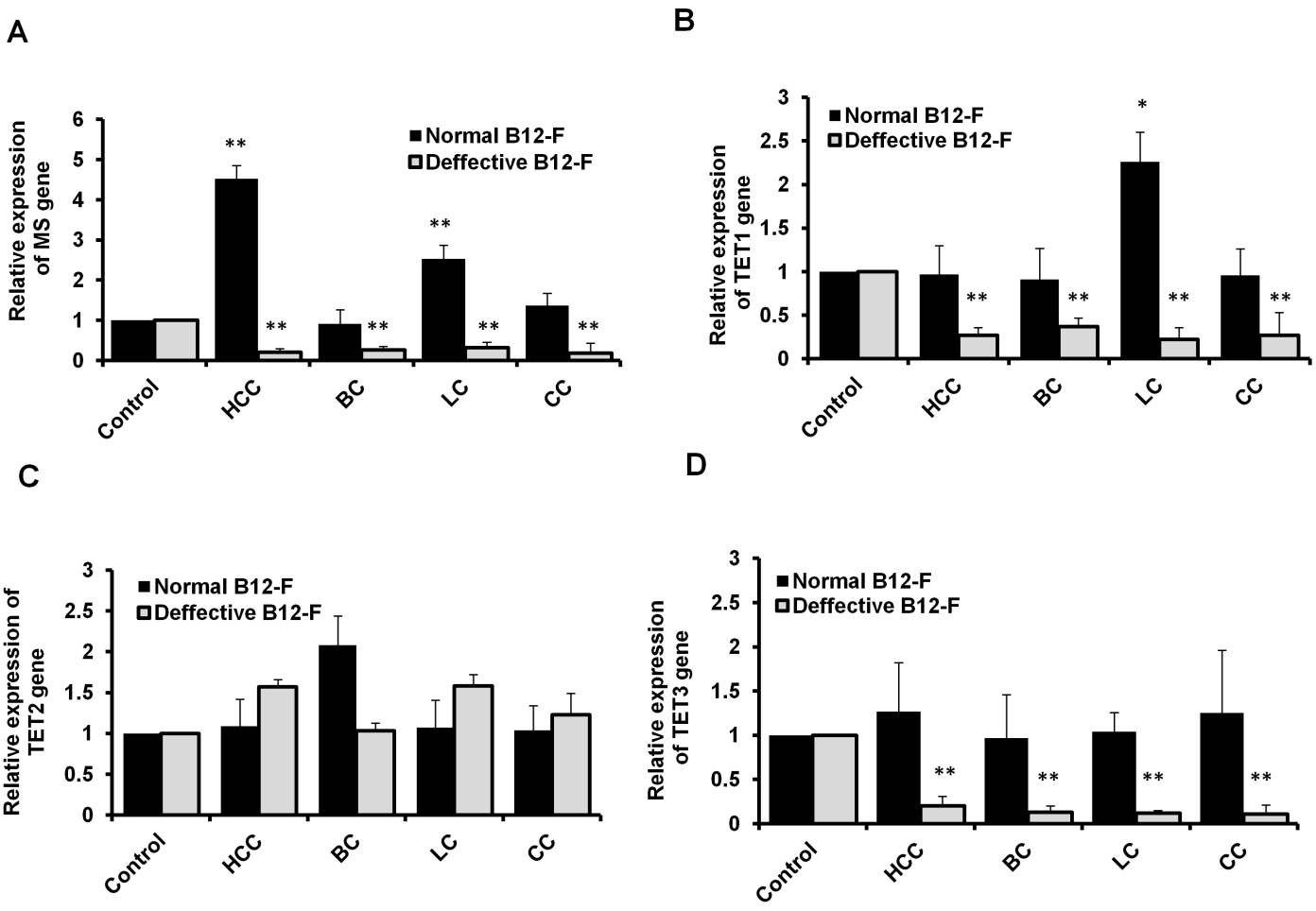

Figure 2

Quantification analysis of methylation-related effectors in blood samples derived from cancer patients based on level of vitamin B12/ferritin (A) Quantification analysis of MS gene expression in derived samples from cancer patients with a deficiency of vitamin B12/ferritin in comparison with patients with a normal level of vitamin B12/ferritin and control-obtained samples using qRT-PCR assay. (B) Quantification analysis of TET1 gene expression in obtained samples from cancer patients with a normal level or low level of vitamin B12/ferritin using qRT-PCR. (C) Quantification analysis of TET2 gene expression in samples derived from cancer patients with a normal level or low level of vitamin B12/ferritin using qRT-PCR. (D) Quantification analysis of TET3 gene expression in samples derived from cancer patients with a normal level or low level of vitamin B12/ferritin using qRT-PCR. Error bars indicate the SD of 3 independent experiments. Student two-tailed t-test was used for statistical differences of Ct values in different groups. (*) indicates P-values ≤0.05 and (**) indicates the P ≤0.01

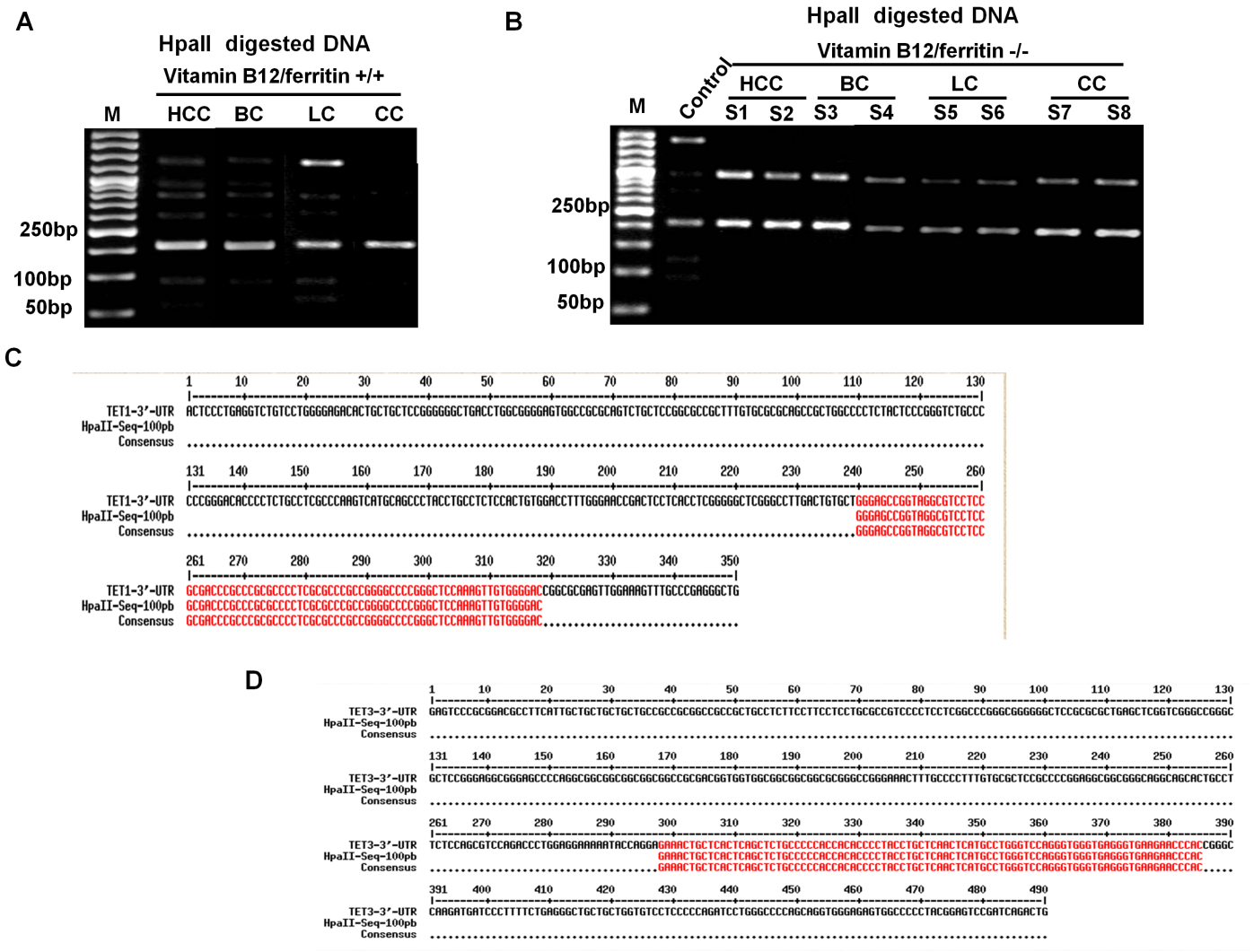

Figure 3

CpG methylation change in genomic DNA of cancer patients (A) Agarose gel electrophoresis represents genomic DNA that was isolated from indicated cancer patients with the normal level of vitamin B12/ferritin and digested with methylation-sensitive HapII for 2 hours. (B) Agarose gel electrophoresis represents genomic DNA that was isolated from indicated cancer patients with a deficient level of vitamin B12/ferritin and digested with methylation-sensitive HapII for 2 hours. (C and D) Alignment between the resulted sequences of the low-intensity HpaII-cleaved fragment and 3'-UTR sequences of TET1 and TET3 indicated the location of consensus sequences between them, respectively.

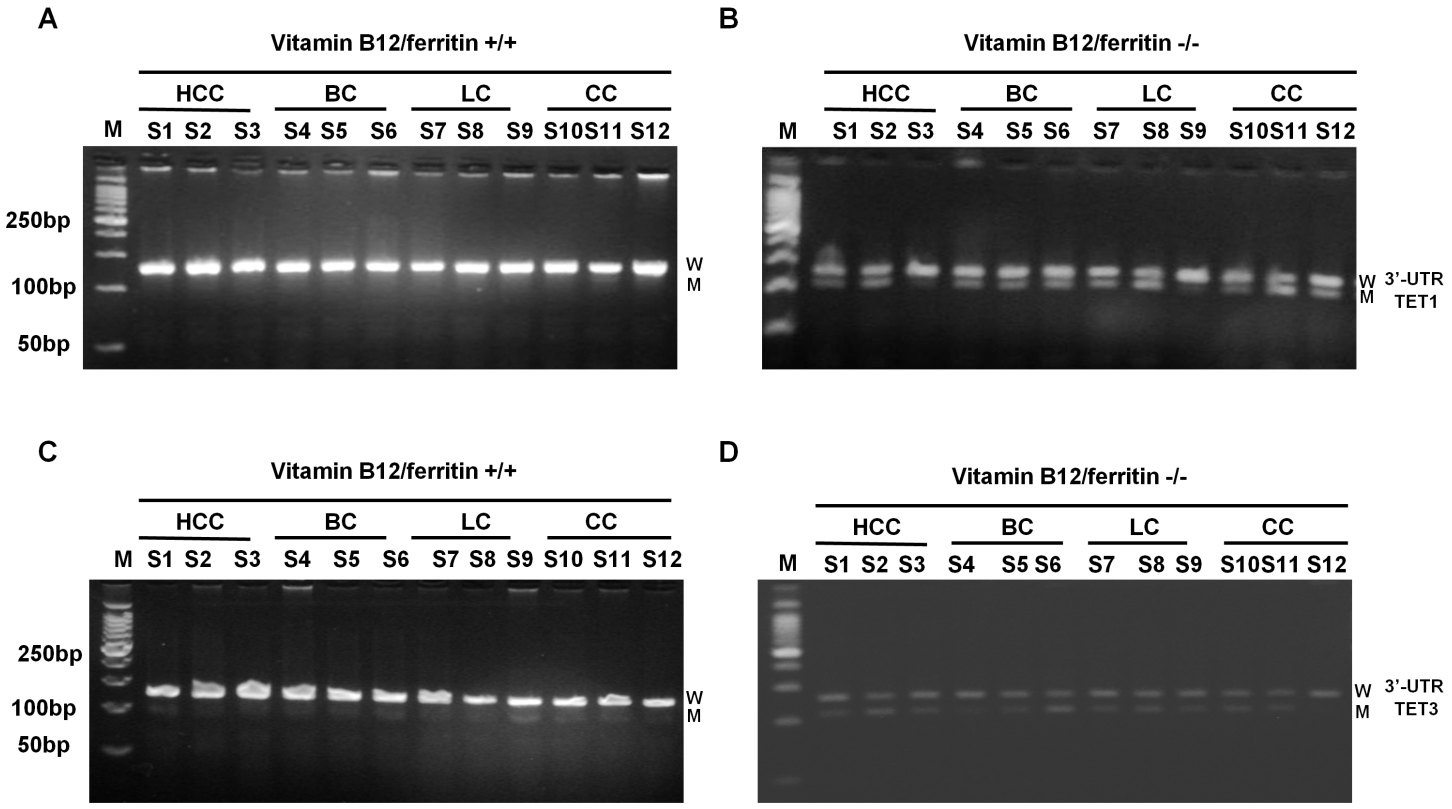

Figure 4

Bisulfite PCR amplification of converted DNA using tripe specific primers for TET promoter region (A and B) Agarose gel electrophoresis of bisulfite PCR products revealed the amplified fragments using the wild-type primer and methylated primer specific for TET1 promoter region and genomic DNA isolated from the indicated cancer patients with a normal level of vitamin B12/ferritin and a low level of vitamin B12/ferritin, respectively. (C and D) Agarose gel electrophoresis of bisulfite PCR products exhibited the amplified fragments using the wild-type primer and methylated primer specific for TET3 promoter region and genomic DNA isolated from the indicated cancer patients with a normal level of vitamin B12/ferritin and a low level of vitamin B12/ferritin, respectively.