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Unmethylated promoter DNA correlates with p53 expression and apoptotic levels only in Vitamin B9 and B12 deficient megaloblastic anemia but not in non-megaloblastic anemia controls

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ABSTRACT

Cyanocobalamin (Vitamin B12, VB12) and Folic acid (Vitamin B9, VB9) deficiency leads to anemia in women. We have recently shown low VB12 and VB9 levels in the serum of megaloblastic anemia (MBA) patients. Further, our study demonstrated elevated homocysteine and p53, respectively, in the serum and bone marrow aspirates of MBA patients but not in non-MBA subjects. However, it is unknown whether any gender specific variation in VB12 and VB9 level exists in MBA and non-MBA patients? In addition, it is unclear whether low VB12 and VB9 has a role in the regulation of p53 expression in MBA patients? And whether elevated p53 is functionally active? If so, does bone marrow aspirates of MBA patients show elevated apoptosis. Hence, we have analyzed VB12 and VB9 levels in MBA patients and compared with non-MBA subjects. Next, methylation status of p53 promoter was determined and correlated with p53 expression. Furthermore, the level of apoptosis in bone marrow aspirate paraffin blocks was estimated using TUNEL staining. In conclusion, low VB12 and VB9 in male and female patients directly correlate with p53 promoter unmethylation status, but, inversely correlate with p53 protein expression and its activity, only in MBA cases but not in non-MBA controls.

1. Introduction

Megaloblastic and non-megaloblastic anemias are two different classes of macrocytic anemia and are usually distinguished by the examination of bone marrow [1]. The megaloblastic anemia (MBA), which is more common, is characterized by increased erythrocyte mean corpuscular volume (MCV) as well as pancytopenia in the peripheral blood smears [2,3]. In addition, the bone marrow of MBA patient's exhibits increased number of large and early stage hematopoietic precursor cells (erythroblasts) with more chromosomal breaks [3]. However, the non-megaloblastic anemia (non-MBA) is a rare type and is usually associated with severe liver or bone marrow diseases [1]. Moreover, the non-MBA is characterized by the presence of mature and large erythrocytes, and no faulty DNA synthesis [1].

Very low levels of folate (Vitamin B9) and/or cobalamin (Vitamin B12) causes megaloblastic anemia in male and female population [4,5]. Whereas the peripheral blood of MBA patients show pancytopenia and elevated erythrocyte mean corpuscular volume (MCV), the bone marrow exhibits the predominance of large early stage hematopoietic precursor cells with increased chromosomal breaks [1,6–8]. In general, p53 transcription factor repairs these chromosomal breaks by, first arresting cells in G1 phase of cell cycle through the up regulation of p21 followed by inducing DNA repair enzymes [9,10]. However, if p53 fails to repair the damage, it promotes the expression of proteins such as Bax, Noxa, and p53 upregulated modulator of apoptosis (PUMA) thereby trigger apoptotic cell death [11,12]. Hence, p53 is a key mediator of apoptotic death in the damaged megaloblasts that are usually observed in MBA [3].

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Tumor protein (TP53 or p53) is a well-known tumor suppressor of 53 kDa, and is coded by a gene located on the short arm of chromosome 17 in humans (17p13.1) [13,14]. The gene coding for p53 has 11 exons and a specific 85 bp promoter (to which the transcription factors bind) located between nucleotides 760–844 [14]. Expression of p53 is usually regulated by methylation of its promoter [15]. In general, DNA methylation is carried out by the transfer of a methyl group from S-adenosyl-L-methionine to the 5th carbon of cytosine residues in the CpG island region of the DNA by DNA-methyl transferase enzymes (DNMTs [16,17]. Methylation of CpG islands silences the genes such as p53 [18,19]. Several studies have shown hypermethylation of p53 gene promoter in many cancers [14]. As a result of this hypermethylation, the expression of p53 decreases, leading to failure of apoptosis [20].

Vitamin B12 and B9 plays a key role in the methylation of DNA [21,22]. In addition to VB12 and VB9, other micronutrients such as betaine, choline and methionine also influence the methylation of DNA and chromosomal integrity [23,24]. For example, VB9 and VB12 are the primary source of 5-methyltetrahydrofolate, which play a vital role in the remethylation of homocysteine to methionine [1,23,25,26]. Methionine is required for the formation of S-adenosylmethionine (SAM), a universal methyl group donor, involved in DNA and RNA synthesis [27]. Failure of producing sufficient SAM results in under methylation of gene promoters, leading to their expression in unusual quantities [28–30]. Previously, we have demonstrated that VB12 and VB9 levels had an inverse correlation with p53 expression in MBA patients [3,6]. Therefore, we have hypothesized that elevated p53 expression in MBA patients' bone marrow aspirates is due to no/under-methylated p53 promoter leading to more apoptotic cell death [3]. Hence, in this study first we have estimated the levels of VB9 and VB12 in male and female MBA and non-MBA samples' serum and measured the methylation status of p53 promoter using MS-PCR in bone marrow aspirates. Further, the levels of apoptosis was estimated using TUNEL staining to determine whether the expressed p53is functionally active. Analysis of the data showed presence of unmethylated p53 in all non-MBA (n = 50) as well as MBA samples (n = 50), irrespective of gender. However, very high p53 expression and apoptotic cell death were observed in MBA cases compared to non-MBA controls indicating that VB12 and VB9 have no influence on the methylation of p53 promoter in MBA. Therefore, further studies addressing (a) how methylation of p53 promoter is being regulated in MBA cases; (b) the factors influencing the stability of expressed p53 in MBA cases compared to non-MBA controls are warranted.

2. Materials and methods

The selection and screening of controls (non-megaloblastic anemia) and cases (megaloblastic anemia) was carried out as described earlier [3]. Institutional Ethics Committee, which follows the principles of declaration of Helsinki, has approved the study (JSS Medical College/Institutional Ethical Committee/MC/IEC/1847/2012-2013; Dated 21 st July 2012). Informed written consent was taken from each individual after explaining the study details in their native language.

Screening and selection of control (non-megaloblastic anemia: Male = 23, Female = 27) subjects and cases (megaloblastic anemia: Male = 31, Female = 19) was carried out based on their clinical symptoms, complete blood count, peripheral blood smear reporting, bone marrow aspirate and bone marrow biopsy reporting and, biochemical investigations [3].

2.1. Analysis of the methylation status of p53 promoter using methylation specific polymerase chain reaction (MS-PCR)

Methylation specific PCR was carried out as detailed in [14]. In brief, first, the bone marrow aspirates were collected by making a small incision (at posterior iliac crest) followed by inserting a hollow needle through the bone till bone marrow. The physician withdrew the bone marrow aspirates and biopsies from 50 patients with Megaloblastic Anemia (Male = 31, Female = 19) and 50 control subjects of non-megaloblastic anemia (Male = 23, Female = 27) who were recommended by clinician for suspected bone marrow disorders. The samples were collected in sterile vacutainer tubes containing EDTA (2.5–3.0 mL) and stored at −80° C. 20 μL bone marrow aspirate was used to extract DNA followed by converting unmethylated Cytosine (C) residues with bisulfite in to Uracil (U) using EpiTect Fast LyseAll kit (#59864) from Qiagen. Universal methylated, and unmethylated DNA samples were also processed for bisulfite treatment and used as controls in MS-PCR experiments. Methylation status of p53 promoter was determined using MS-PCR. The primer sequences reported by Chmelarova M et al., were used to determine unmethylated (5′-TTGGTAGGTGGATTATTTGTTT-3′ (sense) and 5′- CCAAT CCAA AAAA ACATATCAC-3′ (antisense); expected PCR product size – 247 bp), and methylated (5′-TTCG GTAGGCGGATTATTTG-3′ (sense) and 5′-AAATATCCCCGAAACCCAAC-3′ (antisense); expected PCR product size – 193 bp) [14]. Each sample was subjected to unmethylation and methylation PCR reactions, at least in duplicates, as detailed below. First, 2.0 μL bisulfite modified DNA (for unmethylated Cytosines) was subjected for PCR reaction, which contains 1× master mix (from EpiTect MSP kit Qiagen # 59305), 0.4 μM forward and 0.4 μM reverse primers. The PCR reactions were carried out in a CG-PalmCycler, with the following reaction conditions: Step#1: *Initial denaturation* at 95° C for 10 min; Step#2: *Subsequent denaturation* at 95° C for 45 s for 40 cycles; Step#3: *Annealing* at 59° C for 45 s; Step#4: *Extension* at 72° C for 45 s; Step#5: *Final extension* at 72° C for 1-cycle of 5 min. A no-DNA control reaction was also performed to rule out the contamination [14].

All PCR products were resolved and analyzed using 2% Agarose gels at 250 V for 40 min. 100 base pairs DNA ladder was used from Qiagen to measure the size of the band and locate the correct product.

2.2. Detection and quantification of apoptosis using TUNEL assay

2.2.1. Preparation of paraffin blocks

Paraffin blocks for TUNEL fluorescence detection were prepared by incubating bone marrow biopsies samples in 10% formalin for 16 h. Next, decalcification was carried out in 10% EDTA treatment for 2–3 days. After this, EDTA was removed by washing the specimens thoroughly in running tap water [3]. The specimens were processed using an automated tissue processor (Thermo Scientific, Shandon Citadel-2000) as detailed below:

1. Step#1: Dehydration was carried out in 70% alcohol (60 min), 90% alcohol (45 min), absolute alcohol (45 min) 2 times, and absolute alcohol (60 min).
2. Step#2: Further dehydration in xylene (60 min) 3 times, followed by infiltration with paraffin wax for 30 min, 60 min and 90 min.
3. Step#3: Embedding of dehydrated specimens in paraffin wax.
4. Step#4: Sectioning by using Leica RM 2255 microtome to generate 4 μm thick sections [3].
5. Step#5: Fixation on poly −L-lysine coated slides

The tissue sections were kept at 37° C for 1 day, which helps in removing the xylene, TUNEL staining of tissue sections was car-