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PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330
Int J Hum Genet, 21(4): 157-166 (2021)
DOI: 10.31901/24566330.2021/21.04.793

Predicting the Role of SERPINA1 DNA Methylation in Chronic Obstructive Pulmonary Disease and Anaemia and Identification of 3 Novel Methylation Sites

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Clinical Trials Registration Number: AUW/IHEC–18–19/HGMB/FHP–21

KEYWORDS Alpha 1 antitrypsin. Altitude. Hemoglobin. Epigenetics. Polymorphism. Respiration

ABSTRACT Anaemia and chronic obstructive pulmonary disease (COPD) are the common blood and respiratory disorders respectively. The proper lung function is maintained by the *SERPINA1* gene predominantly that encodes for alpha 1 antitrypsin protein, which also regulates the iron homeostasis of the human body, whereas imbalance in the iron homeostasis may result in the anaemic condition. The altitudinal variations influence anaemia and COPD. DNA methylation is involved in the early developmental processes, which influences the gene functioning without altering the sequence. The current study has been aimed at analyzing the inter-relationship between anaemia and COPD with DNA methylation of the *SERPINA1* gene under altitudinal changes. The methodology involves the DNA isolation, bisulfite conversion and sequencing of the *SERPINA1* gene. The results of the current study have shown that *SERPINA1* DNA methylation did not significantly involve anaemia and COPD irrespective altitudes, but 3 novel CpG sites cg94377701, cg94389678 and cg94389930 were identified in the *SERPINA1* gene of anaemia and COPD patients.

INTRODUCTION

Anaemia, a predominant blood disorder (Sunuwar et al. 2021; Styszynski et al. 2021) and chronic obstructive pulmonary disease (COPD), a common respiratory clinical condition is being suspected to be interlinked on a genetic basis (Rodrigues et al. 2021). The anaemic prevalence in COPD patients is higher, accounting for around 33 percent (de Miguel Diez Jde et al. 2009; Yohannes and Ershler 2011; Thangavelu et al. 2019). The COPD condition is diagnosed with the ratio of forced expiratory volume (FEV1) and forced vital capacity (FVC). The FEV1 values are being found to be influenced by the anaemic patients (Rahimi Rad et al. 2015). The inflammation in COPD causes cytokines and acute phase reactants like C-reactive protein to be altered, which inhibits

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the red blood cell precursors and absorption of iron resulting in the anaemic condition (Carroz 2007). Altitude influences the erythrocyte production and regulation *via* hypoxia-inducible factors (Sarna et al. 2020), which suggests the correlation with the erythrocyte dependent clinical condition, anaemia. An increase in altitude affects the functioning lungs as the air density, humidity as well as temperature declines with an elevation in altitudinal ranges (Stream 2009).

DNA methylation is one of the epigenetic modifications that are characterized by the addition of a methyl group to the cytosine of the genetic sequence, converting it into 5-methyl cytosine (Ahn et al. 2021; Sedley 2021). Histone modification is the other major epigenetic modification, in which the histone proteins are modified with the addition of methyl, phosphate, acetyl or ubiquitin group after the translation process (Pinel et al. 2019). DNA methylation may be beneficial during the tissue and cell formation and is majorly involved during the stable mitotic process called genomic imprinting (Moore et al. 2013; Jin and Liu 2018). DNA methylation has been reported to be an important process for regulating genome expression and development (Crider et al. 2012; Melanie et al. 2019; Nataliya Petryk et al. 2021). The regulation of DNA methylation involves enzymes like ten-eleven translocation enzymes, which in turn is regulated by iron. The disruption of iron homeostasis may indicate the malfunctioning or improper regulation of DNA methylation (Lien et al. 2019). The methylation of the erythropoietin (*EPO*) gene acts as a risk factor for anaemia (Ingrosso and Perna 2020).

SERPINA1 gene, which encodes for a protein called alpha 1 antitrypsin involves the regulation of protease enzyme called neutrophil elastase (Sangeetha et al. 2020). The neutrophil elastase has an inflammatory property that is capable of damaging the lung tissue when left unregulated (Connolly et al. 2018; Matamala et al. 2018; Leon and Bouchecareilh 2021). The alpha 1 antitrypsin also gets involved in an iron regulatory hormone called hepcidin (Pandur et al. 2009; Gulec et al. 2014; Pfeifhofer-Obermair et al. 2018), which makes the common identity for *SERPINA1* between iron-dependent disorder, anaemia and the inflammatory disease, COPD. The present study involves the comparison of anaemic and COPD patients on the altitudinal and epigenetic basis in correspondence to their respective control samples.

Review of Literature

The synthesis of erythrocytes is predominantly regulated by the hepcidin hormone. A study by (Pagani et al. 2019) has reported that the increased synthesis of hepcidin causes iron malabsorption resulting in iron deficiency anaemia. Around 90 percent of the protection on the lower tract of the lungs has been provided by alpha 1 antitrypsin (Dunlea et al. 2018). Hypermethylation, as well as hypomethylation of the *SERPINA1* gene, has been observed in patients with COPD (Rotondo et al. 2021). Balasubramanian et al. (2021) has reported that the anaemic prevalence of COPD increases the disease progression and the fatality range.

Huang et al. (2021) has reported that the red blood cell parameters are highly influential in the progression of COPD. The hereditary incidences of COPD among the individuals were reported to be approximately 37 percent (Gillenwater et al. 2020). Studies have suggested that the change in altitudinal influences the anaemic condition (Baldwin et al. 2021; Liu et al. 2021). Iron deficiencies resulting in anaemic conditions have been reported to be associated with an increase in altitudinal ranges (DeLoughery et al. 2021). Methylation of *GSDMB* has been reported to be associated with COPD conditions (Moll et al. 2021). The methylation of the *SERPINA1* has been observed to have a role in colorectal cancer (Jaberie et al. 2020). The availability of literature inter-connecting the DNA methylation of the *SERPINA1* gene, anaemia, COPD along differences in altitude is deficient and hence, this present study focuses on the lacking part of the epigenetic studies in anaemia and COPD.

Objectives

The current study has been aimed at analyzing the role of the *SERPINA1* DNA methylation in the anaemic and COPD patients along with the control samples collected from two different altitudinal regions. The comparison and analysis of the influence of change in altitudes on anaemia and COPD by the presence or absence of DNA methylation have also been determined. The present study

has also been extended to identify any novel methylation sites in the *SERPINA1* gene if present.

METHODOLOGY

Sample Collection

The sample collection (n=518) was based on the inclusion and exclusion criteria obtained *via* a questionnaire collected (n=877) from random individuals residing in two different altitudinal regions of ranges around 411m (low altitude) and 2631m (high altitude) above the sea level. The Declaration of Helsinki, which was developed by the World Medical Association, has been followed for the collection of blood samples. Among 518 collected samples 207 (103-low altitude; 104-high altitude) were anaemic 105 (52-low altitude; 53-high altitude) were COPD patients and 206 were healthy control. Among 103 low altitudes anaemic, 62 were male and 41 were female, whereas 32 were male and 72 were female in high altitude. About 27 male COPD samples were collected from both the altitudes and 24 and 26 female COPD samples were collected from low and high altitude regions respectively. Around 52 and 36 male control were from low and high altitude regions respectively, whereas female samples accounted as 51 and 67 in number. On comparing the samples on an age basis, it has been found that age significantly influences COPD condition since all COPD patients were above 30 years of age, whereas anaemic condition was prevalent among all the age groups. Among the 518 samples, around 113 patients were below 30 years of age and 221 were above 50 years and the rest of the samples were between 30 to 50 years of age. Around 87 out of 105 COPD patients were diagnosed with anaemic conditions.

The inclusion criteria were people above the age of 15years, residing in the geographical regions of the desired altitude diagnosed with anaemic and COPD conditions, whereas the exclusion criteria were people with fatal diseases like cardiovascular disease, neurogenetic disorders. The human peripheral blood sample (3ml) has been collected after obtaining a proper Human Ethical Clearance Certificate (Approval No: AUW/IHEC-18-19/HGMB/FHP-21) from Avinashilingam Deemed University for Women, Coimbatore, Tamil Nadu, India and an informed consent form from the individuals.

DNA Isolation and Quantification

The collected whole blood samples were subjected to DNA isolation using Miller et al. (1988) method. The blood sample was centrifuged with 0.9 percent saline initially to induce the osmotic pressure on the blood cells. The supernatant was discarded and the solution containing a mixture of triton X, magnesium chloride and sucrose was added to lyse the cell membranes. The addition is followed by centrifugation at 8000rpm for 5 minutes and the supernatant was discarded. The pellet was then dissolved in tris Cl, tris NaCl and ethylenediaminetetraacetic acid (EDTA) containing solution to maintain the stable pH, followed by the addition of lauryl sodium salt, 5M sodium perchlorate solution, which removes the protein complexes and ice-cold chloroform to enable the efficient separation of the organic and aqueous phase. The mixture is subjected to centrifugation and the uppermost layer (aqueous phase) was collected, which is then added with ice-cold ethanol, followed by centrifugation. The pellet obtained was then air-dried and the presence of genomic DNA was confirmed by 1 percent agarose gel electrophoresis. The isolated DNA was subjected to quantification in Biodrop 2000 at 260/280nm to check the purity of obtained DNA.

Bisulfite Conversion and Gene Sequencing

Followed by quantification of DNA, the ideally pure DNA has been subjected to bisulfite conversion (Kurdyukov and Bullock 2016). Bisulfite solution containing 3M sodium hydroxide, 320mM hydroquinone, 100mM tetraethylammonium chloride, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonsaure and guanidine hydrochloride was prepared at 55°C in a dark tube. The DNA was denatured at 95ºC in the thermocycler for 5 minutes and immediately pre-heated sodium bisulfite solution was added. The tubes were then placed on a water bath for 6 to 8 hours at 55ºC. The incubation was followed by centrifugation and the supernatant containing the residues of sodium bisulfite was removed. The DNA was then washed with 80 percent ethanol and 200µl of 1X TE buffer was added. The converted DNA was subjected

to polymerase chain reaction using the constructed methylation primers at specific polymerase chain reaction conditions (Table 1). During the bisulfite conversion, the unmethylated cytosine is converted into uracil initially followed by the conversion of uracil to thiamine during the polymerase chain reaction. The presence or absence of methylated sequence has been initially visualized in the 2 percent agarose gel followed by the gene sequencing (Edwards et al. 1989). The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequenced gene has been aligned using the local sequence alignment tool.

Table 1: Primers designed for DNA methylation analysis

Forward Primer(5' → 3')	TTAAGTAGTGGATTT AGAGGGGTAA
Reverse Primer(5' → 3')	TAAACCAAATA CAATAACTCCTTTC
Annealing temperature	58.0°C

RESULTS

DNA Isolation and Quantification

The isolated DNA has been confirmed in 1 percent agarose gel electrophoresis (Fig. 1) and on quantification, the absorbance values were between 1.6 to 1.9 with a concentration around 1.0 to 3.0µg/ml indicating the high purity and ideal nature of the DNA for the polymerase chain reaction.

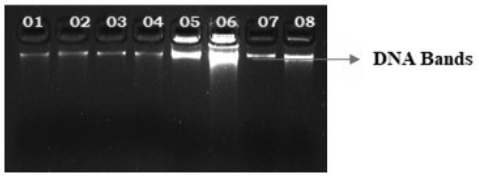

Fig. 1. DNA bands observed under gel documentation system
The figure shows the bands observed in the ultraviolet light in the Gel Documentation System, indicating the presence of DNA in the loaded samples.
Source: Authors, 2021

Agarose Gel Electrophoresis

The methylation analysis of the collected samples (n=518), analysed along with the methylated control for human DNA (Promega Corporation, India) on 2 percent agarose gel. The positive amplification of methylated control and negative amplification of samples has been obtained (Fig. 2) and the amplicons obtained were around 500bp in length. The samples that showed positive amplification with the methylation primers, as well as samples, which did not get amplified with the constructed methylation primer, has been subjected to gene sequencing for analysing the *SERPINA1* gene sequence. Among the analysed samples, only 12 samples showed positive amplification with methylated primer, indicating the presence of DNA methylation, which includes three anaemic comprising two males from high altitude and one female from low altitude region, nine COPD patients comprising two males and three females from high altitude and four males from low altitude regions (Table 2). The DNA methylation analysis did not yield significant results.

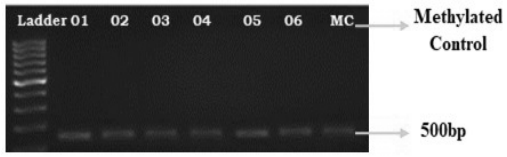

Fig. 2. Methylated DNA bands observed under gel documentation system
The figure shows the bands observed in the ultraviolet light in the Gel Documentation System, indicating the presence of methylated DNA in the loaded samples.
Source: Authors, 2021

Table 2: Number of methylated and unmethylated Samples

Characteristics	*Total*	*Anaemia*	*COPD*	*Control*
Number of samples	518	207	105	206
Methylated	12	3	9	0
Unmethylated	516	204	96	206

Gene Sequencing

The sequencing of methylated sequences and unmethylated sequences yielded the chromatogram shown (Fig. 3). The methylated sample sequences, unmethylated sample sequences have been aligned against the methylated control sample to identify the location of methylated cytosine. Figure 4 shows the three different locations, cg94377701, cg94389678 and cg94389930 where methylated cytosine is present in the *SERPINA1* gene of the human DNA.

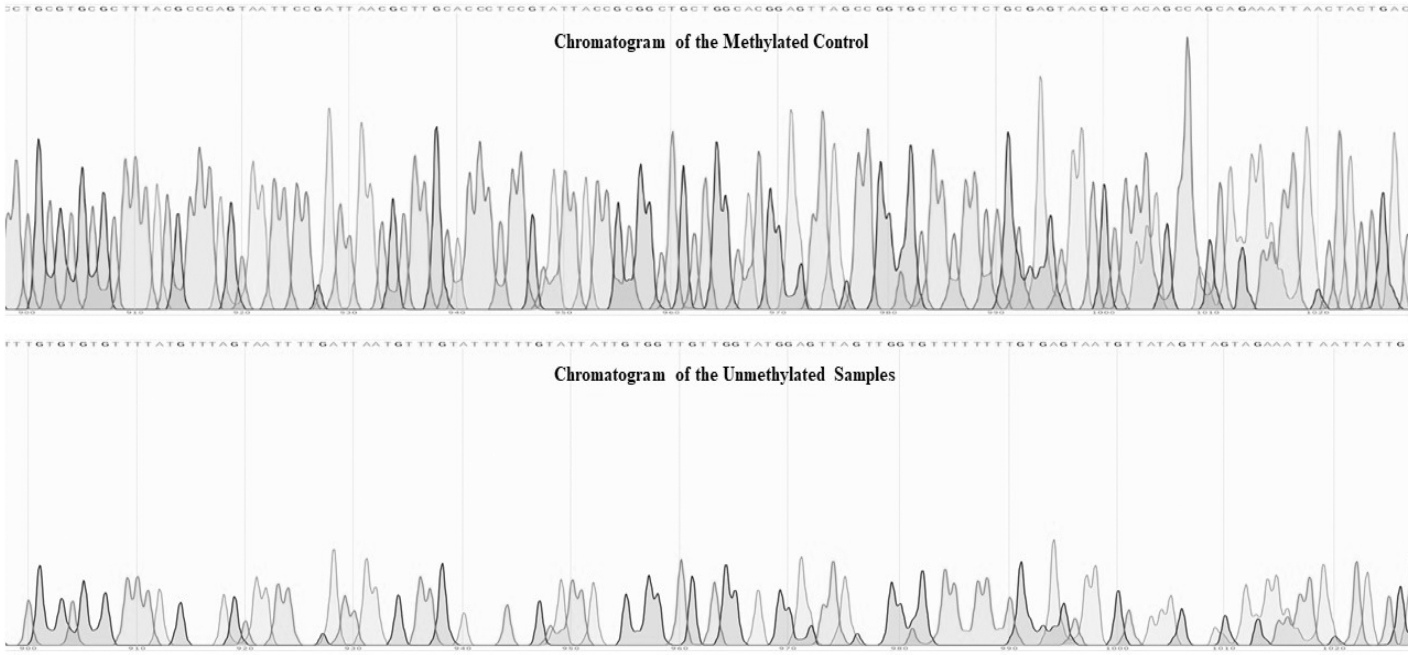

Fig. 3. Chromatogram of the methylated and unmethylated samples
The figure shows the chromatogram obtained from the methylated control and the unmethylated samples. The methylated conatrol shows the presence of cytosine nucleotide indicating the unconverted cytosine due to the presence of methyl group attached to it, whereas the unmethylated cytosine is converted to thiamine that is shown in the chromatogram of unmethylated samples.
Source: Authors, 2021

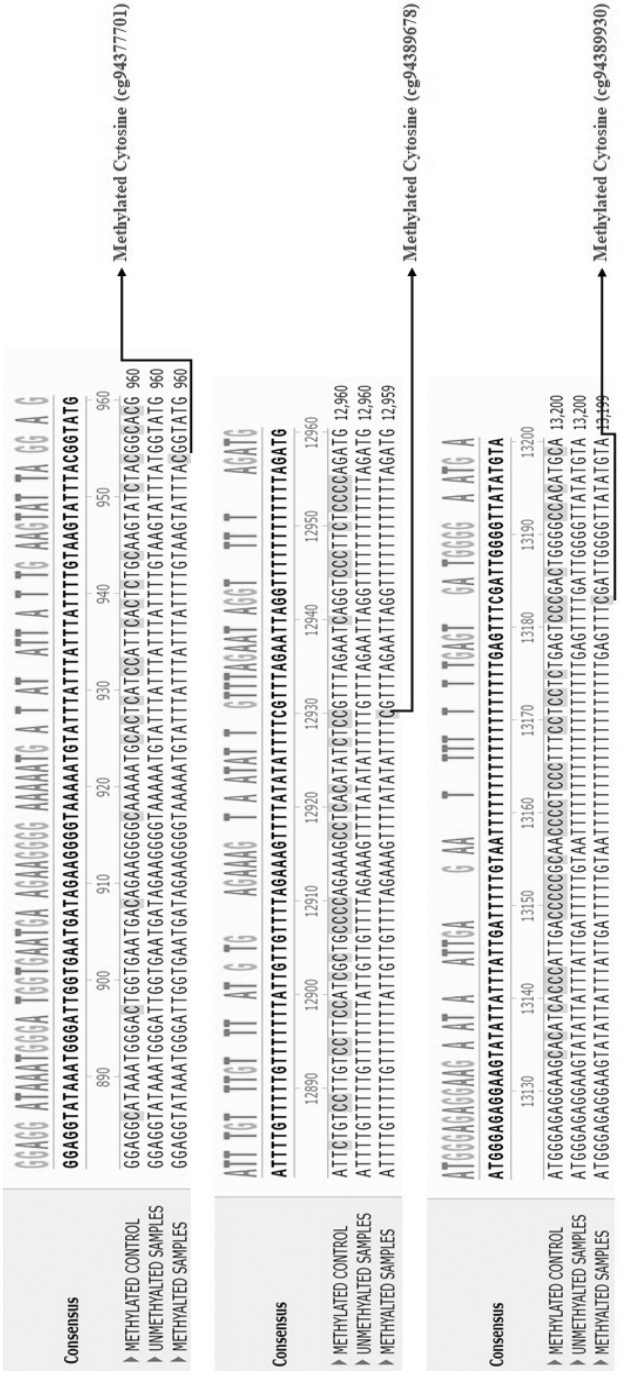

Fig. 4. Local sequence alignment of methylated control and samples indicating the three methylated sites
The figure shows the sequences of methylated control, unmethylated samples and methylated samples aligned via the Local Sequence Alignment tool and the three different methylated sites identified has been noted.
Source: Authors, 2021

DISCUSSION

A recent study has reported that anaemic prevalence in COPD patients increases the mortality rate along with the faster progression of disease (Saxena et al. 2021). Li et al. (2021) has reported that the management of the anaemic condition in COPD patients have shown better progress in the treatment of COPD (Li et al. 2021). The current findings have shown that the anaemic prevalence of COPD is significantly higher ($p<0.001$), which has been supported by the results provided by Saxena et al. (2021) and Li et al. (2021) in their recent reports.

The increase in altitude influences the anaemic condition inversely and elevates the progression of COPD condition as well. The methylated location, cg08257009 of the *SERPINA1* gene has been reported to have lesser significance in influencing the proper functioning of lungs, the respiratory organ (Beckmeyer Borowko et al. 2018). A recent study has reported that the epigenetic modification of the beta-globin gene plays a crucial role in thalassemia (Bao et al. 2020). The current findings have identified three different novel locations, cg94377701, cg94389678 and cg94389930 in the *SERPINA1* gene with methylated sequences in anaemic and COPD patients but the number of patients expressing the methylated sequences was found to be insignificant ($p>0.01$). The methylation of cytosine at the CpG site, cg94377701 results in threonine synthesis instead of methionine amino acids, whereas CpG site, cg94389678 methylation causes the amino acids serine to be coded in the place of phenylalanine. The unmethylated CpG site, cg94389930 codes for stop codon, whereas methylation causes the synthesis of arginine amino acids. The currently identified three CpG sites were not reported elsewhere so far.

The current findings suggesting the presence of epigenetic modification in the *SERPINA1* gene in COPD patients have been supported by the latter studies reporting the underlying genetic relationship between COPD and the *SERPINA1* gene. A recent study by Rotondo et al. (2021) has reported a positive correlation between DNA methylation of the *SERPINA1* gene and the lymphocyte ranges in COPD patients. Methylation analysis of DNA in COPD patients of the *SERPINA1* gene may have a significant role in the early prognosis of COPD (Rotondo et al. 2021). Another study has also reported a suggestion that the *SERPINA1* gene methylation may cause disturbances in the normal circadian rhythm (Clarkson Townsend et al. 2019). Other than COPD, a recent study has reported that an autoimmune disease called Grave's disease is to be positively associated with DNA methylation of the *SERPINA1* gene (Cai et al. 2021). The anaemic condition interlinking with the epigenetic modification of the *SERPINA1* gene has not been reported so far.

Apart from the addition of methyl group to cytosine, the removal of a methyl group from cytosine, called hypomethylation at certain sites may also disrupt normal body functioning. Few studies have reported that the hypomethylation in the *SERPINA1* gene is positively associated with the worsening of the clinical condition, COPD (Qiu et al. 2012; Parris et al. 2019). DNA methylation at two specific locations cg24621042 and cg02181506 of the *SERPINA1* gene have shown a negative impact on lung function and a positive association with the worsening of COPD (Qiu et al. 2012). Few studies have reported that lung functioning is negatively influenced either by the addition of methyl group (hypermethylation) or hypomethylation from the cytosine of the *SERPINA1* gene and the clinical conditions related to lungs are being found to be worsened with the presence of methylated DNA sequences (da Silva et al. 2017; Sidhaye et al. 2018).

The proper lung function requires the methylation of genes to a certain extent and the rate of methylation is found to be associated with the lung function inversely, that is higher the methylation rate, lower the proper lung function (Vucic et al. 2014). DNA methylation of Bcl-2 interacting protein called, BH3-interacting domain (*BID*) worsens the COPD progression (Sundar et al. 2017). DNA methylation at cg02181506 in the *SERPINA1* gene has been observed to be methylated in smokers as well as non-smokers with lung abnormality (Siedlinski et al. 2012). Runt-related transcription factor 1 (*RUNX1*) is another gene involved in anaemic occurrence (Cao et al. 2021).

The current findings did not suggest the positive correlation of DNA methylation with COPD and anaemic conditions, even though the methylation has been observed in the *SERPINA1* gene of COPD and anaemia patients. Because the num-

ber of patients with the presence of DNA methylation among the collected samples was not significant. This study has contrasting results with Qiu et al. (2012) who have suggested that DNA methylation of the *SERPINA1* gene has a significant role in COPD patients in elevating the progression of the clinical condition.

CONCLUSION

The current study failed to report the significant correlation of *SERPINA1*gene methylation with anaemia as well as COPD since the DNA methylation was not observed in a significant number of anaemic and COPD patients. Although the association between anaemic, COPD patients and DNA methylation of the *SERPINA1* gene was not significant, our study reports three new methylated CpG sites (cg94377701, cg94389678 and cg94389930) of the *SERPINA1* gene of anaemia and COPD patients, which is a novel finding since the CpG sites were not methylated in the control samples. On comparing the low and high altitude regions, the high altitude patients were prone to methylation more than low altitude regions but the significance was also negligible. Hence, the authors suggest that the role of methylation in the *SERPINA1* gene of anaemia and COPD patients was insignificant.

RECOMMENDATIONS

The authors recommend the analysis of methylation in the *SERPINA1* gene with respiratory conditions other than COPD and blood disorders other than anaemic conditions since the DNA methylation does influence the functioning of the respiratory organ, lungs and synthesis of red blood cells. The current study may be extended to correlate DNA methylation in the *SERPINA1* gene with other respiratory diseases either or under anaemic conditions.

LIMITATIONS

The limitation of the current study lies in the fact that the *SERPINA1* gene alone has been considered for the epigenetic modification analysis. A wide range of genes related to lung function and anaemia may provide a significant answer for the inter-relationship of anaemia with COPD on the methylation basis.

ABBREVIATIONS

BID - BH3-interacting domain
COPD - Chronic Obstructive Pulmonary Disease
EDTA - Ethylenediaminetetraacetic acid
EPO - Erythropoietin
FEV - Forced Expiratory Volume
FVC - Forced Vital Capacity
RUNX1 - Runt-related transcription factor 1

ACKNOWLEDGEMENT

The authors sincerely thank Bharathiar University, Coimbatore, India for providing the laboratory facilities and fellowship to perform the present study. The authors also extend their thanks towards, Nilgiris Laboratory, Cunoor, India for helping in sample collection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paper received for publication in October, 2021
Paper accepted for publication in November, 2021