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A Pilot Study Investigating the Impact of High Altitude on Myostatin and Irisin Levels

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ABSTRACT

Many people visit and stay at high altitude due to adventure or occupation. The high-altitude environment comprises many factors alien to sea residents and detrimental to physical and mental health. Myokines are peptides and cytokines secreted from muscles and have a prime role in regulating skeletal muscle growth and myo-degradation. Therefore, the present study investigated the function of myokines in regulating muscle mass during acute and chronic high-altitude exposure. The study was conducted on Indian healthy subjects (n=29) who were distributed into three groups: Control (sea level (SL; n=15), acute high altitude stayed subjects (stayed at high altitude for less than ten days (AHA; n=7); chronic high altitude stayed subjects (stayed at high altitude for 15 days to 3 months (CHA; n=7). Irisin levels were also increased in AHA group compared to SL group, depicting inclined myogenesis. However, CHA group showed an increase in myostatin levels but a non-significant change in irisin content in relation to SL group, suggesting enhanced myo-degradation. These findings generated a unique role of myokines, including myostatin and irisin, in managing skeletal muscle health with reference to high altitude.

Key Words: High altitude; Myostatin; Irisin; Muscle mass; Fat-free mass

NOMENCLATURE

HA	High Altitude
HH	Hypobaric hypoxia
SL	Sea Level

1. INTRODUCTION

Skeletal muscle is an essential endocrine organ that maintains muscle strength, locomotor activity, muscle mass, and physical performance. Recently, it has been reported myokines are produced and secreted from skeletal muscle, and in nature, these are proteins and cytokines.[1] Skeletal muscle-secreted myokines perform their functions via autocrine, paracrine or endocrine fashion. Myokines also regulate myogenesis and functions of other organs[2]. Among all the secreted myokines, myostatin and irisin are prime myokines that engage in protein metabolism (synthesis and degradation) in skeletal muscle[3].

Mountaineers and low-land residents must perform up to the mark even in high altitude-associated adverse conditions such as hypobaric hypoxia, UV radiation exposure, chilled wind, humidity, cold, etc. Among all these adverse effects, hypobaric hypoxia (low oxygen level) is the primary stressor responsible for compromised physical performance. Extensive research in this direction suggests that skeletal muscle protein loss is the main culprit for hampered physical performance[4]. To understand the severity of the issue, Hypobaric Hypoxia (HH) induced muscle protein loss mechanism has been solved because protein is the main component of skeletal muscle (approx. 70 % of the muscle weight)[5].

Deep investigation into the issue suggested a decrease Fat-Free Mass (FFM) under severe high-altitude exposure.[6,7] Krzywicki,[8] *et al*.,described a loss of body protein, fat, minerals, water in 2 weeks high altitude exposed human subjects. Rose,[9] *et al*.,observed a significant reduction in the upper arm, thigh muscles, and body weights in Mt. Everest exposed subjects. Hoppler,[10] *et al*.,described a decline in muscle volume due to decreased Cross-Sectional Area (CSA) of myofibres. Simulated extreme altitude reduces type I and type II muscle fibres (26 % and 25 %, respectively)[11]. Bharadwaj,[4] *et al*.,conducted the first study on Indian soldiers, concluding that prolonged stay at high altitude results in loss of skeletal muscle mass.

Ergo, our group raised the curtain with the fact that altered skeletal muscle protein homeostasis with excessive protein degradation in relation to protein synthesis is responsible for Hypobaric Hypoxia (HH) linked muscle protein loss in rats[12]. A detailed analysis evidenced that the disrupted proteostasis network was tightly conjugated with multiple signaling pathways, including enhanced degradative pathways, inflammation, altered anabolic signaling, and apoptosis under chronic hypobaric hypoxia and resulted in hypobaric hypoxia associated skeletal muscle atrophy[13]. Recently, our lab

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also established that skeletal muscle-secreted myokines, mainly myostatin and irisin, control myodegradation and regeneration. Further, an in-silico study also established myostatin might be a predictive biomarker for high altitude associated skeletal muscle loss and performance[14]. However, till date no study is available that establish the function of myokines on skeletal muscle health during a lengthy stay at high altitude. Therefore, the current work investigated the changes in irisin and myostatin levels in physically active subjects at different durations of stay at high altitude.

2. MATERIALS AND METHOD

2.1 Subject Groups and Sample Collection

The present cross-sectional study was performed on twenty-nine human volunteers as per the ethical guidelines of the Indian Council of Medical Research (ICMR). Institutional human ethical committee approval was taken for the study, and signed consent was obtained from all volunteers of the study.

Each subjects were physically active male subjects divided into three study groups.

Group 1: Sea level subjects, collection was done in Patiala (SL; n=15).

Group 2: Subjects stayed for less than 10 days at Leh (Altitude: 3500 meter) (AHA; n=7).

Group 3: Subjects stayed for 15 days to 3 months at Leh (Altitude: 3500 meter) (CHA; n=7).

2.2 Sample Processing

Venous blood samples were collected at sea level and high altitude in the morning at fasting conditions. Plasma was separated by centrifugation at 1000 g for 15 minutes, kept at -20 °C during transport, and stored at -80 °C till the biochemical assay was done.

Myostatin (Cloud Clone), Irisin (Cloud Clone), and 3-Nitrotyrosine (Cloud Clone) were estimated in plasma using commercially available ELISA Kits, and the manufacturer's instruction was followed.

2.3 Statistical Analysis

Data were denoted as mean±SEM. One-way analysis of variance (ANOVA) followed by *post hoc Bonferroni* analysis was performed to analyse the statistical significance among groups. GraphPad Prism ver 8.00 software (GraphPad, CA, USA) was exploited for analysis. The p-value of ≤0.05, with a 95 % confidence interval, was considered significant. Correlations between quantitative variables were analyzed using the Spearman coefficient.

3. RESULTS

The mean age, height, body mass, Fat-Free Mass/Body Weight (FFM/BW), muscle mass (MM/BW), and fat percentage/body weight (fat%/BW) of all the participants are provided in Table 1. FFM/BW, MM/BW were significantly increased in AHA subjects with reference to SL group, which was further decreased in CHA group in comparison to SL group. However, fat%/BW decreased in AHA group and CHA in relation to SL group.

Table 1. Morphological parameters for SL, AHA and CHA group. Data are presented as mean ± SEM. BW, body weight; FFM, fat free mass. *p < 0.05, significant change from sea level (SL).

	SL Group	AHA Group	CHA Group	AHA Group	CHA Group
Age (yrs)	41.33±3.124	31.48±1.28	33.28±2.31		
Height (Cm)	170.91±1.19	172.71±2.17	174.85±1.58		
Weight (Kg)	73.64±2.259	67.87±1.532	76.27±1.681	Δ%	Δ%
FFM/BW	0.846±0.013	0.89±0.023	0.843±0.007	5.2	-0.354
Muscle Mass/BW	0.803±0.013	0.865±0.027*	0.799±0.007	7.72	-0.498
Fat %/BW	0.204±0.013	0.193±0.008	0.205±0.008	-5.39	-0.49

Myostatin levels were enhanced significantly in CHA group compared with SL controls. Irisin levels were also increased significantly in AHA group than SL group, which was declined considerably in CHA subjects with comparison to AHA subjects. While, non-significant change was noted between SL group and CHA group. Further, a non-significant difference was observed in 3-nitrotyrosine (3-NT) levels in all the groups (Figure 1).

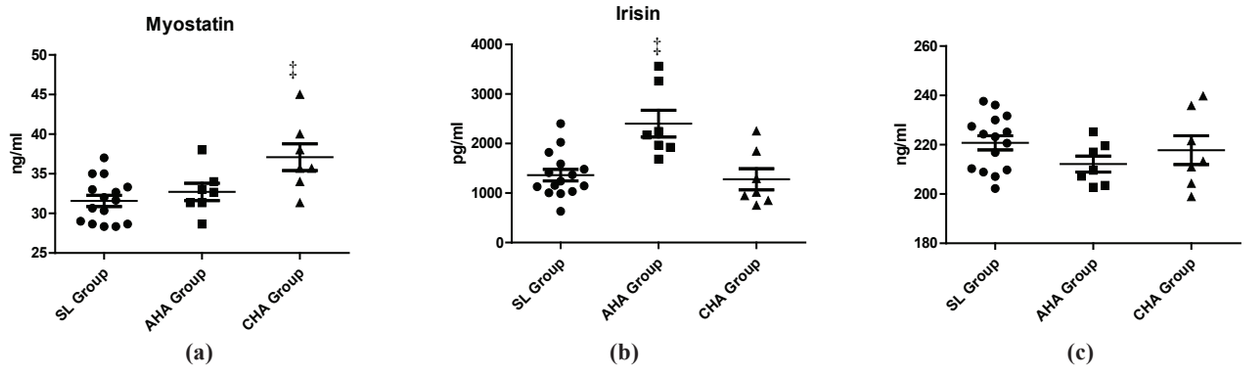
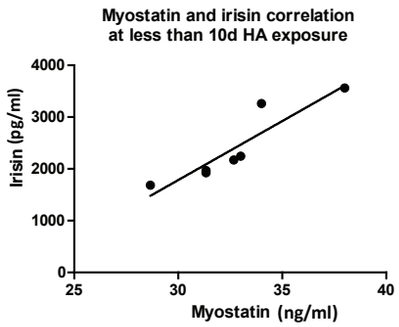


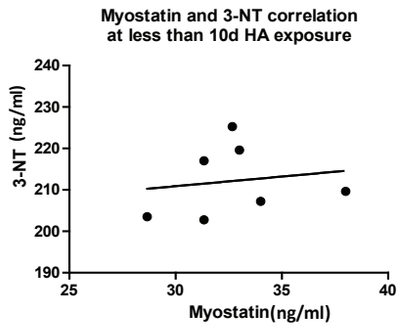
Figure 1. Changes in A: Myostatin, B: Irisin and C: 3-Nitrotyrosine (3-NT) levels in SL group, AHA group and CHA group. Values are means ± SEM. *p < 0.05, significant change from sea level.

The correlation curve was plotted in AHA group among myostatin, irisin, and 3-NT. The analysis showed a significant correlation between myostatin and irisin in group 2 (r=0.9910, P=0.0002). At the same time, no correlation was detected between myostatin and 3-NT (r=0.3243; P=0.2488) and between irisin and 3-NT (r=0.6429; P=0.0694) in group 2 (Figure 2).



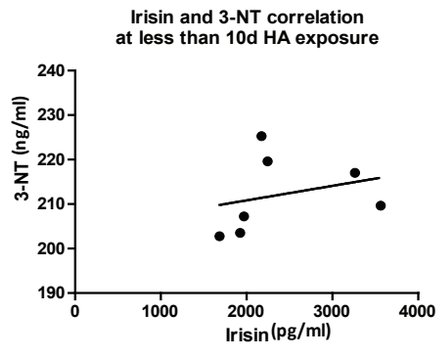
Spearman r	0.9910
P value (one-tailed)	0.0002
P value summary	***

(a)



Spearman r	0.3243
P value (one-tailed)	0.2488
P value summary	ns

(b)

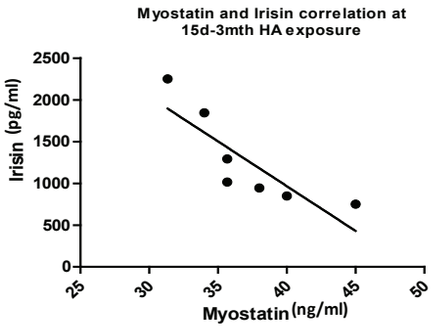


Spearman r	0.6429
P value (one-tailed)	0.0694
P value summary	ns

(c)

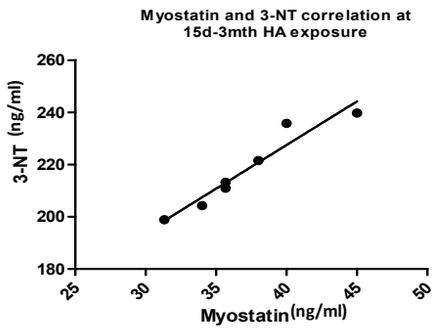
Figure 2. Correlation between (a) myostatin and irisin (b) myostatin and 3-NT and (c) irisin and 3-NT in AHA stayed subjects.

The correlation curve was also constructed in CHA group among myostatin, irisin, and 3-NT. A negative correlation was found between myostatin and irisin (r=-0.9910; P=0.0004) and irisin and 3-NT (r=-0.8214; P=0.0171). However, a positive correlation was noted between myostatin and 3-NT (r=0.9910; P=0.0004) (Figure 3).



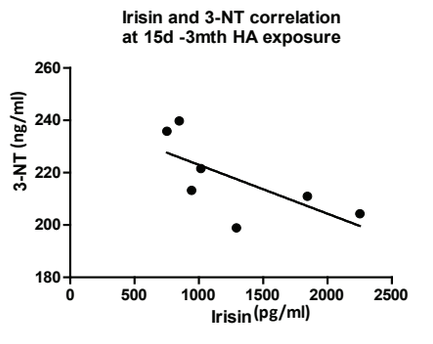
Spearman r	-0.9910
P value (two-tailed)	0.0004
P value summary	***

(a)



Spearman r	0.9910
P value (two-tailed)	0.0004
P value summary	***

(b)



Spearman r	-0.8214
P value (one-tailed)	0.0171
P value summary	*

(c)

Figure 3. Correlation between (a) myostatin and irisin (b) myostatin and 3-NT and (c) irisin and 3-NT in CHA stayed subjects.

4. DISCUSSION

The current study demonstrated morphological and biochemical indices regarding myokine secretion during high altitude exposure (acute and chronic exposure). Myokines are proteins and cytokines that are synthesised and secreted from myocytes of muscle cells. Myokine plays a prime role in maintaining the quality of life via muscle-organ communication[15]. Additionally, myokines are responsible for governing muscle mass via managing muscle contraction, cell proliferation, and differentiation[16,17].

Myostatin is a member of the TGF-β superfamily, abundantly present in skeletal muscle in high amount[18,19]. It is known for its negative regulation for muscle growth[20,21]. Distinctly, myostatin over-expression leads to various muscular disorders[22,23,24]. Myostatin-mediated Smad signaling stimulation suppresses protein synthesis in muscle tissue by hampering Akt-mTOR signaling path as well as activating FoxO-dependent protein degradation pathways like ubiquitin-proteasome pathway in mature myofibers (Figure 4)[25,26]. Recently, our group also predicted myostatin as a probable biomarker for hypobaric hypoxia-associated muscle atrophy[14].

Skeletal muscle secrets irisin, a cleave form of fibronectin type III domain-containing protein 5 (FNDC5). Few recent researches suggested its role for muscle hypertrophy via activating IL-6 signaling and promoting the protein synthesis pathway[27]. The latest research evidenced low circulating irisin levels in postmenopausal sarcopenic women[28]. A bunch of studies revealed a promising function of irisin in many metabolic diseases like diabetes, obesity, and managing energy metabolism[29,30]. Skeletal muscle-secreted irisin is a positive regulator of mitochondrial biogenesis. Irisin also boosts the Akt/mTOR/NRF2 pathway and enhances myogenesis process (Figure 4)[31-32]. Our recent study also demonstrated the improved irisin level during acute HH exposure, which was comparable to control rats under chronic hypoxic exposure[14].

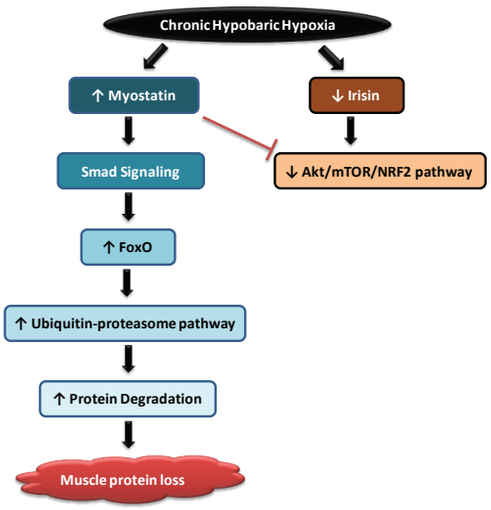


Figure 4. A schematic diagram to demonstrate the effect chronic hypobaric hypoxia on myostatin and irisin level and its co-relationship with skeletal muscle mass loss.

As oxidative stress progresses, 3-nitrotyrosine (3-NT) is produced due to oxidation of tyrosine. 3-NT signifies a marker of oxidative stress that results in protein misfolding and dysfunction[33]. A high degree of Tyr-nitrated proteins was observed in numerous diseases, including asthma, renal complications, sepsis, metabolic syndrome, rheumatoid arthritis, and joint injury[34,35,36]. Likewise, 3-NT and protein carbonyl content were also enhanced in HH exposure in rats[14].

In the present study, AHA subjects (Group 2) showed inclined FFM/BW and MM/BW, accompanied by significantly elevated irisin levels, compared with SL subjects (Group 1). Correlation analysis depicted a significant positive correlation between myostatin and irisin during acute HA exposure. In the case of CHA subjects described a decrease FFM/BW and MM/BW, which was escorted by a significant incline in myostatin level. Irisin levels were not altered in CHA subjects, compared with SL subjects. Ergo, the irisin level decreased considerably in CHA subjects in relation to SL subjects (Figure 4). As per our best knowledge, the present study, for the first time, indicated the role of myokines during acute and elongated stay at high altitude.

Interestingly, the current human data harmonized with our earlier in-vivo studies in which acute hypobaric hypoxia exposure enhanced protein synthesis and degradation. In contrast, chronic hypobaric hypoxia exposure leads to inclined protein degradation with no change in protein synthesis[12,13]. Further, the present data exhibited that acute HA exposure leads to enhanced myogenesis and degradation like myostatin and irisin increment significantly. However,no change in irisin level was noted during chronic HA exposure and this was accompanied by a significant rise in myostatin levels, which depicts protein degradation while myogenesis was impaired. Additionally, muscle mass, fat percentage, and fat-free mass in the same subjects were also decreased, which also supported this hypothesis.

5. LIMITATION

The present pilot study investigated the effect of acute and chronic high-altitude stay on skeletal muscle-secreted myokines. The study is unique as till date no Indian human data is available to depict the impact of HA stay on myokines. These prime molecules manage skeletal muscle health and quality of life. Nevertheless, the survey also comprises few limitations: firstly, it is a cross-sectional study, and the sample size is small and unequal. Ergo, validation of these results in a bigger number of subjects is required.

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Computational Investigation of Regulatory Region SNPs of Autophagy Gene *BECN1*

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ABSTRACT

The autophagy process plays a cytoprotective role and ensures the healthy survival of a cell. The role of autophagy has been implicated in various diseases, making it an essential candidate for therapeutic interventions. Beclin 1, a candidate autophagy protein, plays a critical role during autophagy initiation and maturation by interacting with various other autophagy proteins. Beclin1 has been reported to be involved in various human diseases. This study uses a computational approach to study the effect of non-coding region single nucleotide polymorphisms (SNPs) of gene encoding beclin1. RegulomeDB, SNP2TFBS, and PROMO ALLGEN were used to predict the effect of promoter region variants on transcription factor binding sites. SNPs located within 3'UTR were analysed by miRdSNP, PolymiRTS Database 3.0, miRNASNP-V3, MicroSNIPER, and miRmap. Nine promoter region variants that alter the transcription factor binding sites and 4 variants in 3'UTR were identified that either create a new target site for miRNA or disrupt an existing one. The functional analysis of these identified SNPs could be done experimentally to unravel their relation with a particular disease and the genetic predisposition of human subjects for a disease.

Keywords: Hypoxia; BECN; Single nucleotide polymorphism; Promoter variants; 3'UTR

1. INTRODUCTION

The human body possesses an intricately capable and advanced intracellular system to manage and facilitate responses to acute stressors and dangerous situations. Under certain extreme conditions, specific intracellular pathways activate to promote optimal conditions for sustaining health and ensuring survival. Macroautophagy (hereafter referred to as autophagy) is a conserved intracellular, pro-survival mechanism that is fundamental for ensuring the healthy survival of a cell when exposed to stressful conditions. This intricately orchestrated process holds a central position in removing the elements that have the potential to cause harm to the cells[1]. Autophagy encompasses three fundamental stages. Initiation begins with activating a protein complex, which initiates the formation of the phagophore, a precursor structure. This phagophore expands and encapsulates targeted cellular components in the elongation phase, culminating in an autophagosome with a double-membraned structure. Subsequently, during maturation, autophagosomes merge with lysosomes to create autophagolysosomes, where the enclosed cellular contents are subjected to degradation, leading to the recycling of vital biomolecules essential for maintaining cellular health and homeostasis[1]. Each step of autophagy is precisely controlled and intricately operated by the actions of proteins encoded by autophagy-related genes (*ATGs*)[2,3]. The basal autophagy functions to uphold cellular homeostasis, consequently, any aberration in the process can inflict damage upon the cell[3]. Autophagy is a well-reported contributing factor in a range of diseases, including neurodegenerative disorders, cancer, hepatic, muscular, infectious, and cardiovascular diseases as well as lung diseases and asthma[3-5].

India is mandated to deploy a substantial military contingent in the western Himalayan region primarily to safeguard national security interests and preserve territorial integrity. Except for a minority representing hill tribes, the majority of these troops hail from lowland regions and have been nurtured in tropical and subtropical environments. Consequently, they confront significant challenges arising from rigorous environmental conditions, prevalent in the western Himalayas. These challenges encompass extremely frigid temperatures, hypobaric hypoxia (characterised by low oxygen pressure at elevated altitudes), pronounced aridity, elevated levels of solar ultraviolet radiation, and formidable winds[6,7]. To tackle such conditions, both external and physiological factors play a significant role. Autophagy is reported to be induced in alveoli cells during exposure to high-altitude hypoxia. Moreover, autophagy is also a reported contributing factor for high-altitude hypoxia-induced lung injury[8]. The genetic makeup of each individual plays a critical role in their ability to respond to various scenarios, including

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both resilience and vulnerability. The genetic diversity among individuals contributes to both advantageous and disadvantageous traits. Certain genetic variations can confer advantages in specific scenarios. To date, more than 40 *ATGs* have been identified, demonstrating a high degree of conservation across eukaryotes. Within this array of autophagy-related genes, *BECN1* emerged as a prominent candidate due to its pivotal role in orchestrating the autophagy process. *BECN1* is situated on the long arm of chromosome 17 at locus 17q21.31[9]. It encodes a 450-amino acid protein known as Beclin 1, characterised by three well-conserved domains: the Bcl-2-homology-3 (BH3) domain spanning amino acids 105 to 130, the coiled-coil domain (CCD) from amino acids 175 to 264, and the evolutionarily conserved domain (ECD) spanning amino acids 248 to 450[9]. Beclin 1 is a candidate autophagy protein that functions as a scaffold for the multiprotein assemblage during the autophagy process[10]. Beclin 1 interactome with multiple proteins modulates the autophagy initiation and maturation stages and thus Beclin 1 acts as central and crucial for the process. High levels of beclin 1 have been correlated with enhanced autophagy and beclin 1-mediated modulation of autophagy has been reported to be involved in high-altitude hypoxia[8,10,11].

Single nucleotide polymorphism (SNP) is variation at a single position in DNA sequence in more than 1 % population. SNPs affect the structure and function of genes depending upon the region in which these are present. Single nucleotide variations have been reported to play pivotal roles in various diseases. Gene regulatory region variants are important to study as these can directly influence gene expression. Thus, SNPs present in the non-coding regions such as promoters and 3'untranslated region (UTR) are important to study. These regulatory region variants can affect the expression of a gene in various ways such as alteration of binding sites for transcription factors and altered microRNA (miRNA) binding sites[12-14].

The promoter polymorphisms of the beclin 1 gene were reported to enhance *BECN1* expression and were related to the early onset of Machado-Joseph's disease[15]. Despite its significant importance, the Beclin 1 gene has not been fully elucidated to check the impact of SNPs present within it. Given the pivotal role of Beclin 1 in autophagy and its associations with various diseases, it becomes imperative to investigate the effects of SNPs situated within the regulatory domains of its gene. In this study, we employ a computational approach to elucidate the effects of SNPs located within the *BECN1* promoter and 3'UTR.

2. METHODOLOGY

The study is covered under three major sections: (1) Detection of promoter and 3'UTR SNPs, (2) Analysis of promoter region SNPs to check their effect on transcription factor binding sites, and (3) Analysis of 3'UTR SNPs to check their effect on alteration of miRNA target binding site.

2.1 Data Mining

The information about non-coding region variants of human *BECN1* was obtained from NCBI (http://ncbi.nlm.nih.gov/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and ENSEMBL (https://asia.ensembl.org/) database. The flow chart is depicted in Fig. 1.

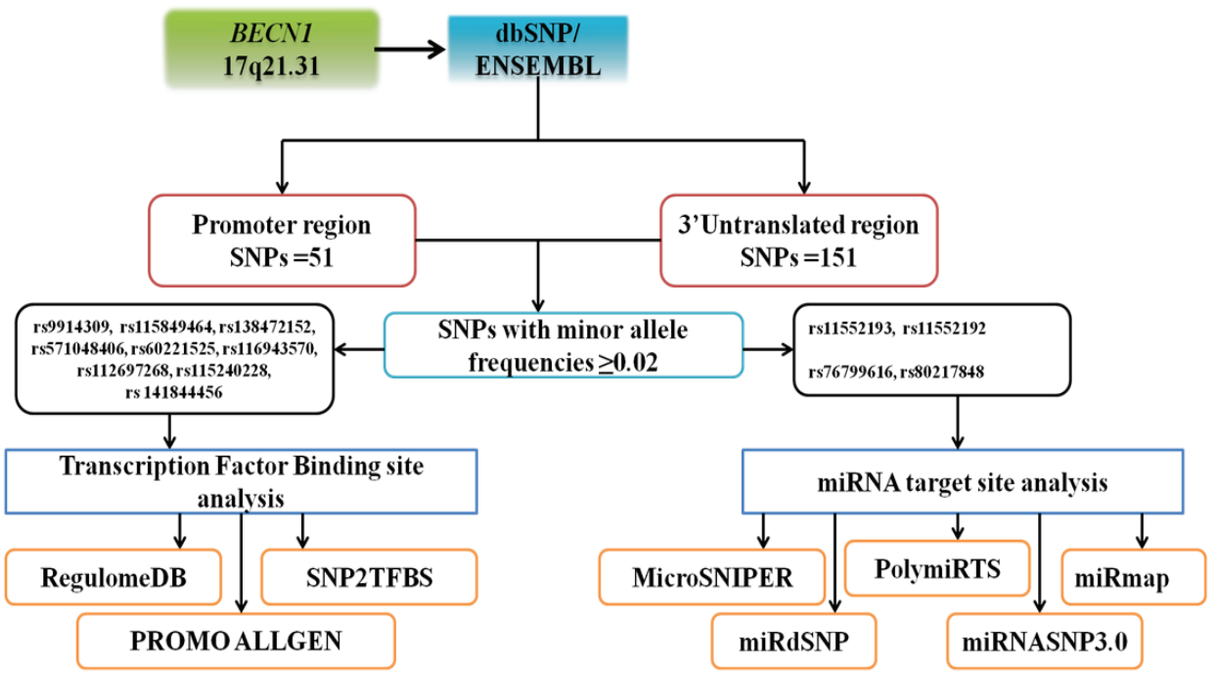

Figure 1. Flowchart depicting selection and analysis of regulatory region SNPs.

2.2 Prediction of Effect of *BECN1* Promoter Region SNPs RegulomeDB

(https://regulomedb.org/regulome-search/) database was used for the annotation of SNPs with known and predicted regulatory elements in the non-coding regions of the *beclin 1* gene[16]. **SNP2TFBS** (http://ccg.vital-it.ch/snp2tfbs/) was used to predict the effect of SNPs on the binding affinity of transcription factors[17]. dbSNP IDs of variants were provided as input. **PROMO** (http://alggen.lsi.upc.es/) was used to predict the effect of SNPs on transcription factor binding sites[18]. A string of nucleotides containing mutant and reference alleles was given as input. The default dissimilarity threshold used (the parameter that controls how similar a sequence must be to the matrix to be reported as hit) was 15 %.

2.3 Analysis of Potential 3'UTR Variants of *Beclin 1*

miRdSNP (http://mirdsnp.ccr.buffalo.edu) was used to predict the disease-associated SNPs and the miRNA binding site in the 3'UTR of *BECN1*[19]. PolymiRTS Database 3.0 (Polymorphism in microRNAs and their TargetSites database version 3.0) (http://compbio.uthsc.edu/miRSNP) was used to predict the variants altering miRNA binding site[20]. miRNASNP-v3 (http://bioinfo.life.hust.edu.cn/miRNASNP/) tool provided a lot of information regarding SNPs in the miRNA target site. It also predicted the effect of SNPs on the binding of miRNAs[21]. rsID of variants or the gene symbol *(BECN1)* was given as an input in these three in silico tools. MicroSNiper (http://cbdb.nimh.nih.gov/microsniper) another tool that also predicts the effect of SNPs on miRNA binding sites was used to predict the creation or loss of the miRNA binding site[22]. The binding efficiency of miRNAs predicted by the above tools was calculated by miRmap (http://cegg.unige.ch/mirmap)[23]. The name of the gene was provided in input and *beclin 1* targeting miRNAs predicted by the above-mentioned tools were searched one by one. Further, the site lost or gained was checked with the results provided by these servers.

3. RESULTS

3.1 Data Retrieval

SNPs of the non-coding region of *BECN1* were obtained from NCBI, dbSNP, and ENSEMBL. There were 51 upstream gene variants, 2372 intronic region variants, and 151 variants in 3'UTR. Since the minor allele frequencies (MAF) of most of the SNPs were not available, variants with a given MAF (≥0.02) and available literature were selected for analysis. In total, nine promoter region SNPs and four 3'UTR SNPs were analysed computationally. We have also performed in silico analysis of intronic region variants and genotyped one of the SNP (data submitted for publication). So, the current study is limited to promoter region variants and 3'UTR variants.

3.2 *Beclin 1* Promoter Region SNPs are Predicted to Alter Transcription Factor Binding Sites

RegulomeDB cross-references variant genomic coordinates with functionally relevant regions from assays like TF ChIP-seq and DNase-seq and also considers transcription factor footprints and QTL data from the ENCODE database. This comprehensive approach helps assess a variant's functional significance by analysing its proximity to gene-regulating regions, aiding in understanding its impact on gene expression and phenotypic traits. The results obtained were given as ranks and scores. RegulomeDB probability score is determined using a random forest model and ranges from 0 to 1. A score of 1 signifies a higher likelihood of a variant being regulatory, while lower scores indicate decreasing probabilities of being a regulatory variant. The scoring scheme assesses the likelihood of a location or variant being functional based on supporting evidence. More available supporting data results in a higher rank, with 1 indicating a higher likelihood of functionality and 7 indicating a lower score, reflecting decreasing functional potential. Among the studied variants, rs138472152 and rs116943570 had RegulomeDB scores closest to 1 (0.82 and 0.81, respectively), depicting that these were most likely to be regulatory variants, whereas the rest of the variants had scores in the range of 0.6- 0.61 (Table 1). rs9914309 had a high rank and least probability score (comparative to the rest of the variants) of 0.55. SNP2TFBS computed the PWM (position weight matrix) score in both reference (hg19) and alternate human genome assemblies. The alternate genome assembly was generated by incorporating the alternate alleles of common genetic variants from 1000 genome projects. SNP2TFBS also provides high score and low score threshold values. The SNPs that change the PWM score above the threshold were retained.

Variant rs571048406 altered the binding sites of PAX4 and ERG2. However, this SNP had a score above the threshold for ERG2 only and hence it is considered to be retained. rs115849464 altered the binding sites for EGR1 and SP2 and the score was above the threshold in both cases, hence the SNP is considered to be retained. rs60221525 affected the binding sites for transcription factor EGR2, however, there was no score difference between reference and alternate genome. PROMO uses version 8.3 of TRANSFAC to construct specific binding site weight matrices for TFBS prediction. The variants having a low dissimilarity index show a high probability of binding. All of the studied variants were predicted to either create or remove the TFBS. The dissimilarity index indicating a high similarity score was less than 15 % in the case of all variants. The results are summarised in Table 1. Variants rs141844456, rs9914309, and rs112697268 were predicted to alter the maximum number of transcription factor binding sites.

3.3 *BECN1* 3'UTR Variants Alter the Binding Site for MiRNAs

miR*dSNP* provided the feature to search the position of an SNP with respect to the miRNA binding site in the 3'UTR. The position of conserved miRNAs and their target sites was obtained as an output. The variants rs11552192 (G>A) and rs11552193 (T>A) were shown

Table 1. List of the promoter variants that alter the binding site of transcription factor

		RegulomeDB		SNP2TFBS		PROMO		
S. No.	**Variant id**	**Rank**	**Score**	**Match**	**Score difference**	**Transcription factor**	**Loss/ gain of site**	**Dissimilarity index (%)**
1.	rs571048406	2b	0.61	EGR2 PAX4	-88 -45	Egr-3 RXR-alpha	Gain of site	11.37 5.27
2.	rs115849464	2b	0.61	EGR1,SP2	>259 -145	p53 PAX5	Loss of site	6.19 9.55
3.	rs115240228	4	0.60	-	-	E2F	Loss of site	13.89
4.	rs116943570	2b	0.81	-	-	MAZ	Gain of site	14.13
5.	rs138472152	2b	0.82	-	-	Ik-1	Gain of site	11.87
6.	rs141844456	4	0.60	-	-	STAT4 cETS-1 Elk-1 HNF-1C HNF-IB cETS-2	Loss of site	4.4 0.26 4.2 9.58 10.21 5.16
						TFII-I	Gain of site	1.82
						AP-2alphaA	Loss of site	3.97
7.	rs9914309	1f	0.55	-	-	SRY TCF-4E LEF-1	Gain of site	13.35 12.60 9.94
8.	rs60221525	2b	0.61	EGR2	0	MAZ	Gain of site	14.13
9.	rs112697268	4	0.60	-	-	PAX5 p53	Loss of sites	1.54 1.27
						GR-alpha AP2-alphaA T3Rbeta1	Gain of sites	6.26 2.55 4.48

but none of the variants was located within the most preferential binding site of highly conserved miRNA that is within the site of miR-30a-5p, miR124-3p-2/506-3p, miR142-5p, and miR-17-5p/93. PolymiRTS Database 3.0 predicted the effect of variants on the miRNA binding site (Table 2). The results were given under two function classes – 'D' and 'C'. Function class 'D' refers to the disruption of the miRNA binding site, whereas 'C' refers to the creation of a new miRNA site, whereas, the context score predicts the likelihood of the outcome (Table 2). Variant rs11552193 led to the disruption of 2 miRNA binding sites and the creation of 3 new sites. Variant rs11552192 created a new site for miRNA hsa-miR-590-3p, whereas, rs76799616 created a new site for 2 miRNAs and rs80217848 resulted in the loss of site for 2 miRNAs (Table 2). Disruption of the miRNA binding site leads to the modulation of expression whereas the creation of a new site has an inhibitory role in gene expression[20]. Context score change predicted the likelihood of the event of creation or disruption of the site. Its more negative value suggests higher chances of site creation or disruption. The miRNASNP-v3 predicted the effect of SNP on the miRNA target binding site as loss or gain of the site (Table 3). Along with that, free energy change values were given in Table 3. rs76799616 only caused the gain of miRNA binding sites whereas the rest three variants resulted in both gain and loss of sites (Table 3). Moreover, most of the sites predicted by miRNASNP-v3 were also predicted by PolymiRTS Database and MicroSNiper. miRmap computed the highest seed match sequence probability for miRNAs hsa-miR-4684-5p and hsa-miR-32-3p with scores of 50 and above. Both of the sites were however predicted to be lost due to variants rs11552192 and rs11552193 (Table 3).

4. DISCUSSION

The investigation of SNPs within non-coding regions carries substantial scientific significance, as it

Table 2. 3'UTR SNPs alter the miRNA binding site predicted by PolymiRTS Database 3.0

S. No.	Variant id	Variation	PolymiRTS Database 3.0			
			miR ID	miR site	Function class	Context+ score change
1.	rs11552193	G>A	hsa-miR-3675-3p	gtgtTA**G**AGATAt	Disruption of site	-0.288
			hsa-miR-520f-5p	gtgTTA**G**AGAtat	Disruption of site	-0.093
			hsa-miR-302b-5p	gtGTTA**A**AGAtat	Creation of site	-0.196
			hsa-miR-302c-5p	gTGTT**A**AAgatat	Creation of site	-0.003
			hsa-miR-302d-5p	gtGTTA**A**AGAtat	Creation of site	-0.0207
2.	rs11552192	T>A	hsa-miR-590-3p	ctaatT**A**AAATTt	Creation of site	-0.015
3.	rs76799616	G>A	hsa-miR-3140-5p	aaATTC**A**GGtat	Creation of site	-0.317
			hsa-miR-4680-3p	aAATTC**A**Ggtaat	Creation of site	-0.195
4.	rs80217848	A>G	hsa-miR-320a-d	catcta**A**GCTTTA	Disruption of site	-0.035
			hsa-miR-4429	catcta**A**GCTTTA	Disruption of site	-0.103

Table 3. Effect of 3'UTS SNPs on miRNA binding site predicted by miRNASNP-v3

			Effect		
S. No.	Variant	Variation	Gain/Loss of miRsite	miRNA	dG binding (Kcal/mol)
1.	rs11552193	G>A	Gain	hsa-miR-302b-5p	-15.07
			Gain	hsa-miR-302c-5p	-10.04
			Gain	hsa-miR-302d-5p	-17.88
			Gain	hsa-miR-552-5p	-8.62
			Loss	hsa-miR-3765-3p	-9.15
			Loss	hsa-miR-520f-5p	-10.63
			Loss	hsa-miR-4684-5p	-9.28
2.	rs11552192	T>A	Gain	hsa-miR-590-3p	-3.33
			Loss	hsa-miR-32-3p	-3.36
3.	rs76799616	G>A	Gain	hsa-miR-4680-3p	-7.86
			Gain	hsa-miR-183-3p	-4.67
			Gain	hsa-miR-4427	-8.55
			Gain	hsa-miR-3140-5p	-11.86
			Gain	hsa-miR-4452	-5.13
			Gain	hsa-miR-5187-3p	-6.69
4.	rs80217848	A>G	Gain	hsa-miR-7109-3p	-11.32
			Gain	hsa-miR-3135a	-10.05
			Loss	hsa-miR-320a-d	-8.66
			Loss	hsa-miR-4429	-7.12

elucidates essential regulatory elements governing gene expression, exerting influence over a broad spectrum of biological phenomena. In the current study, we have shortlisted and predicted the possible impact of SNPs present in *BECN1* non-coding regions computationally. Analysis of promoter region variant predicted the nine potential variants altering *BECN1* transcription factor binding sites which could eventually lead to altered expression of *beclin 1*. Earlier, Kazachkova et al., (2017) studied *BECN1* promoter region variants rs60221525 and rs116943570 and checked their correlation with Machado Joseph's disease. Apart from their finding of the alteration of TFBS due to these variants, they further checked that the variants affected the expression of *BECN1* which is correlated to early onset of the disease [15]. The *BECN1* gene expression is under the regulation of various transcription factors and levels of expression are directly correlated with autophagy initiation[3]. The effect of SNPs on TFBS predicted by these in silico servers could be further experimentally validated by checking the gene expression. The miRNAs modulate gene expression by binding to 3'UTR[24,25]. Most of the miRNAs downregulate gene expressions, yet few reported miRNAs can act as positive regulators of gene expression[25]. The computational examination of the 3'UTR of *Beclin 1* has revealed that the investigated SNPs either introduce or eliminate binding sites for miRNAs. In mammalian genes, the seed sequence is widely recognised as the paramount feature for miRNA-mediated target recognition. Moreover, 3'UTR SNPs were predicted to eliminate the sites for two miRNAs with the highest seed match probability. SNPs that alter miRNA sites have been reported to be prognostic and predictive cancer biomarkers[26]. So, these SNPs could be studied further for functional validation and could be correlated with a particular trait such as stress response. Expression of *Beclin 1* is directly correlated with induction of autophagy[27]. Altered autophagy due to altered expression of *beclin 1* contributes as a major factor in the case of various diseases _ENREF_28[28]. Furthermore, a recent study has reported that autophagy plays a significant role as a mechanism in the development of lung injury induced by high-altitude hypoxia[8]. Additionally, *Beclin 1* expression was seen to be enhanced in the tissues exposed to hypoxia. Thus underlying factors that enhance the expression of candidate genes such as *beclin 1* under various conditions need to be thoroughly investigated. SNPs can confer protection or susceptibility towards physiological traits such as stress responses.

SNPs within the human genome are a rich source of genetic diversity, impacting our understanding of traits, diseases, and personalised medicine. SNPs enhance genetic comprehension and hold the potential for improved healthcare. In silico analysis is vital for initial SNP analysis, facilitating the identification of functionally significant variants, saving time and resources, and offering insights into the genetic foundations of traits and diseases.

This is a preliminary study that predicts regulatory region *beclin 1* SNPs that affect the transcription factor and miRNA binding sites which can be a reason for the altered expression of *beclin 1* and this could be further studied and correlated with the susceptibility of individuals towards environmental stress such as hypoxia. Thus, comprehending an individual's genetic predispositions can provide valuable insights for tailoring personalised healthcare strategies and interventions to optimize well-being while mitigating potential risks. Our study is limited to preliminary computational analysis and lacks experimental validation. However, this study forms a basis for further functional studies such as the study of the effect of only these shortlisted SNPs with predicted impact on *beclin 1* expression. Furthermore, most importantly association studies on these SNPs could be done to check the genetic predisposition of individuals such as troops posted or likely to get posted to high altitudes to ensure their physiologic fitness to face such extreme environmental conditions.

In conclusion, this computational approach has provided significant results of analysis of the regulatory region of *BECN1* that could further help find out the underlying mechanism of its malfunction in various diseases.

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