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Expression of *hTERT* and *Lp-PLA(2)* genes is increased in primary hypothyroidism.

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ABSTRACT

Objective: Sightly low serum free T4 levels are associated with longer life expectancy. However, hypothyroidism is associated with increased risk of inflammation and vascular complications. Therefore, relation of thyroid hormones (THs) with life expectancy seems to be complex. This study was carried out in an effort to understand the contribution of THs in inflammation and aging.

Methodology: 15 cases of treatment naïve primary hypothyroidism and 15 age and sex matched euthyroid controls were recruited. mRNA expression of Telomerase (*hTERT*), leucocyte telomere length (LTL) and Lipoprotein associated phospholipase A2 (*Lp-PLA2*), a marker of vascular inflammation and risk predictor of cardiovascular events were measured by qPCR in whole blood.

Result: Expression of *hTERT* was found to be 6.95 times higher in the cases as compared to the controls. mRNA expression of *Lp-PLA(2)* was also 3.3 times higher in cases. LTL in euthyroid was approximately 81% of the length in hypothyroid patients.

Conclusion: A higher expression of *hTERT* indicates that hypothyroidism confers better cell survival in peripheral leucocytes inspite of the presence of vascular inflammation. However causal relationship cannot be ascertained with these results.

1. Introduction

Thyroid hormone (TH) which is an important regulator of a wide range of metabolic processes have been studied for their role in aging and longevity (Gauthier et al., 2020; Jansen et al., 2015a). It is reported that higher serum thyroid stimulating hormone (TSH) levels and/or low T4 levels are associated with longer life expectancy (Zambrano et al., 2014). Thyroid hormone spurs cells to senescence by acting through Thyroid Receptor-B (THRB) (Zambrano et al., 2014). It is observed that thyroid hormones increase mitochondrial activity and production of Reactive Oxygen Species (ROS). This surge in ROS leads to DNA damage precipitating cellular senescence (Zambrano et al., 2014). It is reported that slightly high TSH and low T3 levels resulting in subclinical hypothyroidism are also linked to improved longevity (Gauthier et al., 2020). This indicates important role of TH in aging.

Cellular aging is marked by progressive shortening of telomere length (La et al., 2016)·Telomeres are composed of repetitive TG-rich sequences (TTAGGG)n and protein complexes which shield the ends of chromosomes in eukaryotic cells, and promote chromosomal integrity and genome stability (Blackburn, 2001). Human telomerase has important role in overcoming shortening of telomeres. Telomerase is a ribonucleoprotein consisting of two essential components: a catalytic unit, telomerase reverse transcriptase (*hTERT*), and the ubiquitously expressed RNA template, hTR (Blackburn, 2001). Gene for *hTERT* is located at chromosome 5p15.33 (Bryce et al., 2000). Reverse transcriptase (hTERT), a catalytic subunit of human telomerase enzyme, has been used as a molecular marker for cellular senescence (La et al., 2016)· In addition, levels of *hTERT* mRNA expression has been recognised as a primary mechanism of telomerase regulation (Liu et al., 2004). Though reports suggest that thyroid hormones affect aging, to the best of our knowledge no study has been conducted to evaluate the effect of thyroid status on *hTERT* expression.

Hypothyroidism is also associated with inflammatory state, atherogenic lipid profile, and endothelial dysfunctions resulting in increased risk of atherosclerosis and cardiovascular diseases (Sertić et al., 2010;

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List of non-standard abbreviations

ATA	American Thyroid association
hTERT	human telomerase reverse transcriptase
Lp-PLA(2)	Lipoprotein associated phospholipase A2
LTL	leucocyte telomere length
ROS	Reactive oxygen Species
THRB	Thyroid hormone receptor B
THs	Thyroid Hormones

Ichiki, 2010; De Lange, 1998) which has detrimental effect on longevity. An important marker for vascular inflammation and cardiovascular risk is Lipoprotein-associated Phospholipase A2 [Lp-PLA (2)] (Corson et al., 2008; Khakpour and Frishman, 2009). The enzyme is encoded by the gene *PLA2G7* and is secreted by neutrophils, macrophages, activated platelets, mast cells and T cells etc (Madjid et al., 2010). Lp-PLA(2) is associated principally with LDL particles and to lesser extent with HDL particles in blood (Madjid et al., 2010). Lp-PLA*(2)* hydrolyses oxidised phospholipids and release lyso-phosphatidylcholine and free oxidised fatty acids causing upregulation of inflammatory mediators (Sertić et al., 2010; Madjid et al., 2010).

While inflammation results in cellular insult, telomerase and long telomeres are considered anti-aging. Cellular aging will thus depend on the balance between these factors. Therefore, this study is designed to evaluate the effect of thyroid status on cellular aging as well as the systemic inflammation in an effort to understand the interplay of these factors in hypothyroid individuals.

2. Methodology

The study is carried out in the department of Biochemistry and in department of Endocrinology & Metabolism after obtaining ethical clearance from the institutional ethical committee for human research.

2.1 Study Population: Since no study is available which has evaluated these parameters, sample size could not be calculated. This is a pilot study with 30 participants.

Patients of more than 18 years of age and either gender, diagnosed with primary hypothyroidism (n = 15) were recruited from the outpatient department and were designated as cases. The diagnosis of hypothyroidism was established according to the ATA guidelines (Garber et al., 2012). Only treatment naive patients (those who have not received any treatment or received treatment for <2 weeks) were included in the study. Euthyroid, apparently healthy age and gender matched controls (n = 15) were also recruited from relatives and accompanying persons and were designated as controls.

Participants with history of fever, respiratory diseases, ongoing infections, renal and hepatic disorders as assessed by history and lab investigations and individuals with subclinical hypothyroidism or those taking treatment for >2 weeks for hypothyroidism were excluded from the study.

Blood samples were drawn from the individuals recruited for the study after obtaining informed consent. The serum/plasma was used for routine investigations to rule out any existing disease e.g diabetes mellitus, renal or hepatic disorder and for analysis of thyroid function. All biochemical investigations were carried out by spectrophotometric method in SynchronX 800 (Beckman, USA). fT3, fT4 and TSH were analysed by chemilumniscence based method in Immulite (2000) XPi (Siemens, Germany). Whole blood in EDTA was used for studying mRNA expression of *Lp-PLA(2)* and *hTERT* genes as well as leucocyte telomere length (LTL).

2.2 *Relative mRNA expression:* mRNA was extracted using RiboZol™ RNA extraction reagent (Amresco, USA) by manufacturer protocol. Isolated mRNA quantification was done using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). Reading was taken at 260 nm and 280 nm. 260 nm/280 nm value of more than 1:1.8 was taken as appropriate and the concentration of RNA was calculated.

*Synthesis of cDNA:*cDNA was synthesised using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per manufacturers protocol. To 500 ng of RNA, 1 μL of a mixture of OligodT and Random Hexamer (1:3) were added. cDNA synthesis was carried out in Peqlab Thermal Cycler (VWR, USA) by incubating at 25 °C for 10 min, followed by 45 °C for 60 min and further incubation at 70 °C for 10 min. 20 μl of cDNA obtained per reaction was diluted by adding 60 μl of fresh nuclease free water (NFW). 2 μl of diluted cDNA was used per 10 μl of qPCR reaction.

qPCR: The *q*PCR was carried out in CFX Connect Real Time System (BIO-RAD, USA). Maxima Hot Start PCR Master Mix (Thermo Scientific, USA) was used along with SYTO 9 dye (Invitrogen, USA). The 10 μL reactions were set up in duplicates using 5 μL of PCR master mix (Thermo Fischer), 0.5μLof SYTO9 dye and 2 μL of cDNA. 0.4 μL of each forward (20 μM) and revere (20 μM) primers (Table 1) were added and volume was made up by adding Nuclease Free water (NFW). Non-Template Control (NTC) reaction was also set up by using NFW in place of cDNA. Activation of Taq DNA polymerase was done at 95°C for 3 min and then further for 20 s more at 95°C (Repeat for 34–39 cycles) followed by annealing at the respective annealing temperatures (*hTERT:* 58 °C, *Lp-PLA(2):* 58 °C). Fluorescence acquisition was done at 72 °C to calculate Ct values.Fold change in the expression of *Lp-PLA(2)* and *hTERT* (Target Genes) was calculated using ΔΔ Ct (Rao et al., 2013) method taking *18S* as normaliser (housekeeping gene).

2.3 *Leucocyte telomere length*: DNA was extracted from the whole blood by using DNA extraction kit (Zymo Research, USA). Telomere length was measured by Real Time-PCR (BIO-RAD, USA) using specific primers (Table 1). β-globin was used as control single-copy gene/reference gene. To prepare reaction volume 10 μL the following reagents were added: 5 μL of Hot Start PCR Master mix (Thermo Scientific, USA), 2 μL of DNA; 0.9 μL of forward and reverse primers (working 10 μM each) of Telomere (Table 1), 0.5 μL of forward and reverse (working 10 μM each) Primer of β-globin (Table 1) and 1.6 μL of Nuclease Free water (NFW) were added. Activation of Taq polymerase was carried out at 90 °C for 7 min. The reaction was allowed to proceed at 94 °C for 15 s and then at 49 °C for 15 s (for 2 cycles) followed by repeat for 39 cycles at 94 °C for 15 Seconds and at 62 °C for 10 s and at 74 °C for 15 s and at 84 °C for 10 s and at 88 °C for 15 s, then holding the reaction at 72 °C for 10 s.The ratio of the two Ct values (Ct telomere (T)/Ct reference gene (S)) gave a value corresponding to relative length of the telomere (Cawthon, 2002).

3. Analysis

Results of the quantitative variables are expressed as mean ± SD. Since this is a pilot study with small sample size, no test of hypothesis

Table 1
Sequence of primers used.

Genes	5′ to 3′ Sequence
*hTERT*F	GCAAGTTGCAAAGCATTGGA
*hTERT*R	ACCTCTGCTTCCGACAGCTC
Lp-PLA(2) F	CCACCCAAATTGCATGTGC
Lp-PLA(2) R	GCCAGTCAAAAGGATAAACCACA
18sF	GTAACCCGTTGAACCCCATT
18sR	CCATCCAATCGGTAGTAGCG
Telomere F	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
Telomere R	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
β-globin F	CGGCGGCGGGCGGCGCGGGCTGGGCGGCTTCATCCACGTTCACCTTG
β-globin R	GCCCGGCCCGCCGCGCCCGTCCCGCCGGAGGAGAAGTCTGCCGTT

F=Forward, R=Reverse.

was carried out. However post analysis sample size calculation was carried out from ΔCt values of *hTERT*. Considering the mean and SD values in both groups and keeping α at 5%, the power of the study came out to be 99.8%. Therefore the sample size was adequate. The variables were compared using unpaired students' *t*-test. Correlation analysis was carried out using Pearson correlation coefficient. A p value < 0.05 was considered statistically significant.

The relative expression of both the genes *hTERT* and *Lp-PLA(2)*was analysed by ΔΔCt method and fold change among the two groups was calculated using $2^{-\Delta\Delta Ct}$ method (Rao et al., 2013). For this △Ct for the gene of interest was estimated for both groups by subtracting Ct value of target gene from the Ct value of normaliser gene. Average △Ct was calculate for both groups. △△Ct value was obtained by subtracting average △Ct of patient from that of control. Fold change was calculated using formula $2^{-\Delta\Delta Ct.}$.

LTL analysis: LTL is expressed as a ratio Ct telomere (T)/Ct reference gene (S) and values expressed as mean of the 15 samples per group that were analysed (Cawthon, 2002).

4. Result

The study was conducted on 30 participants who were designated into 2 groups (cases & controls), with 15 participants each. Both groups were matched for age and sex. There were 13 females and 2 males in each group. The mean age, clinical and biochemical parameters of the participants of both cases and controls are depicted in Table 2. 02 participants in control group and 01 participant amongst cases were found to be hypertensive according to the guidelines (The Seventh Report of the, 2003). As per the criteria based on International Diabetes Federation for Asia pacific region (The IDF consensus worldwide definition), all participants were found to be obese. None of the participants were taking any drugs. All patients of hypothyroidism were treatment naive.

We calculated the fold change in mRNA expression of *hTERT* and *Lp-PLA(2)* genes from the Ct values obtained from qPCR. Average ΔCt values for each gene using 18S as normaliser are depicted in Table 3. Expression of *hTERT* and *Lp-PLA(2)* was found to be increased in cases. The results are represented graphically in Fig. 1 mRNA expression of *hTERT* was 6.95 times higher and *Lp-PLA(2)* was 3.3 times higher in cases as compared to controls. Leucocyte telomere length was comparable in the both the groups, although it was slightly lower in cases (81.5%) as compared to that in controls (Table 3).

Pearsons correlation coefficient was calculated to determine if any correlation existed between TSH values and mRNA expression of the genes and LTL. The results are given in Table 4. No statistically significant correlation was observed among the said variables in the study. However we observed that expression of *Lp-PLA(2)* significantly correlated positively ($r = 0.711$, $p = 0.003$) with expression of hTERT in patients of hypothyroidism.

Table 2
Clinical and biochemical parameters of Cases and Controls.

Parameter	Cases n = 15	Controls n = 15	p value
Age (years)	35.6 ± 10.1	33.9 ± 10.8	0.644
Weight (kg)	62.8 ± 8.5	63.6 ± 11.4	0.84
BMI	25.20 ± 2.90	25.7 ± 2.59	0.721
Waist circumference (cm)	92.7 ± 3.6	91.9 ± 3.8	0.780
SBP (mm Hg)	124.3 ± 9.8	121.2 ± 6.3	0.718
DBP (mm Hg)	70.9 ± 10.5	74.5 ± 8.5	0.218
FBS (mg/dl)	86.9 ± 8.3	84.1 ± 5.3	0.291
B urea (mg/dl)	30.4 ± 15.3	25.5 ± 6.4	0.264
S çreatinine (mg/dl)	0.8 ± 0.2	0.7 ± 0.2	0.687
AST (IU/L)	22.1 ± 12.7	21.0 ± 8.1	0.267
ALT (IU/L)	23.2 ± 13.2	21.8 ± 11.2	0.405
fT3 (pg/mL)	2.08 ± 0.97	3.28 ± 1,56	0.011*
fT4 (ng/dL)	0.75 ± 0.29	1.11 ± 0.25	0.001*
TSH (mIU/mL)	24.34 ± 31.41	2.23 ± 0.98	0.0.007*

Values are expressed as Mean ± SD. *P value < 0.05 is taken as statistically significant.
SBP=Systolic Blood Pressure; DBP = Diastolic Blood Pressure; FBS=Fasting Blood Sugar.
B Urea = Blood Urea; S creatinine = Serum creatinine; AST = Aspartate Transferase; ALT = Alanine Transferase; fT3 = Free triiodothyronine; ft4 = Free Thyroxine; TSH= Thyroid Stimulating Hormone.

Table 3
Average ΔCt values for *hTERT* and *Lp-PLA(2)* genes and Leucocyte Telomere Length in Cases and Control.

	Average ΔCt keeping 18S as Normaliser		LTL
	hTERT	Lp-PLA(2)	
Cases	−7.89 ± 3.38	−6.81 ± 3.14	8602.91 ± 3639.99
Control	−10.78 ± 1.88	−8.53 ± 3.32	10576.9 ± 3754.16
p value	0.011[a]	0.183	0.118

Values are expressed as Mean ± SD.
hTERT = Human telomerase reverse transcriptase; *Lp-PLA(2)* = Lipoprotein-associated phospholipase A2; LTL = Leucocyte Telomere length.
[a] P value < 0.05 is taken as statistically significant.

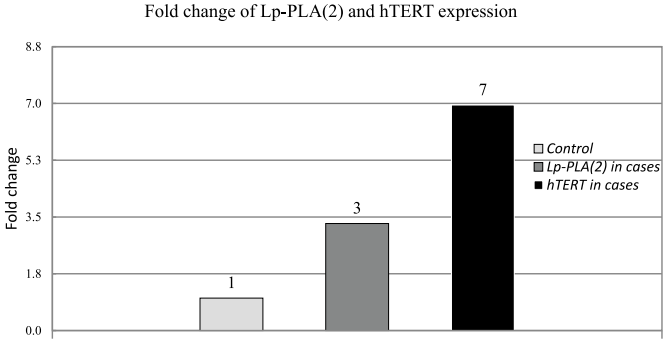

Fig. 1. Fold change in expression of *Lp-PLA(2)* and *hTERT* genes in cases as compared to controls.
The mRNA expression of *Lp-PLA(2)* gene was 3.3 folds and that of *hTERT* gene was 6.95 fold higher in hypothyroid patients. (The expression in control group is considered as 1).

5. Discussion

This study was carried out on the hypothyroid patients who were not taking any treatment. In our study we found that mRNA expression of *hTERT* is 6.95 times more in patients of hypothyroidism suggesting that telomerase expression is higher in peripheral leucocytes in hypothyroidism. Previously it has been reported that slightly high levels of TSH are associated with longer lifespan (Jansen et al., 2015a, 2015b; Zambrano et al., 2014; Rozing et al., 2010). Reports have indicated that

Table 4
Correlation analysis of mRNA expression of *hTERT* and *Lp-PLA(2)* genes and Leucocyte Telomere Length with TSH.

	Cases (n = 15) Pearson Correlation *(p value)*	Controls (n = 15) Pearson Correlation *(p value)*
hTERT expression	−0.469 *(0.078)*	−0.088 *(0.775)*
Lp-PLA(2) expression	−0.498 *(0.058)*	−0.426 *(0.113)*
LTL	0.101 *(0.702)*	−0.459 *(0.085*

TSH levels are taken as dependent variable. ΔCt values of hTERT and Lp-PLA(2) are taken as variable for the analysis. p value < 0.05 is taken as statistically significant.
hTERT = Human telomerase reverse transcriptase; *Lp-PLA(2)* = Lipoprotein-associated phospholipase A2; LTL = Leucocyte Telomere length. TSH= Thyroid stimulating hormone.

many old individuals are diagnosed with subclinical hypothyroidism and might even have certain physical function advantages and lower mortality when compared to euthyroid individuals (Simonsick et al., 2016). Therefore we expected that thyroid hormones and TSH may have some role in cellular aging also. Although the mechanism proposed for this longevity is slow metabolic rate together with low oxidative stress (Gauthier et al., 2020), the effect of thyroid hormone on telomerase activity has not been studied in humans. A higher expression of *hTERT* in our study indicates that thyroid hormones may also be acting by regulating telomerase enzyme.

We also observed that leucocyte telomere length (LTL) were comparable in both groups although LTL was slightly shorter in hypothyroid patients. From our study we cannot ascertain whether thyroid hormones have any direct effect on h*TERT* expression and telomere length, however, it seems probable that a high expression of *hTERT* may have been the result of compensation for short LTL in peripheral leucocytes.

We also found that expression of *Lp-PLA(2)* enzyme is increased in these patients. Though various studies have attributed Lp-PLA(2) as a marker of inflammation, some anti oxidative and anti inflammatory role has also been attributed to Lp-PLA(2) (Silva et al., 2011) which depends on its interaction with LDL and HDL (Silva et al., 2011). Since *Lp-PLA(2)* is the marker of inflammation, it is considered a risk factor for atherosclerosis (Gong et al., 2011)' An increase in *Lp-PLA(2)* expression observed in our study is in consistence with the reports that hypothyroidism is an inflammatory state (Ichiki, 2010; De Lange, 1998). Similar results are also reported earlier where sPLA(2) levels were found to be higher in subclinical hypothyroidism indicating it to be an inflammatory state (Mohammad et al., 2014). Therefore a higher expression of *Lp-PLA (2)* enzyme also suggests an increased risk of cardiovascular diseases in untreated hypothyroid individuals.

While high *hTERT* expression seems to indicate better cell survival, higher *Lp-PLA(2)* expression suggests ongoing inflammation which is detrimental to health. This contrast in the results can be explained by the fact that both genes were studied in whole blood where PBMCs are major source of RNA. Inflammatory cells undergo extensive cell division and clonal expansion to effectively generate an inflammatory response. Repeated cell division results in telomere shortening. After a critical shortening is achieved, the cells undergo apoptosis. Telomerase enzyme takes care of chromosomal shortening and thus improves chromosomal stability and longevity of cells. A plausible explanation for an increase in *hTERT* expression in our study is consistent with the inflammatory response mounted by the PBMCs. This is further corroborated by positive correlation observed between *Lp-PLA(2)* and *hTERT* expression. In addition, it has been also been proposed earlier that telomerase/telomere dysfunction coexist with inflammation. (Kordinas et al., 2016). Nonetheless, a higher expression of *hTERT* in our study provides evidence that to maintain a steady inflammatory response, there may be a compensatory rise in the telomerase enzyme.

Though *hTERT* expression analysis in our study indicates better cell survival in hypothyroidism, at least in PBMCs, it will be worthwhile to study the same in other tissues also. A larger sample size is also required to ascertain the findings of this study.

Strength of the study: To the best of our knowledge best knowledge, there are no studies which have evaluated expression of *hTERT,* leucocyte telomere length and expression of *Lp-PLA(2)* in primary hypothyroidism previously. Moreover our study has been conducted the treatment naive patients. It is noteworthy that the clinical and biochemical parameters are also similar in both groups. This design has its own advantage as the effect of drugs and other confounding variables is not present in this study.

Limitations: Our study has certain limitations. For the purpose of statistical evaluation of variables, particularly correlation analysis, sample size is relatively small. This may raise some concerns on the statistical precisions of the estimates. In addition this study has not evaluated the relation between the expression and activity of *hTERT* and *Lp-PLA(2)*. Therefore, a large-scale study is warranted to validate our findings.

6. Conclusion

The results of our study indicate that *hTERT* and *Lp-PLA(2)* expression is higher and LTL is slightly shorter in hypothyroid individuals. The results establish that in primary hypothyroidism the expression of telomerase enzyme is higher indicating better survival of PBMCs. However, our study also indicates an inflammatory state in hypothyroid patients. Though causal relationship of thyroid hormones with increased cellular survival cannot be ascertained with these results, this study indicates an inflammatory state and hitherto better survival of PBMCs probably as a result of clonal expansion of immune cells in hypothyroidism individuals. A larger sample size is required to ascertain the findings of this study. It will also be worthwhile to study the same in other tissues as well.

CRediT authorship contribution statement

Seema Garg: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation. **Parul Gupta:** Writing – review & editing, Writing – original draft, Formal analysis. **Satyam Wahi:** Writing – original draft. **Mohit Mehndiratta:** Writing – review & editing, Resources, Methodology. **S.V. Madhu:** Writing – review & editing. **Edelbert Almeida:** Methodology, Conceptualization. **Rajarshi Kar:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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