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Telomerase reverse transcriptase and telomerase RNA component gene expression as novel biomarkers for Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a neurological, age-related condition that causes cognitive decline and memory loss; it induces dementia in the elderly. Telomerase is a reverse transcriptase ribonucleoprotein that adds nucleotides to the end of DNA. This study aimed to compare human telomerase reverse transcriptase (hTERT) and telomerase RNA component (TERC) expression in different phases of AD and healthy cohorts. Sixty participants were divided into 30 who had dementia and 30 who did not. After collecting blood samples, total RNAs were extracted from the plasma. Screening for hTERT and TERC gene expression was carried out by quantitative reverse transcriptase real-time polymerase chain reaction (RT-qPCR) using the relative quantification method to estimate the expression changes in hTERT and TERC. The RT-qPCR results show that hTERT and TERC gene expression was significantly down-regulated in Alzheimer's patients compared to the health subjects (P-value= <0.0001,0.005), respectively. The area under curve AUC was 0.773 for hTERT and 0.703 for TERC. The Mini-Mental State Examination scores revealed a significant difference between dementia and non-dementia subjects (P=<0.0001). We conclude down-regulations in both hTERT and TERC gene expression in AD patients, which supports our hypothesis that the telomerase expression gene in the blood of AD patients can serve as a non-invasive, early, and novel diagnostic marker of AD.

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Introduction

Alois Alzheimer is the first to define AD (1), a neurodegenerative condition marked by cognitive degeneration, beginning with memory loss and progressing to affect behaviour, visuospatial orientation, speech, and the brain's motor functions (2). Alzheimer's disease is one of the most common kinds of dementia, and it is expected to impact about 131.5 million people by 2050 if no new treatments are developed (3). Genetic, epigenetic, and environmental factors all contribute to the progression of Alzheimer's disease (4). The number of AD sufferers grows as the population ages(5). AD is caused by damage in the AD patient's brain due to the aggregation of injurious protein amyloid-β (Aβ) (6). The hallmark of AD pathology is the aggregation of hyperphosphorylated and truncated microtubule-associated tau protein in the neuropil threads and neurofibrillary tangles (7, 8)

The telomere is a nucleoprotein structure at the end of the terminal eukaryotic linear chromosome and capping it. In mammalian cells, the telomere sequence is 5′-TTAGGG-3′ repeat (9, 10). It is essential to maintain genomic stability (11, 12) and protect end-to-end fusion, degradation, and cytogenetic abnormalities (9, 10).

Telomerase is a ribonucleoprotein with the activity of reverse transcriptase, which adds nucleotides at the DNA ends (13-15). Holoenzyme of telomerase comprises three subunits: catalytic protein subunits contain hTERT, and RNA subunits consist of hTERC and telomerase-related protein (TEP). In humans, hTERT and TERC are the most essential (16-18). TERT and TERC play an indispensable role in facilitating telomere synthesis; furthermore, TERT and TERC are joined together to create the active telomerase enzyme. The Telomerase enzyme is a reverse transcriptase, producing telomeric repeats via hTERC as a template (19). With every cell division, telomeric DNA progressively erodes in most human cells without the telomerase enzyme (20). Biologically, telomere and telomerase highly correlate with developing many neurodegenerative diseases like Alzheimer's (21).

Human TERT is a part of the telomerase structure and functions in telomeres by adding a repeated specific sequence, TTAGGG (22). TERT has performed telomere elongation and other processes, including mitochondria translocation and oxidative stress reduction resulting in reactive oxygen species ROS accumulation (23). The Telomerase reverse transcriptase gene encodes the TERT (24) on human chromosome 5p15.33. The length of the TERT gene is about 42 kb. The Telomerase RNA Component TERC is located on the 3q26.2 chromosome. The TERC gene encodes TERC; TERC, a short RNA, noncoding, conserves as a template for DNA synthesis to preserve telomere length. Further, it participates in holoenzyme telomerase localization, catalysis, and assembly (24, 25)

This study investigated the differences in the expres-

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sion levels of hTERT and TERC genes directly from the blood of Alzheimer's patients relative to the non-dementia cohort. The aim was to detect the association between AD and telomerase gene expression and the possibility of using hTERT and TERC as non-invasive, early diagnostic biomarkers for Alzheimer's disease.

Materials and Methods

Samples were collected from a few private neurological clinics in Erbil city. The present study recruited 60 participants, 30 patients diagnosed with dementia divided into 11 MCI and 19 AD by neurologists according to the minimantel state examination MMSE (26), and 30 healthy controls (non-dementia) were approximately of the same age and gender. The Mini-Mental State Examination MMSE scores were utilized to estimate the cognitive stage of the disease. MMSE is a cognitive test used in epidemiologic studies and clinical practice (26); The healthy non-dementia subjects had no sign of any neurological functions and features reflected by laboratory examination, and MMSE scores were higher than 24. Samples were collected during the period between December 2021 and March 2022. The information was obtained from AD patients and their caretakers by inquiring about their medical history. A questionnaire was used to identify both cohorts by asking about family history of Alzheimer's disease, cardiovascular diseases, diabetes mellitus, cancer, infectious diseases, types of medication, and other neurological diseases such as different types of dementia, Parkinson's disease, and multiple sclerosis. Neurologists give information about computerized tomography (CT) scans of the brain in AD cases.

Blood sampling

Peripheral venous blood was collected from all participants using EDTA K3 tubes. The blood samples were centrifuged within 2 hours for 8 min at 1300 g at 4°C. Total RNA isolation was conducted directly from supernatant plasma, and replicates were stored in sterile DNA/RNA- Free Eppendorf tubes at -80°C for future investigation until used.

RNA isolation and purification

Total RNA was isolated from plasma and performed using a specified kit to extract both mRNA and microRNA, GenElute™ Total RNA Purification Kit (Sigma, Aldrich, Germany). The RNase-Free DNase I kit (Norgen, Biotek Corp, Canadian) was applied to remove DNA traces. Each sample's isolated RNA purity and quality were assayed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, USA).

The RT-qPCR detection

Real-time qPCR was performed directly following the total RNA extraction. An equal amount of RNA concentrations was taken from all purified RNA samples. Five microliters of isolated RNA were reverse transcribed into cDNA in the one-qPCR reaction; the first step is reverse transcribing the isolated RNA into cDNA, followed by qPCR amplification in the same reaction wells. Reactions were performed using SYBER® Green mix (One-Step SYBR® Green RT-qPCR kit) (Genecopoeia, USA). All reactions were run in duplicate. The amplification was carried out by Primer Pro 48 Real-time PCR (TECHNE, UK). Primers used in this study targeted the hTERT, TERC, and ACTB (an internal control reference gene for normalization). The primers were designed by NCBI and manufactured by (Genecopoeia, USA). The RT-qPCR primers sequence was used (Table1.).

A one-step qPCR cycle profile was performed for all primers:

1. Reverse transcription of mRNAs into cDNA at 42 °C for 10 min, followed by initial denaturation at 95 °C for 3 min, then followed by 45 cycles (denaturation at 95 °C /10 s and extension at 60 °C /30 s).
2. Melting curve analyses were conducted at 0.3 °C intervals from 72 °C to 95 °C.
3. The cycle threshold (Ct) valves for each sample were normalized using (ACTB) as an internal control (reference gene), relative quantification method was used to calculate the fold change in expression.

The relative quantification (2^-ΔΔCt) RT-qPCR

The RT-qPCR data were analyzed using the relative quantification (RQ) method 2-ΔΔCt, and RQ were calculated. First, the CT value is compared to a housekeeping gene (endogenous control). Then, the formula ΔCT = (Ct AD mRNA gene-Ct control reference gene) was used to normalize the Ct target gene to the Ct of the ACTB reference gene. Then, ΔΔCt = (ΔCt of dementia -ΔCt of non-dementia) was used, normalizing the test sample's ΔCt to the control ΔCT to obtain expression ratio = 2^-ΔΔCt fold change (27, 28).

Statistical analysis

The data were analyzed utilizing a package of statistical programs (GraphPad Prism, version 8.0.1) and (SSPS, version 25). The Chi-square test of association was utilized to compare proportions. Mann-Whitney test was used to compare data and non-normal distribution variables of the two subjects. An unpaired student's t-test of two independent cohorts was used to compare the two groups. Finally, the receiver operating characteristic (ROC) was applied to

Table 1. Primer sequences and their amplicon sizes were used in the RT-qPCR reactions.

Genes	Primer sequence	Amplicon size	Catalogue number
hTERT	F: 5'-TGTCAAGGTGGATGTGAGGG-3' R: 5 '-GCAGTACGTGTTCTGGGGTT-3'	97	Cat.No. HQP018017
TERC	F: 5'-AACCCTAACTGAGAAGGGCG-3' R: 5'-AGAATGAACGGTGGAAGGCG-3'	114	Cat.No. CSHQP1933L
ACTB	F: 5'-CCAACCGCGAGAAGATGA-3' R: 5'-CCAGAGGCGTACAGGGATAG -3'	97	Cat. No. HQP016381.

estimate the area under curve AUC for each mRNA gene. Youden's index (sensitivity+specificity-1) (29) was used to estimate the cutoff point of the screening variables where the highest index was selected as it gives the test's most heightened sensitivity and specificity in differentiating dementia from non-dementia and evaluating the diagnostic effect. A P-value ≤ 0.05 was considered significant.

Results

Participant's characteristics

The total participants in the present study were (60 subjects), and were divided into the dementia group (30) consisting of MCI (11) and AD (19) and the non-dementia group (30). The participants' distribution of gender and age is shown in (Table 2.). The statistical analysis shows no significant differences in age and gender between the AD cases and non-dementia cohorts, with P-value= 0.107 and 0.795, respectively.

Demographic characteristics of participants

Family history, infectious disease, the presence of another neurological disease, cardiovascular disease, diabetes mellites, smoking, and cancer; the statistical analysis revealed no significant differences in those variables between the two groups P-value= 0.052, 0.237, 0.1120.136, 0.542, 0.795, 1.00, respectively. The clinical and demographic characteristics of demented and non-demented subjects are presented in (Table 3.).

Table 2. Patient characteristics distribution of dementia cases and healthy controls.

Variables	Dementia	Non-dementia	Total	
	No. (%)	No. (%)	No. (%)	P-value[a]
Age	73.7±9.0	71.3±8.2		0.107
< 65	2 (6.7)	8 (26.7)	10 (16.7)	
65-74	13 (43.3)	9 (30.0)	22 (36.7)	
>75	15 (50.0)	13 (43.3)	28 (46.7)	
Gender				0.795
Male	13 (43.3)	14 (46.7)	27 (45.0)	
Female	17 (56.7)	16 (53.3)	33 (55.0)	
Total	30 (100.0 %)	30 (100.0 %)	60 (100.0 %)	

[a] Chi-square means ±SD.

Table 3. Clinical and demographic characteristics of demented and non-demented subjects regarding family history and many neurological and physiological factors.

Variables	Dementia	Non-dementia	Total	
	No. %	No.%	No.%	*P*-value[b]
Family history of AD				0.052
Yes	5 (16.7)	0 (0.0)	5 (8.3)	
No	25 (83.3)	30 (100.0)	55 (91.7)	
Cardiovascular disease				0.136
Yes	10 (33.3)	5 (16.7)	15 (25.0)	
No	20 (66.7)	25 (83.3)	45 (75.0)	
Diabetes mellitus				0.542
Yes	6 (20.0)	8 (26.7)	14 (23.3)	
No	24 (80.0)	22 (73.3)	46 (76.7)	
Other neurological diseases				0.112
Yes	4 (13.3)	0 (0.0)	4 (6.7)	
No	26 (86.7)	30 (100.0)	56 (93.3)	
Infectious disease				0.237
Yes	3 (10.0)	0 (0.0)	3(5.0)	
No	27 (90.0)	30 (100.0)	57 (95.0)	
Smoking				0.754
Yes	6 (20.0)	7 (23.3)	13(21.7)	
No	24 (80.0)	23 (76.7)	47 (78.3)	
Cancer				1.00
Yes	1 (3.3)	0 (0.0)	1 (1.7)	
No	29 (96.7)	30 (100.0)	59 (98.3)	
Total	30(100.0)	30 (100.0)	60 (100.0)	

[b] by Chi-square.

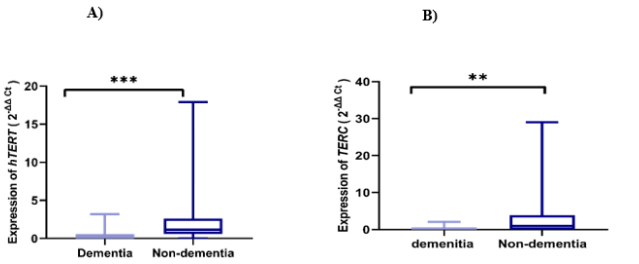

Figure 1. Telomerase gene (hTERT, TERC) expression level, (A) hTERT was a down-regulated expression in the dementia group compared with no dementia group, with a significant difference in the relative expression level of the hTERT gene in patients with dementia and non-dementia subjects (P= <0.0001). (B) Fold change in TERC gene expression level between two groups (P=0.005) dementia cohort decreased relative to healthy control (non-dementia).

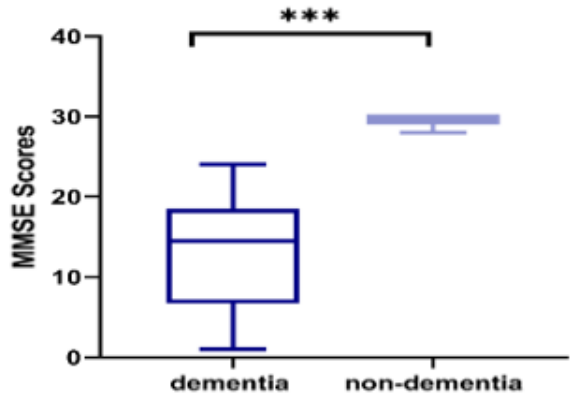

Figure 2. Shows that the demented group has lower MMSE scores than non-demented individuals P=<0.0001.

Evaluation of hTERT and TERC expression via RT-qPCR

The Ct value of hTERT and TERC was normalized to ACTB as a reference gene to calculate the 2^-ΔΔCt (fold change in expression); the Ct value of ACTB after RT-qPCR amplification analysis revealed no significant difference between the two cohorts, and the average was 24.1 for AD and 23.8 for healthy control.

hTERT and TERCR gene expression were analyzed to show the differences in expression levels between the two groups. The level of hTERT was significantly down-regulated P=<0.0001 and about 7- fold lower than in the non-dementia cohort (Figure 1A). The same with TERC gene expression validation by RT-qPCR relative quantification confirmed a significant decrease in TERC gene expression in plasma concentration of AD cases close to non-dementia P=0.005. (Figure 1B) is displayed the differences in fold change expression TERC gene. The MMSE scores consider abnormal when being less than 24 (26), whereas higher than 24, which means average cognitive impairment, P= <0.001, was significant among cohorts (Figure 2), which shows the difference between AD patients and healthy aged individuals in scores of cognitive test of MMSE.

McNemar test was used to test the validity of telomerase genes when the results of dementia were compared to non-dementia, as shown in (table 5). The ROC curve predestined the diagnostic value of significant alteration of telomerase gene expression (hTERT, TERC). The test was applied to evaluate the AUC, sensitivity, and specificity of hTERT and TERC mRNA genes as a diagnostic biomarker of AD between two cohorts (30) (table 5, Figure 4. A, B),

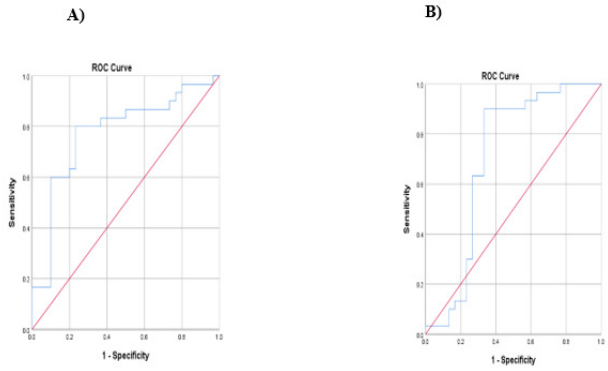

Figure 3. Receiver operating characteristic ROC curve for hTERT, TERC to predict dementia for discriminative ability between dementia and non-dementia (A) ROC curve for hTERT, AUC=0.773 and cutoff=< 0.665 (B) ROC curve for TERC, AUC=0.703 and cutoff=< 0.671.

presenting the AUC for hTERT TERC to predict dementia.

Tables 4 and 5 is shown the analysis of the ROC curve used to estimate the AUC to estimate the sensitivity and specificity of hTERT and TERC genes as diagnostic biomarkers of AD between two cohorts. ROC analysis of hTERT revealed AUC=0.773, P-value=<0.0001, SD=0.063, 95% confidence intervals = (0.649-0.898), sensitivity=80 % and specificity=76.7 %, the validity of hTERT in predicting of dementia, while TERC gene was AUC=0.703, P-value=0.0061, SD=0.074, 95% confidence intervals = (0.559-0.848), sensitivity 90 %, and specificity 66.7 %, the validity of TERC in predicting dementia. The hTERT and TERC are considered good non-invasive dia-

Table 4. The area under the hTERT, TECR ROC curve.

Area Under the Curve					
Test results variables	Area	Std. Error	Asymptotic Sig. P-value	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
hTERT	0.773	0.063	0.000	0.649	0.898
TERC	0.703	0.074	0.007	0.559	0.848

Table 5. Validity of hTERT, TERC in predicting dementia.

Telomerase genes	Cutoff value	Sensitivity	Specificity	PV+	PV-	agreement	P-value*
hTERT	< 0.665	80%	76.7%	77.4%	79.3%	78.3%	1.000
TERC	< 0.671	90%	66.7%	73%	87%	78.3%	0.092

*McNemar test, PV+ predictive value positive, PV- Predicative value negative.

gnostic biomarkers for the early detection and prediction of Alzheimer's disease.

Discussion

Many scientists have been dedicated to exploring tools to find the relationship between ageing and Alzheimer's disease, and many variables have been hypothesized in the etiopathogenesis of Alzheimer's (31). In the brain, the telomeres' activity characterizes during development and plays a role in neuronal survival and differentiation (32). During early embryonic development in somatic cells, the TERT gene and telomerase activity levels decrease (33). Telomerase expression during neuronal differentiation is essential in promoting neuron survival (34). Knockdown experiments and mutational analysis revealed that telomerase deficiency led to telomere loss, resulting in system organ failure (35, 36). The telomerase level plays a significant role in modulating the damage to DNA and repair during neural development. Moreover, it has been known to protect the cell against insults such as amyloid peptides and excitotoxins, which have a role in neurodegenerative disease pathogenesis (34, 37, 38).

Furthermore, it suggested that a decline in the telomerase level in mature neurons contributes to various neurodegenerative conditions (33). Telomerase prevents telomere shortening; it acts as an anti-ageing enzyme (35). The current study examines hTERT and TERC genes as early diagnostic biomarkers for the prediction of Alzheimer's disease. Moreover, the ROC curve revealed that hTERT and TERC have the sensitivity and specificity for typical early biomarkers of AD; our results indicate that both hTERT and TERC have good non-invasive and early diagnostic values to differentiate AD from healthy subjects. Alzheimer's is a popular form of dementia in ageing people (39). It is a progressive form of the disease, and multiple factors like genetic, environmental, and epigenetic factors contribute to its progress; oxidative stress plays a pivotal function in the pathogenesis of AD (4, 31). In humans, the end replication problem is due to the absence of DNA polymerase at the 3' end of the strand, resulting in the telomere not fully replicated at the 3' end leading to telomere shortening with each replication cycle (40). They discovered that telomerase is essential in maintaining the telomere length after each replication cycle by adding six tandem repeated sequences (19). Previous studies have found a relative telomere shortening with age-related disease and lifespan (41-43). The mechanical telomere short detected in the AD brain was associated with the decline in the expression of telomerase. These data gave a novel automated insight into the pathophysiology of Alzheimer's disease(44).

Interestingly, it has been found that the blood-brain barrier BBB is partly destroyed in Alzheimer's patients (45), and the function of BBB is to protect the central nervous system from the unregular passage of molecules to the circulation (46). Therefore, the BBB breakdown, considered a novel finding in neurological diseases, could be a tool for developing effective diagnostic strategies for treating neurological disorders (45). Previous studies revealed the human telomere length in the blood cells of AD patients. They detected the length of telomere and telomerase activity decline in MCI and AD cases compared to healthy individuals(47-50). In addition, another work detected hTERT and TERC genetic polymorphism in human Alzheimer's cases and revealed that it might influence AD occurrence(51-54).

Statistical analysis revealed that the hTERT gene was down-regulated in dementia cases, including MCI and AD compared to the non-dementia subject; the TERC gene shows lower in dementia patients than in healthy control. MMSE scores in dementia cases were lower than in non-dementia, showing significant differences. The present study detected no significant differences regarding age, gender, diabetes mellitus, infectious disease, smoking, cancer, family history of AD, cardiovascular disease, and other neurological diseases.

Moreover, when comparing the 30 dementia subjects (MCI and AD), we detected that hTERT and TERC were down-regulated significantly and differed from 30 healthy controls. RT-qPCR was used to estimate the expression concentration relative to the healthy cohort. Specifically, the hTERT and TERC were substantially lower in the dementia cohort. However, there is very little information concerning telomerase expression in human blood cells and brain tissue. Few studies support our finding, which in tissues of animal models were investigated; our result agrees with another study reported by Franco and Blasco (55), who revealed a decrease of TERT levels in AD subjects relative to the control (55). The present work is a line of results presented by Tsoukalas and Buga (56), who detected low TERT expression levels in 12 months rats compared with six-month-old rats. Other researchers have investigated telomerase activity by analyzing different tissues of aged animal models. They found that TERT and TERC were relatively expressed at lower levels than other tissues and controls, which correlates to the decreased telomerase activity in the same brain tissue (57). Many studies have detected a decline in telomerase activity and protein expression levels in Alzheimer's patients (58).

Moreover, previous studies discovered that increasing telomerase expression via engineering-modified and utilized anti-ageing drugs might be a novel tool for treating neurological diseases. Shim and Horner (59) first detected an increase in amyloid-β (Aβ) accumulation in AD cases and a decline in TERT expression when modifying AD mice to maintain the physiological expression level of the TERT gene and reduction Aβ accumulation (59). Further, Otgaar and Ferreira (60) the effect of anti-ageing drugs resulting in elevated telomerase activity and hTERT level; LRP::FLAG might be a novel anti-ageing drug through the cellular process of ageing (60).

Alzheimer's is shared among the ageing population, creating medical crises and effects enormous personal and family burdens. The problem increases with inadequate tools for early diagnosis and identifying AD patients. In this study, we investigated telomerase mRNA expression genes in blood. We concluded that the expression of hTERT and TERC genes are down-regulated in dementia samples relative to the non-dementia cohort. Our findings could represent a starting point for future researchers to investigate telomerase genes directly in blood/plasma as an early diagnostic tool for AD. Further, the present results will provide a motive for feature investigation of the clinical value of plasma telomerase mRNA expression in AD progress and therapeutic efficiency.

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Interest conflict
The authors declare no conflict of interest.

Consent for publications
The authors read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are presented in this published article.

Authors' Contribution
AN designed the study, supervised the experimental works, and reviewed and edited the final version of the manuscript; RKY conducted the laboratory works, writing and editing the manuscript drafts.

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Ethics approval and consent to participate
The study design was approved by the Ethics Committee of Salahalddin University-Erbil for clearance since it involved human participants. The authors ensured that this work was carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All participants obtained informed consent before participating in the present study.

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