Original article

Investigating the Role of *BACE1* and *SORL1* as Exosomal Biomarkers in Dementia among Type II Diabetic Individuals

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ABSTRACT

Background: Type II diabetes is the major cause of several microvascular and macrovascular complications found to alter the neurological balance within the brain and involve in cognitive impairment. *BACE1* and *SORL1* genes are the focus of the study, which may be involved in the cross linkage of Type II diabetes disease and dementia. This study aimed to investigate the expression of these two genes in Type II diabetic patients suffering from dementia and its correlation.

Patients and methods: Blood samples from a total of 47 subjects divided into four groups (diabetes, diabetes with dementia, brain tumors patients) and 12 healthy controls were collected from Lahore General Hospital (LGH). An internationally preferred minimal mental state examination (MMSE) scoring was used (English and Urdu validated translation). The data was analysed by GraphPad Prism 9.0 and one way ANOVA was performed to find variance amongst the samples with respected to expression analysis.

Results: This study included 12 healthy controls and 47 patients (22 males and 25 females) with the mean age±SD (55.9±6.2) during 8 months. *BACE1* expression was significantly elevated in the dementia with diabetes group compared to healthy controls (mean difference: 2.1, 95% CI = 1.0 to 3.2, $p<0.05$). Similarly, *SORL1* expression showed a significant increase in the diabetes group compared to healthy controls (mean difference = 1.8, 95% CI = 0.5 to 3.1, $p<0.05$). However, no significant differences were observed in either *BACE1* or *SORL1* expression between the diabetes and brain tumor groups ($p>0.05$ for both comparisons).

Conclusion: *BACE1* and *SORL1* genes expression levels were significantly higher in diabetic (Type II) patients with dementia and with dementia alone. It can be concluded that *BACE1* and *SORL1* genes expression detected through exosomal analysis can be used as early diagnostic marker in diabetic individuals with dementia.

Keywords:

Neurology, diabetes mellitus, dementia, cognitive impairment, *BACE1*, *SORL1*, expression analysis

INTRODUCTION

Diabetes is the 6th leading cause of death worldwide, is a complex metabolic disorder that presents itself with several microvascular and macrovascular complications.[1] Type II diabetes mellitus (DM), the most common form of diabetes is associated with mild cognitive decrements leading to severe form of dementia which is a group of neurological disorders.[2] The major risk factors involve is the presence of genetic variants mainly *ApoE ε4* allele, *APP, PSEN1/PSEN2*, depression, smoking, hypertension, and diabetes mellitus.[3] Genome wise association studies (GWAS) revealed several candidate genes that are linked directly or indirectly with dementia. Among these genes, *BACE1* and *SORL1* are found as potential genes.[4]

Beta scretase-1 *BACE1* gene, a β-secretase enzyme, constitutively expressed in neuronal cells. It is responsible for cleaving APP and processes it via two pathways i.e. amyloidogenic and non-amyloidogenic pathway. It generates Aβ depending upon the type of processing pathway it adopts.[5] APP cleavage by *BACE1* in amyloidogenic pathway follows sequential proteolytic steps in which *BACE1* performs the first cleavage to generate sAPPβ and C99 which then undergoes a second cleavage by γ-secretase to yield Aβ and APP intracellular domain AICD.[6] In contrast to this, non-amyloidogenic route first undergoes cleavage by α and γ-secretase to yield C83 and P3 fragments respectively. This highlights that *BACE1* is a rate limiting step in producing Aβ and is regarded as an important element in prevention and alleviating dementia like symptoms.[7] Sortilin-related receptor L, *SORL1* gene, an endocytic receptor, is found to encode for a protein.[8] Owing to its multiple roles as an endocytic sorter and retromer based retrograde-trafficking, one of its major roles is trafficking and sorting APP intracellularly via recycling

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pathways. A loss or decrease in protein's expression is evidently reported to be associated with the onset endosomal pathology.[9] Hence, any defect in protein would ultimately lead to functional defects in sorting Aβ in the cells. Exosomes micro-vesicles carry specific cargo within themselves like RNAs (lncRNAs, miRNAs, etc.), proteins, nucleic acids, and other bioactive molecules involved in the pathogenesis of dementia.[10,11] These cell derived vesicles are evidently found to carry Aβ and tau (mediator proteins). *BACE1* which is involved in generating amyloid oligomers, exerts its action on APP within the early endosomal structures. Tau protein also makes it way successfully into the extracellular vesicles.[12] Hence both *BACE1* and *SORL1* are identified as potential risk genes in not only inducing neurodegeneration in individuals but also cognitive impairments in diabetic individuals. This study aimed to analyse the exosomal derived expression of *SORL1* and *BACE1* in diabetic patients with dementia and to correlate the expression of *SORL1* and *BACE1* genes with severity of cognitive impairment.

PATIENTS AND METHODS

A total of about 47 EDTA blood samples of patients with diabetes, diabetes with dementia, brain tumors and 12 healthy controls were collected after written informed consent and according to predefined inclusion and exclusion criteria, from diabetic clinic and Neurology OPD of Lahore General Hospital (LGH). These samples were transported to the labs of Kauser Abdullah Malik (K.A.M) School of Life Sciences, Forman Christian College University (FCCU) for storage until further processing. Inclusion criteria was age (>40 years), both genders, healthy controls, Type I and Type II DM, Patients of diabetes and dementia. Patients with no clinical history and diagnostic tests record or those who refused informed consent or with secondary diabetes, were excluded.

RNA extraction was performed via TRIzol Method (Invitrogen™ Catalog #15596026, USA). In summary, protocol consisted of homogenization (500µl of blood from each sample mixed with 750µl of TRIzol and vortexed until the cells were lysed and homogenised). For phase separation (addition of 200µl chloroform with mixing followed by centrifugation at 4°C for 10 mins at 13,000 rpm and transparent aqueous RNA layer was obtained). For RNA precipitation (500µl of isopropanol was added to the separated aqueous layer and mixed thoroughly followed by centrifugation at 4°C for 15 mins at 14,000 rpm). Finally, RNA pellet formed at the former step was washed with 1000ul of absolute ethanol (100%) via centrifugation and repeated twice. The RNA pellet was air dried for about 8-10 mins and finally dissolved in about 30µl of nuclease free water. Plasma was obtained from blood samples via centrifugation at 20000 rpm for 15 min. Exosomes were isolated from plasma commercial kit following manufacturer protocol (Thermo Scientific, Catalog # 4484450).

RNA was quantified using nanodrop method. The extracted RNA of each sample was analysed for its purity and integrity. About 1.5-2.0µl of sample was picked and placed onto the sampling arm of nanodrop analyser. Considering higher purity, maximum cDNA yield and minimal proteins contaminations, only RNAs giving 260/280 and 260/230 ratio more than 1.5 along with depiction of clear bands on agarose gel electrophoresis were processed for cDNA synthesis. Otherwise, re-precipitated or extracted again. Quality and quantity of RNA was checked by Nanodrop 2000/2000c™ spectrophotometer (Thermo Scientific™).

For cDNA synthesis the reaction mixture consisted of MasterMix per reaction (template RNA (5µl), Oligo dT primer (1µl), nuclease free water (6µl), reaction buffer 5X (4µl), riboLock RNAse inhibitor (20U/µl), 10mM dNTP mix (2µl), and revertaid 200U/µl (1µl)) was prepared. The cDNA was synthesized by using a Thermo Scientific™ kit (RevertAid First Strand cDNA Synthesis Kit: Catalog # K1622, USA).

Brief protocol for gel electrophoresis for RNA extracted samples comprised of gel preparation. A 1.5% agaros gel was prepared by mixing 1.5g of agarose in 100 ml of 1X TAE buffer with gentle mixing under heat. After setting up castor and combs 1X TAE was added into the gel tank. A 7.0µl of ethidium bromide was added into the gel and mixed to distribute it evenly. The gel was poured into the castor plate and set aside for at least 20 minutes to solidify. After solidification of the gel the comb were removed carefully without distorting the wells. The gel was placed inside the gel tank. The equalized samples were loaded into the wells. After loading all the samples, 1Kb ladder was loaded as a reference marker to determine the location of the desired bands. The gel apparatus conditions were set on 70 volts for about 40 minutes. Finally, the results were analysed on a Gel Documentation System™).

For real time PCR reaction, a master mix per reaction included Taq plus (7.5µl), nuclease free water (4.5µl), template cDNA (1µl), forward primer (1µl), and reverse primer (1µl) (ThermoFisher Scientific™ SYBR

Table 1: Sequence of forward and reverse primers used in this study

BACE 1	**Forward**	5' F-ACATCCTGGTGGATACAGG3'
	Reverse	5' R-CAGGATGCCTTCCCAGTT 3'
SORL1	**Forward**	5' F-GAGATAGCCTGGCATTGG 3'
	Reverse	5' F-GCCTTGAAGAGTGTTGCA 3'

Green qPCR Master Mix: Catalog # 4309155, USA). The reaction mixture tubes were placed in thermocycler machine and the amplification was done. After reaction termination, the PCR tubes were put in ice for immediate processing or stored at -20°C for later analysis. RT-qPCR was performed by using Bio-Rad's CFX 96™ q-PCR thermocycler. It was carried out to examine the expression levels of mRNA of S*ORL1* and *BACE1* genes.

The data was entered and analysed in GraphPad Prism 9.0. Demographic data was plotted through bar charts and pie graphs. Frequencies of relative morbid conditions were plotted as bar charts and expression analysis was performed. One way ANOVA was done to find variance amongst the samples. A p-value less than 0.05 was considered as significant.

RESULTS

Total 47 blood samples of patients enrolled in the study were collected (22 were males and 25 were females) including 12 healthy controls. Medical history of enrolled patients was recorded. Out of 47, 14 (30%) patients had dementia, 17 (40%) had diabetes, 5 (10%) had brain tumours and 11 (20%) were of diabetes with dementia. The demographic and clinical features of patients in four groups are given in Table 2.

The mental status of dementia and diabetics with dementia patients was evaluated using MMSE questionnaire. The examination conducted via this questionnaire provided an idea regarding the severity of the disease within the study groups. To assess the mental state of hospitalised patients, an internationally preferred method called minimal mental state examination (MMSE) scoring was used. The individuals were categorized under severe (0-17 score), mild (18-23 score), less/no cognitive impairment (24 or higher score). Scoring was done according to the scoring criteria pre-established within the questionnaire by the medical professionals who developed the test. Figure 1 shows the scoring interpretation on basis of severity.

Expression of *SORL1* (in diabetic patients) and *BACE1* (in dementia patients) gene was analysed via conventional PCR and gel electrophoresis. *SORL1* gene expression when analysed in all diabetic patients sample via densitometry, it showed an increase in expression in comparison to housekeeping gene i.e. β-Actin. Out of 47, 12 (26%) participants displayed a relatively much higher expression of *BACE1*. On consulting their medical history, those patients were found to be obese with poor compliance to glycaemic control with increased HbA1c levels which could be a plausible reason to such high expression (Figure 2).

In the randomly selected samples (six in the above case), it can be seen that *BACE1* showed a higher expression among all the samples in comparison to *SORL1.* This indicated an inverse action relationship between the two genes among dementia individuals i.e. a reduction in *SORL1* expression leads to an increase in *BACE1* expression level hence, causing cognitive alterations (Figure 3). *BACE1* and *SORL1* expression in diabetic with dementia cohort and brain tumor patients is shown in Figure 4. The data showed an overall high expression of *BACE1* in both patient groups with dementia alone and diabetics with dementia. The levels of BACE 1 changes with increase cognitive impairment whether in dementia alone or diabetic patients suffering from dementia. The relative fold change of *BACE1* in dementia and diabetics with dementia via RT-qPCR is depicted in Figure 5.

SORL1 indicates an overall relative fold decrease in dementia patients whereas, it indicates an inverse case in diabetes cohort where it shows a fold increase. The gene shows a downregulated expression in diabetes with dementia patients and a relative fold increase was observed in brain tumor cohort as shown in Figure 6.

Table 2: Summary the demographical and clinical data of the enrolled subjects

Groups	Frequency (%)	Gender	Age (Mean+SD)	FBS (Mean±SD)	Mean HbA1c (%)	Duration of Diabetes (Mean±SD)	MMSE scores (Mean±SD)
Dementia	30	Male	61±3.3	100± 8	5.8	NA	20±2
		Female	63±3.7	103± 4	5.5	NA	19±1
Diabetes	40	Male	45.5±9.5	115± 7	7.2	7±3	16±3
		Female	51.5±6.5	113± 4	6.9	5±2	18±1
Diabetes with dementia	20	Male	65±5.6	130± 8	7.9	25±5	24±2
		Female	70±3.6	125± 9	7.0	20±4	23±3
Brain tumor	10	Male	65.5±5	95± 8	5.5	5±2	18±2
		Female	55.5±2.1	100± 5	5.4	5±1	19±2

Abbreviations: FBS = Fasting Blood Glucose; **MMSE** = Mini Mental Status Examination

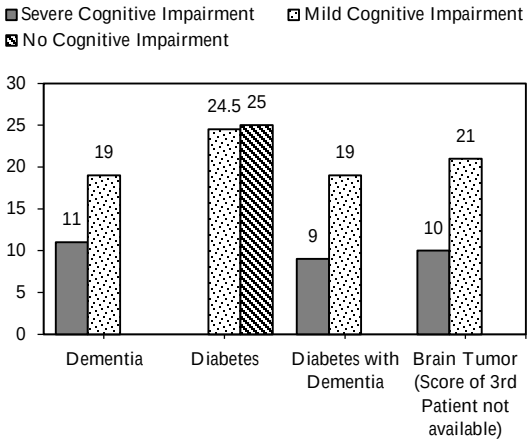

Figure 1: The level and severity of cognitive impairment among enrolled study groups on basis of MMSE scores

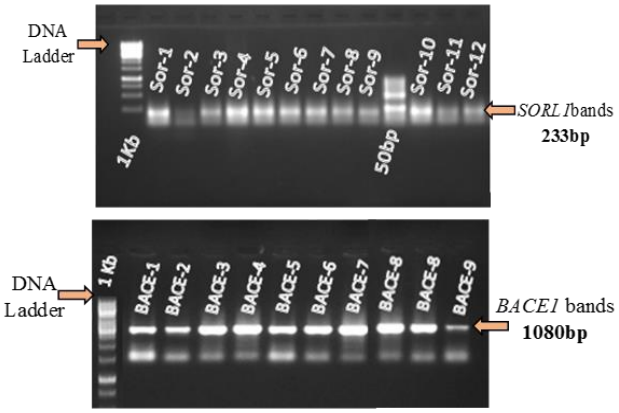

Figure 2: The Gel picture indicates expression of ***SORL1*** **gene in diabetic patients** and ***BACE1*** gene in dementia patients.

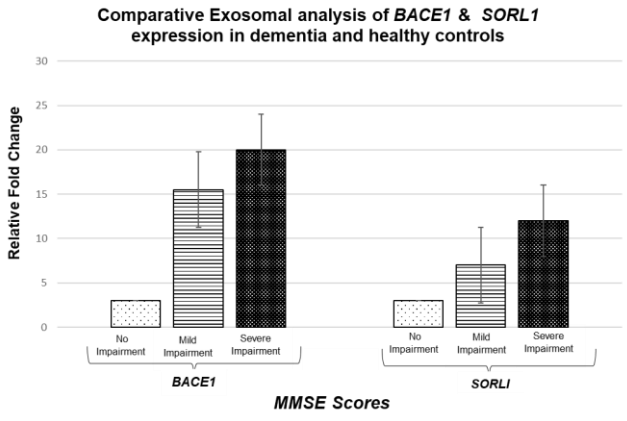

Figure 3: Comparative exosomal analysis of ***BACE1*** **and** ***SORL1*** expression in dementia and healthy subjects.

DISCUSSION

Dysfunctional expression of *BACE1* and *SORL1* genes results in accelerating hyperglycemia and cognitive impairments. In previous studies, diabetic individuals with poor compliance to body glucose showed an increased expression of these genes i.e. *SORL1* could be a plausible reason to such poor compliance to body sugar.[13] Barthelson with a group of researchers suggested that the expression of *SORL1* results in an accumulation of AβPP in the Golgi and prevents it from being sorted to late endosomal compartments where Aβ is produced.[14] Conversely, its overexpression results in accumulation and increased Aβ levels in comparison to controls as indicated by a study conducted by Fronza MG and co-scientists.[15]

With support from another study on pathogenesis of dementia, it has been proven via comparative analysis of *SORL1* with *BACE1* gene that *SORL1* showed a reduced expression in overall cases as compared to *BACE1* that showed an overall high expression in most of the cases as shown in our results.[16] Findings of this study were similar to a research when a group of researchers observed a decreased expression of the protein in mice lead to the generation of amyloid beta metabolism.[17] In addition to this, a group of researchers under also hypothesize that *SORL1* gene has a neuroprotective role.[18] Binkle L and coworkers also found that the diminished expression of S*ORL1* protein can contribute to dementia like pathology. Higher activity of the *BACE1* gene contributes to amyloidogenesis i.e. formation of amyloid protein. In extreme cases, this process generates amyloid plaques within the brain of individuals hence contributing to dementia.[19]

This study identified higher expression of the *BACE1* mRNA within the subjects diagnosed with dementia. A study determined the relative fold change when calculated via real time PCR gave a *BACE1* gene fold increase of about 1.28 in dementia cases which indicated overexpression of the gene as supported by this study.[20] Furthermore it has been proven that 1.2-fold *BACE1* increase in diabetics with dementia also indicated a higher expression of the respective gene. These results were in assimilation with the findings of Zuliani G and coworkers when they reported an increase in activity of BACE1 in AD subjects in comparison to non- AD subjects.[21] One plausible reason to increased BACE1 activity in diabetics with dementia and dementia patients is due to lower levels of Vitamin D.[22] Vitamin D is found to have protective effects on

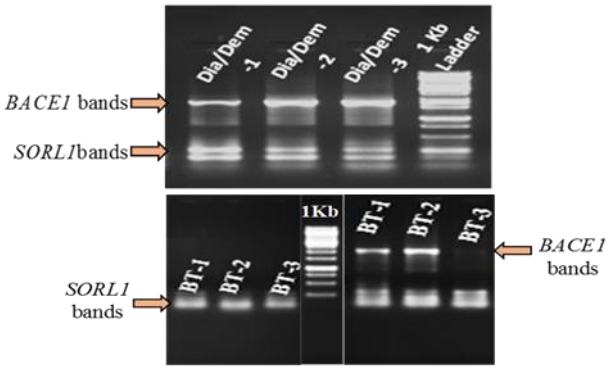

Figure: 4: *BACE1* and *SORL1*expression in diabetics with dementia cohort where, Dia/Dem-1, 2 & 3 indicates diabetics with dementia patients (left). Expression of *BACE1* and *SORL1* in brain tumor patients where, BT-1, BT-2 & BT-3 denotes brain tumor patients (right).

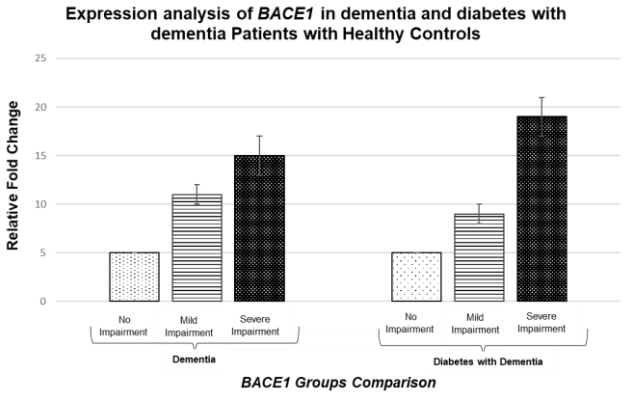

Figure 5: The above figure gives a fold change in dementia (1-9) and diabetics with dementia cases (10-12) calculated by2^-(ΔΔCq).

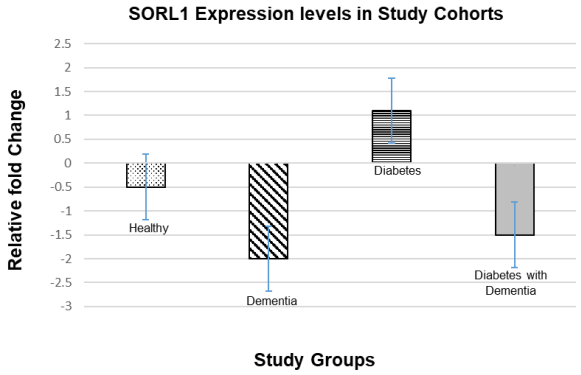

Figure 6: The above figure gives a relative fold change of *SORL1* in all study cohorts.

brain neuronal cells due to its chemical properties. However, a reduction in Vitamin D could lead to serious cognitive irregularities. These findings are similar to findings of current study where some patients with dementia showed a medical history of reduced vitamin D levels when tests were conducted. Hence these lower levels might have induced higher activity of *BACE1*.[23]

However, the isolation of RNA from the vesicles did not yield desirable results. The pellet obtained via isolation of vesicles resulted in precipitation of a lot of impurities and other cell debris which made it harder to isolate the required mRNA of genes. The obtained RNA were of very low quality and hence resulted in low or no cDNA synthesis. Studies conducted previously indicate and report extensive and laborious methodologies to purify RNA from exosomes which could be achieved in our setting due to time constraints and limited resources.

CONCLUSION

This study concluded that the exosomal derived expression of *SORL1* & *BACE1* genes in Type II diabetic patients with dementia plays a significant role in cognitive impairment. Moreover, our study highlights significant alterations in BACE1 and SORL1 expression levels across different patient groups, shedding light on their potential roles in various neurodegenerative and metabolic conditions. Specifically, BACE1 expression was notably elevated in individuals with dementia and diabetes, suggesting a possible synergistic effect of these comorbidities on amyloidogenic pathways implicated in dementia pathogenesis. Similarly, the increased expression of SORL1 in diabetic patients underscores its involvement in metabolic dysregulation and its potential contribution to dementia risk in this population. However, the absence of significant differences in BACE1 and SORL1 expression between diabetic and brain tumor groups suggests distinct underlying mechanisms governing these conditions. Overall, our findings provide valuable insights into the complex interplay between neurodegenerative processes, metabolic disorders, and tumorigenesis, emphasizing the need for further research to elucidate the molecular mechanisms underlying these associations and identify potential therapeutic targets.

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