Archives of Razi Institute, Vol. 77, No. 6 (2022) 2379-2384

DOI: 10.22092/ARI.2022.358615.2265

Original Article

Overexpressed Toll-Like Receptor 2 and the Influence on the Severity of Acute Ischemic Stroke

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Received 7 May 2022; Accepted 8 June 2022
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Abstract

Brain insult caused by cerebral ischemia/reperfusion is a complex pathophysiological process in which inflammatory reaction is thought to play a crucial part. Toll-like receptors are a sort of transmembrane proteins that can initiate inflammatory reactions upon identifying either endogenous stress or changes in the innate immune system's molecular patterns that are damage-associated or even exogenous pathogen-associated molecular patterns. TLR2, amongst all other Toll-like receptors, is recognized to have a more vital role in the pathologic progress of brain insult attributed to ischemia/reperfusion. The current study aimed to address the impact of TLR2 overexpression on the severity of acute ischemic stroke. A case-control study was piloted on two groups of participants; the first group (cases) was 95 patients suffering from acute ischemic stroke, while the second one (controls) was 50 volunteers free of disease. Matching controls to patients was done according to age (within five years) and gender. The study extended from the beginning of February to the end of August 2020. Participants in both groups were collected from Merjan Medical city in Babylon Governorate. Values of Toll-like receptor 5 were calculated with the real-time polymerase chain reaction (RT-PCR) technique. Statistically, a significant difference was found between the patients and controls concerning the values of TLR2 (*P*-value=0.008). Other measured parameters, which are age, gender, and history of hypertension and diabetes, showed no significant differences between the study groups. Overexpressed TLR2possesses a positive impact on increasing the severity of acute ischemic stroke.

Keywords: Acute Ischemic Stroke, TLR2, PCR

1. Introduction

Acute ischemic stroke can be defined as an impairment of neurologic function resulting from a sudden loss of blood supply to a particular area within the brain. The underlying cause is either a thrombus or an embolus that ends in a cerebral vessel occlusion that supplies peculiar territory of the brain. During the process of vaso-occlusion, there is an aspect in which the core tissue damage is not reversible, and another aspect of the so-called "penumbra" is in which the tissue has impaired function due to diminished blood supply yet is still reversibly damaged (1). Globally, one-sixth of the population could have a cerebrovascular accident (CVA) each year; over 13.7 million have CVA, and mortality of around 5.8 million per year as a complication of stroke. Worldwide, patients who survived a CVA are over 80 million. Approximately seventy percent (9.5 million) of new CVA are ischemic in origin, while the remaining are subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ISH); the percentage of ischemic CVA in North America is found to be more, at approximately 86% (2). Toll-like receptors (TLRs) are part of innate immunity through bacterial endotoxin-induced

inflammatory outcomes but are also associated with the pathogenic processes occurring in atherosclerosis and MI (3).

Many pieces of evidence suggest that TLRs are critical factors in ischemic injuries, as they have been shown to identify endogenous proteins released from damaged tissue cells (4). The underlying mechanism in which brain insult is caused by brain ischemia/reperfusion remains unclear, yet the inflammatory process has been shown to play a major role in tissue damage (5). Recent studies-based evidence emphasized that activation of TLRs is mediated by the release of endogenous proteins from destructed tissue of the brain and participates vitally in the mediation of brain insult following ischemia/ reperfusion sequence. Specifically, TLR2 has been shown to play a more vital role than other TLRs in cerebral ischemia/reperfusion progression. Thus, many new studies have targeted TLRs as a promising novel strategy for treating ischemic strokes (6). Various cells of the innate immune system are shown to contain Toll-like receptors, such as monocyte/macrophage, dendritic cells, natural killer cells, and polymorph nuclear neutrophils, which stimulate an immediate pathogen-directed response (7). Inside the brain, their main location is on the astrocytes, glial cells including microglia, and Oligodendrocytes. A large reserve of TLRs is expressed on astrocytes and microglial cells, and considerable production of pro-inflammatory cytokines occurs upon TLR's attachment to their matching ligands.

On the contrary, a minor reservoir of TLRs (such as) can be expressed by Oligodendrocytes. Owning to the theory that TLR2 has a major role in repairing the central nervous system, increasing numbers of researchers have focused on studying TLRs expression in neurons (8). Despite the fact that in normal circumstances, the central nervous system(CNS) is completely aseptic with no microbial contamination, it is shown that overexpression of TLR2, TLR9, and TLR4in the nerve cells during under perfusion insult (9). On the contrary, new studies have found increasing evidence to support the vital role of TLR2 in inflammatory response modulation through its linkage to corresponding ligands (e.g., heat-shock proteins, high mobility group box 1, hyaluronic acid, fibronectin) (10).

The current study aimed to address the impact of TLR2 overexpression on the severity of acute ischemic stroke.

2. Materials and Methods

A case-control study was piloted on two groups of participants; the first group (cases) was 95 patients suffering from acute ischemic, while the second one (controls) was 50 volunteers free of disease. Matching controls to patients was done according to age (within five years) and gender. The study extended from the beginning of February to the end of August 2020. Participants in both groups were collected from Merjan Medical city in Babylon Governorate. The cases were selected according to the decision of their attendant physician in addition to the evidence of acute infarction determined by computerized cranial tomography (CT) within the first 24 hours of the disease appearance. Patients with subarachnoid hemorrhage, hemorrhagic stroke, or initial ischemic stroke attack were excluded. All the cases were incidents, and some had a prior stroke attack. All the controls agreed to participate in the study, and there were no non-respondents. An explicit and valid written and verbal informed consent from each participant in the study was achieved before their inclusion. Short history notes were obtained from each participant in the study, including the name, age, gender, and clinical history of hypertension and diabetes.

2.1. Blood Collection and Serum Preparation

Two milliliters of fresh blood were drawn from both groups of participants. Labeled EDTA tubes were used for molecular studies. Each sample was labeled and given a serial number and the participant's name. For subsequent molecular and biochemical analyses, blood samples were frozen at -20°C.

2.2. Real-Time Quantitative Polymerase Chain Reaction (RT-PCR or qPCR)

Total RNA extraction was done from frozen EDTA blood using the (TRIzol®) reagent kit, and the

procedure was established according to the company (Bioneer, Korea) guidelines. DNase I enzyme was used to treat extracted total RNA to remove the small amounts of genomic DNA present using the DNase I enzyme kit. The procedure was done according to the descriptions of Promega Company, USA. DNase-I treated samples were used to synthesize cDNA using the MMLV reverse transcriptase kit and done according to the company (Bioneer, Korea) instructions. The qPCR was used for quantification of a gene of interest (TLR2) expression that was neutralized by the housekeeping gene (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in the blood samples of patients and controlled by the use of real-time PCR technique and this method was carried out according to the instructions of Promega Company, USA (Table 1). The results of qPCR were processed by the use of relative quantification of gene expression levels (fold change) (the DCT process using a reference gene) that was described by Livak (11).

Table 1. Primers for target gene (TLR2) and housekeeping gene (GAPDH) (12)

Primer	Sequence	Amplicon
TLR2	F TCTGGAGACGACTCAGAAAAGC R TGTTGCTTCCTGCCAATTGC	92 bp
GAPDH	F AATTCCATGGCACCGTCAAG R ATCGCCCCACTTGATTTTGG	104 bp

2.3. Statistical Analysis

Continuous variables were expressed as mean±standard. Deviation (SD), while the other categorical variables were expressed as percentages (no. (%)). The two sets of participants were compared using an independent-samples t-test for the numeric parameters and a chi-square test for the string parameters. All analyses were done using a statistical package of social science (SPSS) version 18.0 software (SPSS Inc., Chicago, IL, USA). An ap-value of less than 0.05 was addressed to be significant statistically (13).

3. Results

Table 2 demonstrates the study's age distribution for patients and control groups. The ages of patients ranged from 41-83 years, and the range for controls was 40-81 years. No significant statistical difference was present between the two participants regarding age (*P*-value=0.213).

Table 2. Age distribution for patients and control groups

Groups / Age	Patients (n=95)	Controls (n=50)	*P*-value
Age (Years) Mean±SD	63.35±8.227	62.33±8.231	0.213

Table 3 shows the gender distribution for the study groups. Forty-five males and 50 females were present among the patients, while the controls contained 23 males and 27 females. No significant statistical difference was present between the study subjects regarding gender distribution (*P*-value=0.657).

Table 3. Gender distribution for patients and control groups

Groups / Gender	Patients (n=95)	Controls (n=50)	Total No. (%)	*P*-value
Males No. (%)	45 (47)	23 (46)	68 (46)	
Females No. (%)	50 (53)	27 (54)	77 (54)	0.657
Total No. (%)	95 (65)	50 (35)	145 (100)	

The distributions of clinical history parameters (hypertension (HT) and diabetes (DM)) are shown in tables 4 and 5. There were no significant statistical differences between the two sets of the study (*P*-values are: 0.673 for HT and 0.552 for DM).

Table 6 demonstrates the value of fold change of target gene (TLR2) and housekeeping gene (GAPDH), which exhibit that TLR2 was highly expressed in patients compared to the controls (*P*-value=0.008).

Table 4. Distribution of hypertensive history for patients and control groups

Groups / HT	Patients (n=95)	Controls (n=50)	Total No. (%)	*P*-value
Present No. (%)	63 (66)	34 (68)	97 (66)	
Absent No. (%)	32 (33)	16 (32)	48 (33)	0.673
Total No. (%)	95 (65)	50 (35)	145 (100)	

Table 5. Distribution of diabetic history for patients and control groups

Groups / DM	Patients (n=95)	Controls (n=50)	Total No. (%)	*P*-value
Present No. (%)	52 (54)	30 (60)	82 (56)	
Absent No. (%)	43 (46)	20 (40)	63 (44)	0.552
Total No. (%)	95 (65)	50 (53)	145 (100)	

Table 6. Values of the fold change of target gene (TLR2) and housekeeping gene (GAPDH) between patients and control groups

Groups / Fold change	Patients (n=95)	Controls (n=50)	*P*-value
Fold change (ng/µl) Mean±SD	58.24±7.772	57.11±6.811	0.008

4. Discussion

As was represented in the results, age distribution exhibited no difference among the study subjects; their mean ages were close, and both were lying in the sixth decade of life, reflecting that the participants in both groups were old. In addition, control selection was made by matching them with the cases by age and gender, so reasonably, no significant difference is expected to be shown between them.

Cerebrovascular disease is the leading cause of death in the US and is strongly associated with age. Over the coming decades, a remarkable expansion in elderly people 65 years and older is anticipated. This growth will result in a noticeable increment in cerebral vascular disorder occurrence, death rate, and a financial burden (14). The distribution of males and females in the current research showed no obvious significant difference between the controls and stroke patient groups. This may be attributed to the fact that in this study, there is no gender-specific affection by AIS and that these conditions affect both males and females almost evenly, which is probably linked to the difference in risk factors effects among men and women between different societies. Added to the effect of matching mentioned in the former section. In order to understand health-associated gender dissimilarity, including the more prevalent acute stroke in males than in females, sex-related parameters in the United States, for example, need to be considered. The cause could partly be attributed to their sex-based tendency to engage in unhealthy lifestyles such as cigarette smoking and alcoholism (15). The impact of traditional risk of atherosclerosis factors such as age, sex, hypertension, and diabetes on the risk of AIS has been established. Moreover, research in epidemiology has shown that diabetic patients are at increased risk for AIS. Eighty percent of all diabetic mortality rates are attributed to atherosclerosis (16). Values of fold change between target and housekeeping genes showed formerly revealed an extremely significant difference between patients and controls regarding them, reflecting that TLR2 plays a significant role and acts as a predisposing risk factor in the occurrence and severity of acute ischemic stroke. Its conventionally believed that the nervous system and immune system as 2 unrelated existences, and any interactive process between them is considered a systemic dysfunction. Nevertheless, in the last decades, clues about interconnections between these systems, especially in nervous tissue insult, have been obtained. While nervous tissue is affected by any injury, many kinds of cells and remarkable release and activation of pro-inflammatory cytokines, no matter where the initial insult is. Native cells, especially the astrocytes and glial cells (microglial cells), mainly regulates the instant nervous tissue injury response; then, there will be recruiting of non-Native cells, namely the macrophages. Blood-brain barrier (BBB) integrity is breached during nervous tissue under perfusion, which

leads to abnormal and often exaggerated interaction between the nervous and the immune system (17). There has been a significant expansion in studies in the last years involving the innate immune system's role in the pathophysiology of nervous tissue ischemic insult. When under perfusion ensue in the setting of acute ischemic CVA, this ends in an acute response to glycocytopenia and underoxygenation that leads to ionic imbalance, calcium derangement, metabolic acidosis, and cytotoxicity (18). The next sequence of reactions will disrupt BBB integrity, leading to peripheral white blood cells and microglial cell recruitment and activation. injury to cell components leads to the expression of damage-associated molecular patterns(DAMPS) from different origins, including from the nucleus and other intra- and extracellular origins, and initiates a pro-inflammatory reaction by activating the Toll-like receptor 2 (19). TLR2 is remarkably over-expressed in the cerebral cortices in response to under perfusion-reperfusion insult, and Toll-like receptor 2-knockout mice showed remarkably less nervous tissue ischemic disruption compared to wild-type mice, thus obviously suggesting a negative role of TLR2 over activation in ischemic stroke (20). Bohacek, Cordeau (21) found that Toll-like receptor 2 underexpression remarkably affected the progression of ischemia size. Specifically, they show that in mice that had Toll-like receptor 2-knockout, they have decreased size ischemia earlier in the course of illness while they notice the larger size of ischemia in comparison to wild type during the following disease course, which concludes that low level of Toll-like receptor 2 regress ischemic insult in the nervous tissue (21). Researchers studying TLR4-deficient mice also found less disruption compared to healthy mice after perfusion-reperfusion or longstanding obstruction of the middle cerebral artery (22). All in all, these researches concluded that Toll-like receptors 2 and 4 had a vital role under perfusion-reperfusion insult and that their overexpression results in worsening nerve injury. Unlike Toll-like receptors 2 and 4, Toll-like receptors 3 and 9 had no participation in ischemic brain insult (23). Finally, a Toll-like receptor act as a surveillance system that contributes to identifying and getting rid of pathogenic factors. Besides, clues from experimental trials found that a Toll-like receptor had a major role in starting the sequence of events leading to ischemic pathology. Especially important preconditioning leads to modulating Toll-like receptor activation, which is considered a neuron protecting factor (24).

Toll-like receptor 2 is highly expressed in the circulating monocytes of patients having an acute attack of ischemic stroke, and this process could have a pathogenic role, also serving as an added indicator of ischemic dysfunction of the brain tissue.

Authors' Contribution

Study concept and design: A. J. A.
Acquisition of data: H. M. S. A. A.
Analysis and interpretation of data: Y. A.
Drafting of the manuscript: H. M. S. A. A.
Critical revision of the manuscript for important intellectual content: A. J. A.
Statistical analysis: Y. A.
Administrative, technical, and material support: A. J. A., H. M. S. A. A. and Y. A.

Ethics

Approval for the research study was obtained from the University of Babylon, Hilla, Iraq ethics board. Informed consent was obtained from all participants.

Conflict of Interest

The authors declare that they have no conflict of interest.

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