The levels of 7,8-dihydrodeoxyguanosine (8-oxoG) and 8-oxoguanine DNA glycosylase 1 (OGG1) – A potential diagnostic biomarkers of Alzheimer's disease☆

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ABSTRACT

Evidence indicates that oxidative stress contributes to neuronal cell death in Alzheimer's disease (AD). Increased oxidative DNA damage l, as measured with 8-oxoguanine (8-oxoG), and reduced capacity of proteins responsible for removing of DNA damage, including 8-oxoguanine DNA glycosylase 1 (OGG1), were detected in brains of AD patients. In the present study we assessed peripheral blood biomarkers of oxidative DNA damage, i.e. 8- oxoG and OGG1, in AD diagnosis, by comparing their levels between the patients and the controls. Our study was performed on DNA and serum isolated from peripheral blood taken from 100 AD patients and 110 controls. For 8-oxoG ELISA was employed. The OGG1 level was determined using ELISA and Western blot technique. Levels of 8-oxoG were significantly higher in DNA of AD patients. Both ELISA and Western blot showed decreased levels of OGG1 in serum of AD patients. Our results show that oxidative DNA damage biomarkers detected in peripheral tissue could reflect the changes occurring in the brain of patients with AD. These results also suggest that peripheral blood samples may be useful to measure oxidative stress biomarkers in AD.

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1. Introduction

According to the WHO Report "Dementia: a public health priority" it is estimated that 35.6 million people suffer from dementia worldwide. Alzheimer disease (AD) is the most widespread cause of dementia in Western societies, especially in people aged 65 years or more [1]. The main feature of AD is a progressive decline in cognitive function, including memory, thinking, language and learning capacity. The diagnosis of AD is difficult due to a lack of specific markers. Currently, AD diagnosis involves a clinical assessment tools for checking signs of cognitive impairments with combination of brain imaging. However, definitive diagnosis can only be based on examining of patients brain tissue. The identification of effective biomarkers for evaluation of risk, diagnosis and monitoring progression of AD has currently been undertaken. Searching for tangible biomarkers should be associated with mechanisms that are involved in the development of AD.

Among numerous hypotheses explaining the pathogenesis for AD, the leading one is the neurodegeneration caused by the oxidative stress [2–7]. Oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) and the efficiency of ROS detoxification. There are many potential sources of ROS which, when overproduced, may contribute to the development of AD. Excessive ROS may be generated by mitochondrial dysfunctions and/or aberrant accumulation of transition metals. Moreover, important source of ROS are amyloid beta, an oligopeptide which is increased in AD [8–10]. Neurons are thought to be prone to oxidative damage because they contain several hundred mitochondria due to their large energy demand. Moreover, neurons are postmitotic cells with limited capacity to regenerate in the adult central nervous system [3]. ROS are highly reactive and can immediately oxidize macromolecules in living cells, including lipids,

proteins, and nucleic acids, leading to various oxidative cellular damages, cell death or mutagenesis.

Numerous studies show increased lipid peroxidation, oxidation of proteins and oxidative DNA damage in brain of patients with AD [11–13]. Moreover, markers of oxidative damage to cellular macromolecules were detected in peripheral blood samples of AD patients [14,15].

Damage to nucleic acid is particularly hazardous for cells because highly altered genetic information present in genomic DNAs often causes cell death. ROS cause various base or sugar backbone modifications in DNA. One of them is 7,8-dihydrodeoxyguanosine (8-oxoG), an oxidized form of guanine, defined as a marker of oxidative stress. The most important DNA repair pathway dealing with oxidative DNA damage is the base excision repair (BER) system. One of BER enzymes responsible for 8-oxoG removing is 8-oxoguanine DNA glycosylase 1 (OGG1), a bifunctional enzyme with DNA glycosylase and apurinic/apyrimidinic (AP) lyase activities [16]. *OGG1* gene is located on the short arm of chromosome 3. This protein has two major isoforms resulting from alternative splicing of the C-terminal region: OGG1-1 located in nucleus and OGG1-2 present in mitochondria. Among various tissues, the expression of OGG1 gene was found to be the highest in the brain [16].

In the present study we aimed to evaluate the level of peripheral markers of oxidative stress, DNA level of 8-oxoG and serum OGG1, in AD patients in comparison to healthy volunteers.

2. Material and methods

2.1. Serum samples and protein preparation

In our study we involved 100 AD patients (44 man and 56 women, mean age 79.2 ± 4.9 years) and 110 healthy volunteers (46 man and 64 women, mean age 77.1 ± 7.3 years). Both AD patients and healthy volunteers (the control) came from Babinski Memorial Hospital. The study group consisted of persons diagnosed with Alzheimer's disease according to DSM-IV criteria. Exclusion criteria for controls were neurodegenerative disorders or a family history of AD. Due to possible false positive results in comet assays, we also excluded patients with dysfunction of kidney, liver and known inflammatory disorders which may increase the level of oxidative DNA damage. The marker of inflammation were elevated levels of C-reactive protein (CRP) and other acute phase proteins such as α2-macroglobulin, haptoglobin C3 and C4 in a clinical tests. Furthermore, in the control group we excluded patients with psychiatric diagnoses, axis I and II disorders (diagnosis based on Structured Clinical Interview for DSM-IV Axis I and II Disorders) and neurological illness. The study was approved by Committee of the Medical University of Lodz (No. RNN/70/14/KE). All AD and control patients gave their informed consent prior to enrollment in the study.

Venous blood samples were collected into 9 mL tubes containing EDTA. The serum was separated by the centrifugation at 5000 ×*g* for 5 min and stored at −20 °C until the protein preparation. For purification of serum from albumin, ProteoPrep Blue Albumin and IgG Depletion kit (Sigma Aldrich, Germany) were used. Specifically, 30 μL of serum sample were taken and purification was performed according to the manufacturer's instructions. To determine the protein concentration the Bradford protein assay was employed. Purified protein samples of known concentration were used to conduct the Western blotting and ELISA assays.

2.2. Detection of 8-oxoG

Genomic DNA was isolated from peripheral blood samples using Genomic Maxi AX Direct kit (AA Biotechnology, Poland). During the isolation, diethylenetriamine pentaacetic acid (0.1 mM) and ascorbic acid (2 mM) were used to prevent background oxidative DNA damage. For DNA strand digestion we used the nuclease P1 (NP1) and alkaline phosphatase (AP) enzymes [17]. Preparation of enzymes solution was as

follow: NP1 from Penicillium citrinum (1 mg 1000 units of 3′-phospho-monoesterase activity) was dissolved in 100 μL 20 mM sodium acetate buffer (pH 5.2). NP1 was further diluted 10 times to a final concentration of 1 U/μL in the acetate buffer. AP from calf intestine (1 U/μL) was stored in 25 mM Tris HCl (pH 7.6), 1 mM MgCl₂, and 50% glycerol (w/v). Digestion was carried out in the following way: 20 μL 5 μg/μL of DNA sample were diluted to 100 μL with high purity water. After acidification with 1 μL 3 M acetate buffer (pH 5.2), DNA was first digested with 1 μL 1 U/μL NP1. After 1.5 h incubation, pH of the reaction mixture was adjusted to pH 7.4 by adding 10 μL 1 M Tris HCl buffer (pH 8), followed by 1 μL 1 U/μL AP for an additional 1 h. Digested DNA samples were used to determine the 8-oxoG level with Oxiselect oxidative DNA damage ELISA kit (Cell Biolabs, USA).

2.3. Western-blot and ELISA analysis of OGG1

For Western blot and ELISA we used protein fraction isolated from blood serum. We analyzed the OGG1 level with two methods: using enzyme-linked immunosorbent assay (ELISA) and immune blotting (Western blot, WB).

Western blot determination of OGG1 level was performed in 12% polyacrylamide gel. Equal amounts of protein (50 μg protein/lane, both protein sample and GAPDH as reference protein) were loaded into the wells. Electrophoresis was conducted in Tris/Glycine/SDS buffer (25 mM Tris, 190 mM glycine, 0.1% SDS, pH 8.3) for 1 h at 120 V in Mini-PROTEAN Tetra Cell apparatus (Bio-Rad, France). Gel-separated proteins were transferred to a Immobilon PVDF membrane (Millipore, USA). Nonspecific binding was blocked by 5% non-fat dry milk for 2 h at room temperature. To estimate the levels of the OGG1 protein, the membranes were incubated overnight with anti-OGG1/2 rabbit polyclonal antibody (ThermoFisher Scientific), and, in the case of GAPDH protein, anti-GAPDH rabbit polyclonal antibody (Santa Cruz Biotechnologies), diluted 1:1000. Next, nitrocellulose sheets were incubated with the second antibody – anti-rabbit conjugated with HRP (horseradish peroxidase) (Cell Signaling Technology, USA) at a dilution of 1:1000. For chemiluminescent reaction we incubated nitrocellulose sheets for 2 min in stable peroxide solution and an enhanced luminol solution in 1:1 proportion (Thermo Fisher Scientific, USA). To stain immunoreactive bands, peroxidase BMB was added (BM blue POD substrate precipitation; Roche, Germany). The surface area of the immunoreactive bands was measured and calculate in ImageJ software (Wayne Rasband, USA).

OGG1 level was also evaluated with enzyme linked immunosorbent assay. The samples were centrifuged at 1000 ×*g* for 20 min, then was performed an immunosorbent assay with ELISA kit for Oxoguanine Glycosylase 1 (Wuhan USCN Business Co., China) according to the manufacturer instruction.

2.4. Statistical analysis

The levels of 8-oxoG and OGG1 were expressed as mean ± SEM. Blinded replicate samples were used for quality control (QC).·The obtained results of experiments were compared using the non-parametric Mann–Whitney's or t-student test for unlinked variables. Test selection was made after verifying normality of studied populations distribution. Form normality examination was used Shapiro–Wilk test, then if the distribution of the population was consistent with normal distribution we conducted paired *t*-test, otherwise Mann–Whitney's test was used. In addition, an area under the receiver operating characteristic (ROC) curve (AUC) analysis was performed to determine the overall accuracy of 8-oxoG and OGG1 as a marker of AD. AUC values was calculated with their 95% confidence intervals. Moreover, we performed a chi-square test to analyze the relationship between obtained results for 8-oxoG and OGG1 and age of patients. Statistical power of samples size was 91% at 5% confidence level.

3. Results

3.1. 8-oxoG level

The level of 8-oxoG detected in peripheral blood samples was significantly higher in Alzheimer's disease patients in comparison to control group (20.5 vs. 9.1 pg/mL; $p < 0.0001$) The results are presented on Fig. 1.

3.2. OGG1 level

Both Western blot and ELISA were used to determine the level of OGG1. We observed that OGG1 protein level measured in the serum was significantly lower in AD patients than in control group. ELISA test (Fig. 2) revealed that the mean concentration of OGG1 was 9.61 ng/mL in AD patients and 14.16 ng/mL in the controls ($p < 0.0001$). The OGG1 level detected by Western blot was expressed as ratio OGG1/GAPDH and showed that AD patients had 14.9 while the controls had 21.4 ($p < 0.05$). The Western blot analysis of OGG1 is shown in Fig. 3. The AUC of 8-oxoG was 0.946 ($p < 0.001$, 0.916–0.977 95% CI) while OGG1 was 0.055 ($p < 0.001$, 0.030–0.80 95% CI). ROC curve is presented in Fig. 4.

There were no statistical association between obtained results and age or sex of patients (p-values < 0.05).

4. Discussion

A large number of studies supports evidences that oxidative stress is a major contributor to neuronal death in Alzheimer's disease. The presence of oxidative stress markers, including oxidative DNA damage, were found in central nervous system of AD patients. Mecocci et al. demonstrated that the level of 8-oxoG was significantly higher in mitochondrial DNA located in three regions of cerebral cortex and cerebellum of AD patients compared to age-matched controls [18]. The level of 8-oxoG was also elevated in nuclear DNA of these AD patients, however to a lower extent than in mitochondrial DNA, suggesting that mitochondrial DNA is more susceptible to oxidative damage. An increased markers of oxidative damage to DNA (8-hydroxyadenine, 8-hydroxyguanine, thymine glycol, Fapy-guanine, 5-hydroxyuracil, and Fapy-adenine) in parietal, temporal, occipital, and frontal lobe, superior temporal gyrus, and hippocampus of AD brains' patients compared to controls was detected by Lyras et al. [19]. Gabbita et al. also found elevated 5-hydroxycytosine, 5-hydroxyuracil, 8-hydroxyadenine, and 8-hydroxyguanine level in AD brain compared with control subjects [5]. Thus, the oxidative damage to DNA detected in brain samples is a tangible diagnostic biomarker of AD
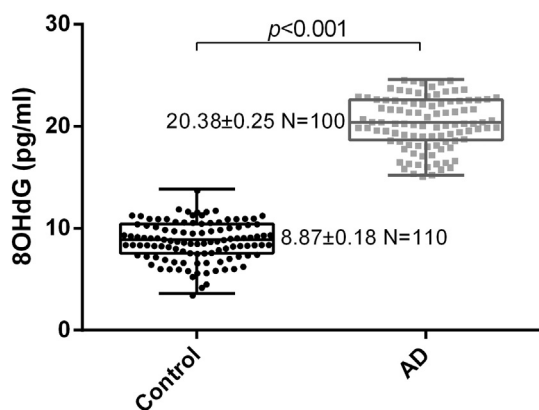
Fig. 2. Enzyme-linked immunosorbent assay results of OGG1 level. Values indicate the mean level of OGG1 $\pm$ SEM, N represents the sample size. We used the unpaired t test for unlinked variables. The difference significant at $p < 0.0001$ as compared to the control.

same as CRP [20]. However, the collection of brain samples is complex and hazardous intervention for patients. For this reason, other tissues should be assessed as a surrogate of oxidative DNA damage. In the present study, we assessed whether peripheral blood samples may be used

Fig. 3. Western blot analysis of OGG1 level. GAPDH was used as internal standard for normalization the results. A) OGG1 and GAPDH expression level. B) The mean of the surface area of the immunoreactive OGG1 bands in relation to GAPDH bands (OGG1/GAPDH ratio) blots. We used the non-parametric Mann–Whitney test for unlinked variables. The difference significant at $p < 0.05$ as compared to the control.

Fig. 1. Enzyme-linked immunosorbent assay results of 8-hydroxydeoxyguanosine (8-oxoG) level. Horizontal line inside box (percentile range) indicate mean level of 8-oxoG $\pm$ SEM. while whiskers indicate lower and upper extremes of studied population. We used the unpaired t-test for unlinked variables to compare control and case samples. The difference significant at $p < 0.001$ as compared to the control.
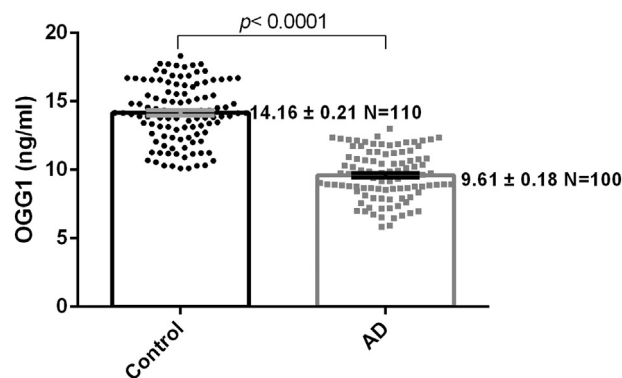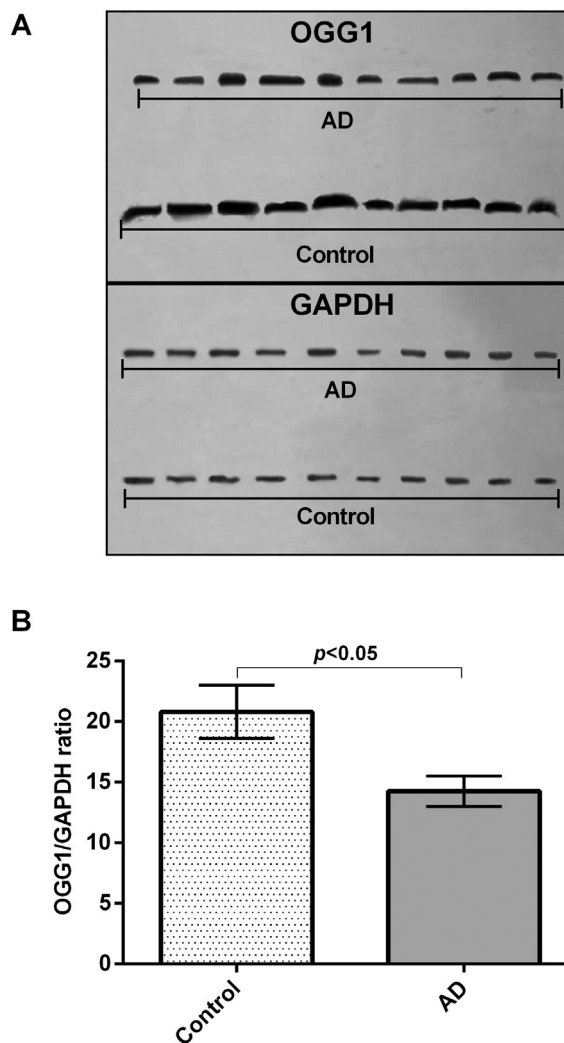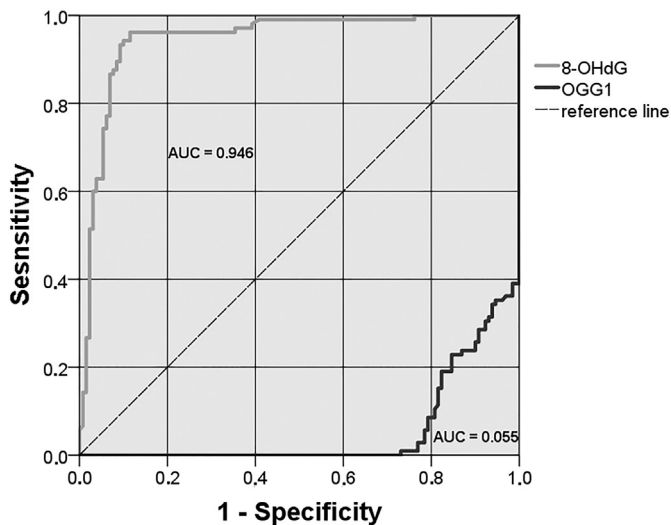
Fig. 4. ROC Curve of 8-oxoG and OGG1. The *p*-values in 8-oxoG and OGG1 were <0.001.

to determine the level of 8-oxoG as an oxidative biomarker in AD patients. Using ELISA test we noted that the level of 8-oxoG was significantly higher in AD patients. This stays in agreement with observation by authors who noted the increased level of oxidative DNA damage determined by the comet assay in peripheral blood mononuclear cells of AD patients [18,21–23]. Moreover, ROC analysis revealed that peripheral 8-oxoG level may be an important biomarker of Alzheimer's disease (AUC = 0.946). Among the reasons for the increased level of 8-oxoG, a decreased plasma level of antioxidants and disturbances in BER pathway seem to be most important [24–26]. A recent study performed by Hatanaka et al. showed that the anti-oxidant potential and levels of endogenous plasma anti-oxidants, such as albumin, total bilirubin and uric acid, were significantly decreased in AD patients [27]. Additionally, the levels of key free radical scavengers, both non-enzymatic (glutathione) and enzymatic (glutathione peroxidase, and catalase), were diminished in plasma of AD patients [28]. Weissman et al. using a set of functional assays showed a significant BER deficiencies in brains of AD patients caused by a limited processing of damaged DNA bases by DNA glycosylases and reduced DNA synthesis capacity of DNA polymerase beta [25]. Moreover, the decreased activity of OGG1 was found in various regions of brain in AD and mild cognitive impairment (MCI) patients compared to control subjects [26].

Future research should examine the use of oxidative DNA damage biomarkers as diagnostic tools to differentiate individuals with MCI from healthy people [26]. The term "MCI" is used to indicate the transition from non-disturbed cognition to symptoms of AD. Now, it is used in a broad sense in patients without diagnosed dementia, but with cognitive defects who have a significant risk of disease progression to dementia. The prevalence of MCI is estimated at 15–30% of the general population over 60 years of age and increases with age (60–70 years: 18.7%, 71–80 years: 27.1%, above 81 years: 29.4%) [29]. It is estimated that approximately 30% of people diagnosed with MCI in the next few years will develop dementia. For the other MCI patients the cognitive deficits will remain stable nature or disappear completely [30,31]. The clinical characteristics of these disorders are heterogeneous and depend on their etiology. Cognitive impairment may encompass memory processes (amnestic form of MCI) or one of the cognitive functions other than memory (i.e. not selective amnestic form of MCI). It may also include aspects of cognitive functioning (generalized form of MCI) [32]. The first of these types, so-called amnestic form, usually leads to the development of the AD (an annual rate of conversion of MCI to AD is estimated at 6–14%) [33,34].

Previous studies showed significantly elevated level of 8-OHdG in mitochondrial DNA compared to nuclear DNA in MCI and late-onset

AD (LAD) brain [35]. A significant negative correlation between OGG1 activity and levels of 8-OHdG was observed. Additionally, in studies of OGG1 in aging, enzyme levels were decreased in peripheral blood lymphocytes [36]. In studies of aging mice, OGG1 activity was significantly lower, as well as, levels of 8-OHdG were higher in aged mice in multiple brain regions [37,38]. For distinguishing between normal aging, MCI and AD, an early, sensitive diagnostic tool should be created, based not only on the 8-oxo and OGG1 levels, but also on genetic and functional aspects of DNA repair. It was also found that A53T and A288V polymorphisms in *OGG1* changed the catalytic and protein-binding activities contributing to increase of the susceptibility to DNA damage of AD brains [39]. Taken together, these observations suggest that decreased OGG1 activity may occur early in the progression of AD and may contribute to elevated 8-oxoG level in brain. In our present study we investigated whether the level of OGG1 is altered in peripheral blood samples of AD patients using both ELISA and Western blot. To our best knowledge this is the first study detecting OGG1 protein level in the serum in context of AD. We reported that OGG1 level was significantly lower in the serum of AD patients in comparison to control subjects. This result stays in agreement with significantly diminished expression of *OGG1* found in peripheral blood samples of AD patients [3,4].

Strengths of this paper is to demonstrate that peripheral blood samples can be used to measure the level of 8-oxoG and OGG1, biomarkers of oxidative damage detected in brains of AD patients. Additionally, the number of sample size was adequate to show that the ELISA is sensitive enough for the detection of both 8-oxoG and OGG1 protein levels. However, patients were treated with cognitive enhancing drugs, which could in theory modulate the results. Nevertheless, there are no publications confirming that these drugs may modify the levels of the biomarkers measured here. Evidently, the assessment of 8-oxo and OGG1 is not an adequate tool to differentiate AD from other diseases that are associated with elevated DNA damage. To develop more specific, diagnostic tests for AD, the biomarkers measured here could be combined with other biomarkers (e.g. the assessment of basal DNA damage, polymorphisms of DNA damage repair genes) that our team established in recent studies [40–42]. Limitations included the use of a non-clinical dementia population which might include participants with other disorders that might impact oxidative damage and level of BER proteins.

5. Conclusion

This study shows increased level of 8-oxoG and decreased OGG1 protein in the serum of patients with Alzheimer's disease, suggesting that oxidative damage to DNA and a decreased DNA glycosylase are involved in the etiopathology of AD. These results also suggest that peripheral blood samples are useful to detect oxidative damage-related biomarkers of Alzheimer's disease. However, further studies on large population are needed to assess the sensitivity of ELISA for measuring effective biomarkers of Alzheimer's disease.

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