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Decreased expression level of BER genes in Alzheimer's disease patients is not derivative of their DNA methylation status

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ABSTRACT

Background: Neurodegeneration in Alzheimer's disease can be caused by accumulation of oxidative DNA damage resulting from altered expression of genes involved in the base excision repair system (BER). Promoter methylation can affect the profile of BER genes expression. Decreased expression of BER genes was observed in the brains of AD patients.

Aim of the study: The aim of our study was to compare the expression and methylation profiles of six genes coding for proteins involved in BER, namely: hOGG1, APE1, MUTYH, NEIL1, PARP1 and XRCC1, in the peripheral blood cells of AD patients and healthy volunteers.

Methods: The study consisted of 100 persons diagnosed with Alzheimer's disease according to DSM-IV criteria, and 110 healthy volunteers. DNA and total RNA were isolated from venous blood cells. Promoter methylation profiles were obtained by High Resolution Melting (HRM) analysis of bisulfide converted DNA samples. Real-time PCR with TaqMan probes was employed for gene expression analysis.

Results: APE1, *hOGG1*, *MUTYH*, *PARP1* and *NEIL1* were significantly ($p < 0.001$) down-regulated in the lymphocytes of AD patients, as compared to healthy volunteers. Expression of XRCC1 didn't differ significantly between both groups. We did not find any differences in the methylation pattern of any of the investigated BER genes.

Conclusions: The methylation status of promoters is not associated with downregulation of BER genes. Our results show that downregulation of BER genes detected in peripheral blood samples could reflect the changes occurring in the brain of patients with AD, and may be a useful biomarker of this disease.

1. Background

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting about 30 million people in the world. Among the many causes of this neurodegenerative disease, neuronal death caused by accumulation of DNA damage has been established. Significantly increased oxidative DNA damage, mainly base lesions such as 8-oxoguanine, both in the brain and peripheral tissues in patients suffering from Alzheimer disease, has been detected (Mecocci et al., 1994; Lyras et al., 1997; Gabbita et al., 1998; Kadioglu et al., 2004; Zivković et al., 2013; Mórocz et al., 2002; Sliwinska et al., 2016). Among the various reasons for increased oxidative DNA damage, disturbances in DNA repair systems play a crucial role. The base excision repair system (BER) is responsible for removing damaged bases. This involves the following steps: (1) recognition and removal of damaged or inappropriate base resulting in the formation of apurynic/apyrimidynic (AP) sites; (2) incision of the phosphodiester backbone at the AP site; (3) 3′end processing; (4) filling of the gap after removal of the nucleotide, and (5) ligation of the DNA nick (Kim and Wilson, 2012). Upon initiation by a DNA glycosylase, the BER process may be performed as 'short-patch' or

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'long-patch'. In short-patch BER, a single nucleotide gap is generated and subsequently filled and ligated. In long-patch BER, a gap of 2–10 nucleotides is generated and filled. It is believed that short-patch BER is generally the prevailing pathway. However, long-patch BER could be the dominant pathway for post-replicative BER (Krokan and Bjørås, 2013). BER is carried out by numerous enzymes, including DNA glycosylases, AP endonucleases, phosphatases, phosphodiesterases, kinases, polymerases and ligases. Growing evidence suggests that an alteration in the expression of BER genes resulting in increased DNA damage may play a pivotal role in the neurodegeneration in Alzheimer's disease (Sliwinska et al., 2016; Lillenes et al., 2013; Jacob et al., 2013; Dezor et al., 2011).

One of the mechanisms responsible for gene expression regulation is methylation of DNA cytosine residue (mainly CpG sites). DNA methylation is one of the epigenetic modifications that alters chromatin structure, and thereby affects the availability of proteins engaged in gene expression (Bird, 2007). Methylation of a gene can occur in the promoter regions or in intron-spanning regions. Silencing of gene expression is a result of promoter methylation, whereas alternative splicing is caused by methylation in the intron region (Jones, 2012). Many physiological processes, such as X-chromosome inactivation, and repression of repetitive elements, are controlled by DNA methylation. However, there is steadily cumulating evidence to show that changes in DNA methylation patterns have been found in neurodegenerative diseases, including AD and Parkinson's disease (Watson et al., 2016; Tohgi et al., 1999; Maunakea et al., 2010; Jin et al., 2011; Rao et al., 2012).

Therefore, the present study was designed to compare the expression and methylation pattern of the promoters of BER genes between AD patients and healthy controls. The following genes were analysed: *hOGG1*, *APE1*, *MUTYH*, *NEIL1*, *PARP1*, and *XRCC1*.

2. Material and methods

2.1. Blood samples and DNA preparation

In our study we included 100 CE patients (44 men and 56 women, mean age 79.2 ± 4.9 years), and 110 healthy volunteers (46 men and 64 women, mean age 77.1 ± 7.3 years). Both AD patients and healthy volunteers (the control) were sourced from the Babinski Memorial Psychiatric Hospital in Lodz. 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, we also excluded patients with dysfunctions of the kidney or liver, and known inflammatory disorders which could increase the level of oxidative DNA damage. The inflammation markers were elevated levels of C-reactive protein (CRP) and other acute phase proteins, such as α2-macroglobulin, haptoglobin C3 and C4 in clinical tests. Furthermore, from 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 the Bioethics Committee of the Medical University of Lodz (Resolution No. RNN/70/14/KE). All AD and control patients gave their informed consent prior to enrolment in the study.

Venous blood samples were collected into 9 mL tubes containing EDTA and stored at − 20 °C until the DNA preparation. DNA isolation was performed using Blood Mini isolation kit (A & A Biotechnology) according to manufacturer's instruction. Quality and quantity of DNA samples was analysed using Pico100 Microliter UV/Vis spectrophotometer (Picodrop).

2.2. Bisulfite conversion

200 ng of DNA was treated with sodium bisulfite, using an EZ Methylation-Gold kit (Zymo Research). The reaction was intended to convert unmethylated cytosines into uracil, leaving 5-methylcytosine unaffected. After bisulfite conversion, the quality and quantity of DNA was analysed as described previously.

2.3. Methylation standards

Human methylated and non-methylated standard DNA (EpiTect Control DNA Set, Qiagen) were also used for further analysis. Additionally both samples were mixed to obtain range of different methylation levels (100, 75, 50, 25, 0% methylation). Standard DNA was bisulfite-converted as mentioned above.

2.4. HRM analysis

Primers were designed according to the recommendations of Wojdacz et al. (2009) in order to minimize PCR bias. Promoter sequences of the analysed genes (*APE1*, *XRCC1*, *NEIL1*, *MUTYH*, *PARP1* and *hOGG1*) were obtained from the Eukaryotic Promoter Database [epd.vital-it.ch]. They were then run through the primer design process using MethPrimer [www.urogene.org/methprimer], which uses 5′—cytosine—phosphate—guanine—3′ (CpG) island prediction for primer selection (Settings: Window: 100, Shift: 1, Obs./Exp.: 0.6, GC content: 50%). The primers were designed to be approximately 25 nt long each, with a melting temperature between 50 and 60 °C. Pairs of primers were expected to give a product in the range of 100–250 nt. The starters obtained, with their specifications, are shown in Table 1. Throughout primers' design procedure we used the National Center for Biotechnology Information the Single Nucleotide Polymorphisms database (NCBI dbSNP) at https://www.ncbi.nlm.nih.gov/projects/SNP/ to avoid presence of single-nucleotide polymorphisms (SNPs) with minor allele frequencies (MAF) higher than 0.05 in primers' annealing sequences. Moreover, in case of all genes, except *PARP1*, no SNPs with MAF > 0.05 were found in the amplicons.

DNA after bisulfide treatment was then used to perform a HRM reaction using KAPA HRM FAST Master Mix (Kapa), in a Bio-Rad CFX96

Table 1
Primers' sequences and specifications.

Gene	Starter sequence	Product size	Tm	Locus (ensembl genome browser, July 2017)	CpGs in product
APEX1	F: TTATTTATTAATTTGTGTTAGGAGG R: CTACAAACTAACCAACTTCCTCTAC	248 bp	64,0	chr14:20454556–20454804	20
XRCC1	F: TTAGTTTGGGTAATATGGTAAGATT R: CCCACTTCAACCTCCTAAATAACTA	123 bp	69,9	chr19:43576134–43576011	6
NEIL1	F: TTAAGTAGTTGGGATTATAGGTGTT R: TTACACTAAATAAATTAATCACAAAAAAAC	213 bp	67,6	chr15:75344375–75344588	7
MUTYH	F: TGTTTTTTGGGAGGATTATTTTTAGT R: AAAACCACAATATCCTCAATCTCAC	173 bp	68,0	chr1:45343275–45343102	10
PARP1	F: GTTTGTAGTTTTATTTTTGAGTTAG R: ACCCCTCCTTAAACAACCTAATAAC	231 bp	66,5	chr1:226425770–226425539	11
hOGG1	F: GTAGATGGAGTATGGAAGAAATGTTTAA R: CTAAAACCTCAAAACATCAAAAACC	101 bp	62,9	chr3:9749391–9749492	6

Real-Time PCR Detection System (Bio-Rad). PCR amplification was performed in 10 µl reactions containing 5 µl of KAPA HRM FAST MasterMix; 0.6 µl of $MgCl_2$; 0.2 µl 10 µM of each primer; 1 µl of 5 ng/µl sample DNA, and 3 µl of distilled water. During the PCR steps, the DNA polymerase pairs adenine with uracil, causing CG to TA transition. As a result, the melting temperature of the PCR product depends on the level of DNA methylation. This reaction was performed on a 96-well plate including non-template control, samples and a set of reference methylation standards. Reaction conditions included initial denaturation 3 min at 95 °C followed by 40 cycles of denaturation (5 s, 95 °C), annealing (30 s, temperature depending on primer) and extension (5 s, 72 °C). The next step was to perform a High Resolution Melting (HRM) Reaction. The thermal profile included 1 min of denaturation at 95 °C, followed by lowering of the temperature to 70 °C, and a gradient of temperatures every 0.2 °C (10 s steps). The last step lets us access the melting curve, based on which we were able to analyse the differences in methylation of the samples, in comparison to the set of methylation standard DNA samples. HRM reaction was followed by analysis of obtained data in the Bio-Rad Precision Melt Analysis Software.

2.5. RNA isolation and reverse transcription

Total RNA from peripheral blood lymphocytes was isolated using a Tempus™ Spin RNA Isolation Kit (Applied Biosystems). The procedure was performed according to the manufacturer's protocol. The concentration of the obtained samples was determined using a Pico100 Microliter UV/Vis spectrophotometer (Picodrop). Total RNA samples were stored at − 80 °C until use. In the next step, 1 µg of RNA was used in a reverse transcriptase reaction with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems).

2.6. Expression profile

Real-time PCR was performed using TaqMan probes for the *APEX1*, *XRCC1*, *NEIL1*, *MUTYH*, *PARP1* and *hOGG1* genes (Applied Biosystems). Additionally, *18 s rRNA* expression data was obtained and used as a reference gene. Reaction was made in 15 µl of total volume, including 7.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 0.75 µl TaqMan probe, 0.5 µl cDNA and 6.25 µl of water. Conditions for the reaction were suggested by the manufacturer of the TaqMan Universal Master Mix – 10 min. of enzyme activation at 95 °C, 45 cycles of denaturation (15 s, 95 °C), and annealing/extension of 60 s at 60 °C. Gene expression analysis was performed in a Stratagene™ Mx3005P QPCR System (Agilent Technologies).

2.7. Statistical analysis

All experiments were performed in 3 independent repeats. Data is expressed as mean ± SD. P values < 0.05 were considered as statistically significant. All statistical tests were calculated using the Statistica software (StatSoft).

3. Results

3.1. Methylation level

The data obtained showed that the promoter methylation level in the AD patients' blood samples did not significantly differ from its level in the control individuals' blood samples (Fig. 1).

3.2. Expression level

Unlike the methylation level, we noted significant downregulation of almost all analysed BER genes in AD patients, in comparison to the healthy controls. Our results showed that only the expression of *XRCC1* did not significantly differ between the two groups, while the expression of *APE1*, *hOGG1*, *MUTYH*, *PARP1* and *NEIL1* was significantly ($p < 0.001$) diminished in lymphocytes of AD patients (Fig. 2).

4. Discussion

It is believed that the reduction of BER efficacy is one of the key factors responsible for the neurodegeneration associated with the development of Alzheimer's Disease. In the present study, we aimed to evaluate whether there are changes in genes encoding BER proteins on epigenetic and genetic levels in the blood samples of AD patients. Therefore, the promoter methylation status and expression of selected BER genes were determined in peripheral blood lymphocytes isolated from AD patients, in comparison to healthy controls.

hOGG1 (8-oxoguanine DNA glycosylase) removes 8-oxoG whereas, MUTYH (MutY homolog) cleaves adenines that are mis-incorporated opposite 8-oxoG. After recognition of the lesion, these mono-functional DNA glycosylases cleave the *N*-glycosidic bond of the damaged base and produce an AP site. We noted that the expression of these DNA glycosylases was significantly diminished in the lymphocytes of AD patients. Similarly, Dezor et al. reported significantly lower hOGG1 in AD patients (Dezor et al., 2011). Moreover, downregulated expression of isoform 1a, 1b, and 1c of *hOGG1* was reported in blood by others (Dorszewska et al., 2009; Mao et al., 2007). However, the methylation pattern of the *hOGG1* and *MUTYH* promoters did not differ significantly between AD patients and control subjects.

NEIL1 (nei endonuclease VIII-like 1) is a bi-functional DNA glycosylases – apart from glycosylase activity, it is also able to cleave the DNA strand by β/δ-elimination – and is responsible for repair of oxidized pyrimidines and ring-opened purines. Interestingly, contrary to most of BER proteins, the NEIL glycosylases were found to be increased in postmitotic neurons (Englander and Ma, 2006). For this reason, it is believed that NEIL glycosylases play a key role in removing oxidative lesions in the adult brain. Our results showed that the expression of *NEIL1* was significantly diminished in the lymphocytes of AD patients. It seems that expression level was not dependent on the methylation of the *NEIL1* promoter.

The incision of the phosphodiester backbone at the AP site is performed by APE1 (AP-endonuclease). We reported a decreased expression of *APE1*, but unchanged methylation of promoter in AD patients, in comparison to healthy subjects.

It is suggested that PARP1 is a DNA damage sensor in the repair of single-strand break (SSBs), and protects against accumulation of SSBs during BER (De Vos et al., 2012). A significant decrease of *PARP1* expression was reported in AD patients.

XRCC1, a scaffolding protein, is responsible for stabilization of Ligase 3, thereby enhancing ligation. Both expression and methylation of XRCC1 did not differ between the studied groups.

It has been suggested that alterations in DNA methylation are part of AD's development, as indicated previously (Maunakea et al., 2010; Jin et al., 2011; Rao et al., 2012; Furuya et al., 2012a, 2012b; Di Francesco et al., 2015; Nagata et al., 2015). However, we did not report any significant differences in the methylation pattern of selected BER genes' promoters in the blood samples of AD patients, in comparison to healthy controls. Although the present study involved only a small number of participants, the results we obtained could indicate that expression of BER genes is not dependent on methylation of their promoters. We therefore suggest that a different mechanism than DNA methylation status determines the expression level of BER genes in AD.

5. Conclusion

Taken together, this is the first study to evaluate the expression levels and methylation patterns of BER components in peripheral blood lymphocytes. Of the genes studied, *hOGG1*, *MUTYH*, *APE1*, *NEIL1* and *PARP1* were diminished in AD patients, with only expression of *XRCC1*

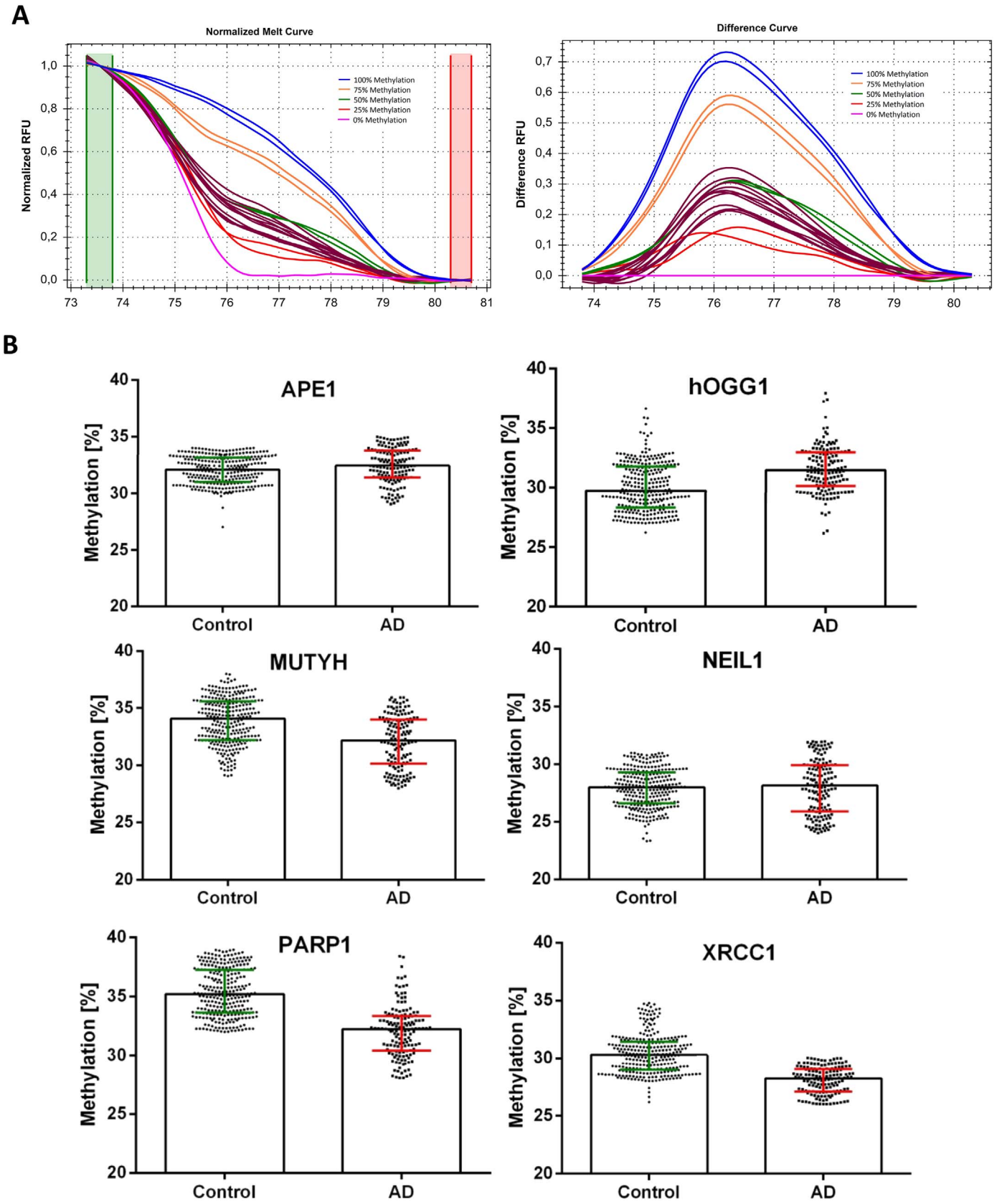

Fig. 1. Panel A: An example of HRM reaction results for the *PARP1* gene in the Alzheimer's Disease (AD) patient group. Representative standard curves are shown for the determination of methylation level in DNA samples. Standard samples were 0%, 25%, 50%, 75% and 100% methylation.
Panel B: methylation level in promoters of *APE1*, *hOGG1*, *MUTYH*, *NEIL1*, *PARP1* and *XRCC1* genes in AD patients, in comparison with healthy controls. All experiments were performed in duplicate. Values are expressed as mean value +/− SD.

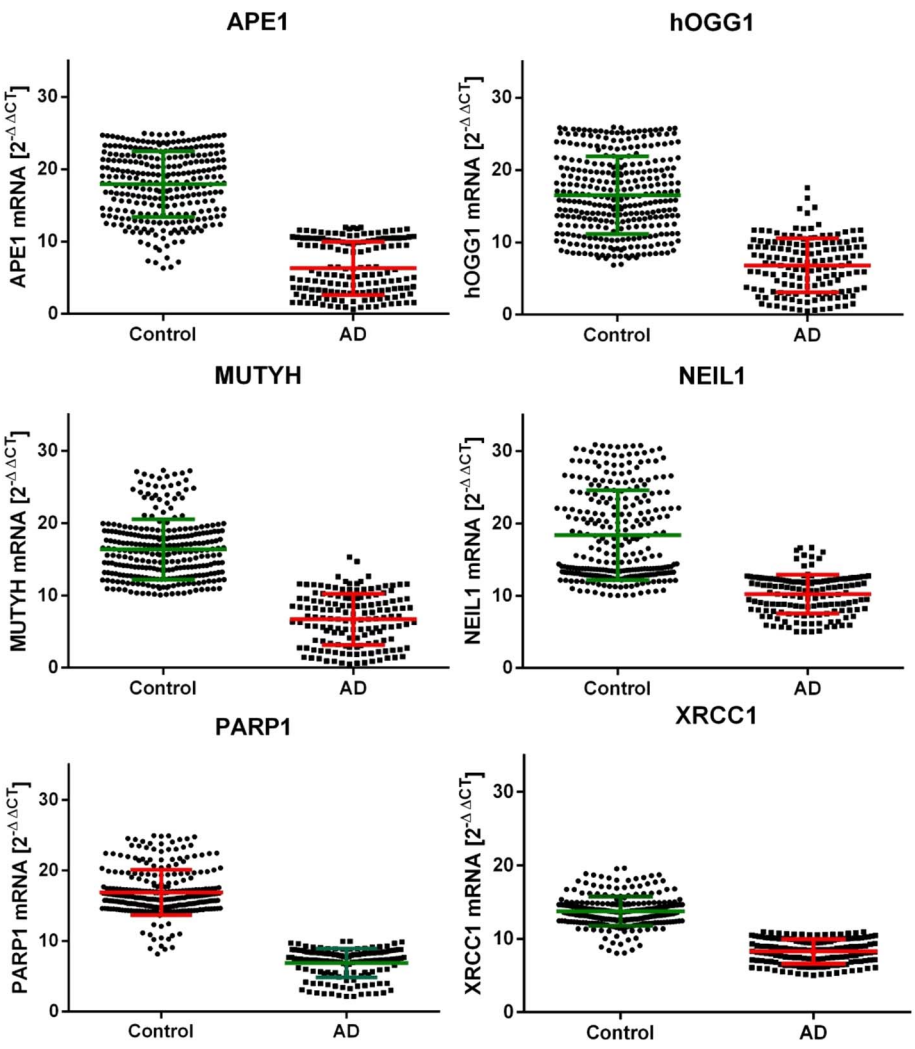

Fig. 2. Expression level of *APE1*, *hOGG1*, *MUTYH*, *NEIL1*, *PARP1* and *XRCC1* genes in Alzheimer's Disease (AD) patients, in comparison with healthy controls. All experiments were performed sets of three. Values are expressed as mean value +/− SD. $P < 0.001$.

remaining unchanged. However, the methylation pattern of promoters seems not to be associated with downregulation of BER genes. In conclusion then, our results show that there is mRNA down regulation of select BER genes in peripheral blood from AD patients.

Conflicts of interest

None.

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