Combined markers for predicting cognitive deficit in patients with Alzheimer’s disease

Dalia Farouk Hussen1*, Ayat Allah Farouk Hussein2, Mahmoud Abdel Moety Monzer3 and Saida Ali Hammad1

Abstract

Background: Alzheimer’s disease (AD) is the most widely recognized type of dementia. It is associated with cell cycle abnormalities including genomic instability and increased micronuclei (MNi) which usually evolve many years before the appearance of the clinical manifestations. Digital electroencephalogram (EEG) has a role in perceiving brain changes in dementia and in early detection of cognitive decline. This study aimed to assess the competency of using neurophysiological markers including absolute power of alpha waves and a cytogenetic marker which comprises scoring of MNi as a step toward early and preclinical diagnosis of AD. The study was conducted on 27 subjects; they were 15 patients diagnosed as sporadic AD and a group of 12 age and sex-matched controls. All subjects were subjected to Mini-Mental State Examination (MMSE), conventional EEG, digital EEG, and cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes.

Results: Conventional EEG showed a normal background activity with no abnormal epileptogenic discharges in both groups. Digital EEG showed significant reduction of the absolute power of alpha waves for AD patients as compared to the control group (\(P < 0.0001\)). Score of MNi showed statistical significant difference between the two groups (\(P < 0.0001\)). By linking scores of both cognitive state using MMSE and MNi among the group of patients, a significant negative correlation was detected (\(r = -0.6066\)). The correlations between cognitive state and the absolute power of alpha wave among the patients revealed a positive correlation (\(r = 0.2235\)).

Conclusions: The combination of both cytogenetic and neurophysiological markers can be beneficial for early detection of cognitive decline and may lead to preclinical identification of individuals at increased risk for AD, where at this stage treatment is constructive. The negative correlation between the scores of MNi and MMSE is suggestive for the impact of genomic instability on the cognitive state.

Keywords: Alzheimer’s disease (AD), Mild cognitive decline, Mini-Mental State Examination (MMSE), Cytokinesis-block micronucleus assay (CBMN), Micronuclei (MNi), Electroencephalogram (EEG), Neurophysiological marker, Cell cycle

Background

Dementia is a regression in the cognitive function which usually appears as a major affection of intellectual abilities to the extent that interferes with social or occupational functions beyond what might be expected from normal aging [1].

Alzheimer’s disease (AD) is the most common form of dementia to date and the most common neurodegenerative disorder [2]. Nowadays, 34 million people are suffering from AD worldwide, and by 2050 this value is expected to increase to 115 million [3]. In low- and middle-income countries, AD is suspected to increase by a considerable higher level [4].

Alzheimer’s disease is a slowly progressive brain disease that includes preclinical and clinical stages, clinical stage starts with mild cognitive decline that does not significantly interfere with the patients daily activities, then...
the mild cognitive decline usually progresses to demen-
tia as patients at this stage are characterized by obvious
memory and behavioral symptoms that impair a person’s
ability to function in daily life. The preclinical stage usu-
ally starts 20 years or more before mild cognitive decline
occurs. Patients at the preclinical stage has no symptoms
while there are usually measurable changes in the brain,
and biomarkers in the cerebrospinal fluid as well as in
blood that indicate the earliest signs of the disease [2].

Detection of biomarkers for early diagnosis and
screening is highly beneficial, where at this stage treat-
ment can be constructive [5].

Genomic instability is the earliest neuropathological event
detected at the preclinical phase of the disease [6–8].

Studies in peripheral lymphocytes of patients with
Alzheimer’s disease (AD), revealed genomic instability in
the form of increased micronuclei (MNi) formation
compared to age and sex healthy controls. Micronucleus
usually originates from a whole chromosome or acentric
chromosome that is retarded in the cell cycle during
anaphase and remains outside the daughter nuclei [9]. It
usually arises as a result of malsegregation due to mitotic
malfonctioning. Thus, it can be used as a biomarker to
investigate and follow genomic instability and deoxyribo-
nucleic acid (DNA) damage. Significant change in the
MNI frequency reflects reduced regenerative ability and
this may prove its usefulness as a potential future diag-
nostic marker to identify individuals of increased risk for
AD [10] as this DNA damage may be associated with
cognitive decline [11].

Although increased MNi formation as a cytogenetic
marker can be an indicator for cognitive affection, this
should be augmented simultaneously with functional
neurophysiological markers using electroencephalogram
(EEG) [12, 13].

EEG is a noninvasive functional neuroimaging method
that can be used for early detection of cognitive decline.
Moreover, it is widely available and faster than other im-
gaging techniques [14].

This study is an attempt to detect the frequency of
micronuclei formation within patients of AD and to test
the correlation between micronuclei frequency, EEG
changes, and the cognitive state. As genome integrity
loss could be associated with an increased risk for neu-
rodegeneration, coalescing genetic markers including
micronuclei frequency with neurophysiological marker
in the form of absolute power of alpha wave may be suc-
cessful for detecting early cognitive decline.

Methods

Patients

This study was conducted on 27 subjects; they included
15 patients diagnosed clinically as sporadic AD. They
comprised 10 females (66.7%) and 5 males (33.3%), their
ages ranged from 66–71 years with mean age of 67.73
years. All selected patients were fulfilling specific inclu-
sion criteria; they were above 65 years of age and con-

fined between mild and moderate stage of AD according
to the Diagnostic and Statistical Manual of Mental Dis-
orders, 5th ed. (DSM V) [15]. All patients were on cho-
linesterase inhibitors.

We have excluded patients with some criteria as age
below 60 years, smokers, diabetics, history of medical or
other neuropsychiatric disorders which may be the cause
of his/her cognitive decline. Patients with motor disabil-
ity and patients with a disturbed level of consciousness
or late stages of AD have been also excluded. A group of
12 age and sex-matched healthy volunteers has been en-
rolled in the study.

All patients and controls were subjected to the fol-
loowing:

Clinical studies

History taking and thorough clinical examination including
neuropsychological examination and cognitive screening
were performed. Screening for cognitive impairment was
performed using the Mini-Mental State Examination
(MMSE) test, which is a brief 30-point questionnaire [16,
17]. It measures various functions including memory, orien-
tation, and arithmetic. A score ≥ 25 points is effectively
ormal. Scores below 25 points can indicate mild (21–24 points),
moderate (10–20 points), or severe (≤ 9 points) affection.

Imaging

All cases underwent magnetic resonance imaging (MRI)
to exclude other causes of dementia.

Conventional encephalogram (EEG) and digital EEG

The EEG was recorded using standardized techniques
with electrode locations based on the international 10-
20 system. A standard 21-channel cup was used; elec-
trode impedance was kept lower than 5 kiloohms (kΩ).
Band pass filter (0.53-35 Hz) (EEG-9200k, Nihon Koh-
den Corporation, Tokyo, Japan).

EEG results were visually scored, background activity
for detection of the alpha rhythm then detection of epi-
leptic discharges either focal or generalized.

Spectral analysis

Artifact free recordings were selected and then ten
epochs of 10 s each were analyzed by applying Fast Fou-
rier Transfer-Spectral EEG, and the mean absolute
power spectrum was calculated for the frequencies of
delta (1-4 c/s), theta (5-7 c/s), alpha (8-12 c/s), and beta
> 13 c/s waves.

Normal reference patterns for EEG were established
from the evaluation of cerebral function in healthy
volunteers.
Cytogenetic studies

A venous blood sample (3-3.5 ml) was taken from each patient and healthy volunteer under aseptic conditions into a sterile heparin-coated vacutainer. Culture tubes were prepared as follows: each 100 ml bottle of RPMI 1640 was added to 25 ml fetal bovine serum, 4 ml L-glutamine, 1.5 ml penicillin/streptomycin solution, and 5 ml of phytohemagglutinin. They were mixed well and distributed in sterile falcon’s flat tipped 5 ml tubes, and then 0.4 ml blood was added for each culture tube. For each individual in the study, 2 culture tubes were set up under laminar air flow to avoid any contamination.

Cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes was done to detect chromosomal malsegregation. After initiation of culture at 37 °C by 44 h (h), blocking of cytokinesis with cytochalasin-B (Cytochalasin B from Drechslera dematioides, Sigma – Aldrich (now Merck)) have been done in which scoring is selectively directed to binucleated cells (BN), as these are cells that can present MNi [18], then the culture was completed for another 24-28 h before harvest and Giemsa staining. Five hundred BN were studied and multiplied by two for each case for scoring of MNi. BN cells with MNi were photographed using computer supported image analyzer (computer-assisted camera system; Applied Imaging, San Jose, CA, USA). Scoring of MNi in BN cells prevents confusing effects caused by altered cell division kinetics [9].

Statistical analysis

Test results were expressed as mean ± SD. Data were evaluated and compared using non-parametric Mann-Whitney test using GraphPad Prism software (version 6.01), GraphPad software, San Diego, CA, USA.

Results

The mean score of MMSE was 18.07 ± 4.026 among the AD patients, while for controls the mean score was 28.73 ± 1.443. MMSE score was done for controls to exclude being in early undiagnosed stage of mild cognitive decline and to allow validation of their both EEG results and their blood samples as control samples. Conventional EEG showed a normal background activity in both the controls and the group of patients, as no abnormal epileptogenic discharges have been detected. Digital EEG showed lower mean absolute power of alpha waves among the patients (6.207 ± 0.3011) (Fig. 1) compared to the controls (17.21 ± 0.5567) (Fig. 2). Statistical analysis revealed a highly significant difference between the two group (P < 0.0001). By linking scores of both cognitive screening using MMSE and MNi using CBMN among the group of patients, a significant negative correlation was detected between the two scores (r = −0.6066) (Fig. 5). The correlations between cognitive state and the absolute power of alpha wave among the patients revealed a positive correlation (r = 0.2235) (Fig. 6), whereas the correlation between the absolute power of alpha wave and the MNi score was non-significant (r = −0.06844).

Discussion

Alzheimer’s disease is a slowly progressive cerebral disease that includes preclinical and clinical stages. The preclinical stage usually starts many years before mild cognitive decline occurs. Patients at the preclinical stage are virtually asymptomatic while there are usually measurable changes in the brain, and biomarkers in the cerebrospinal fluid as well as in blood that indicate the earliest signs of the disease [2].

Although biomarkers in the cerebrospinal fluid are considered more specific, they are only detected through invasive lumbar puncture which has various complications [19]. Blood and neurophysiological imaging could...
serve as sources for detecting biomarkers through non-invasive methods and at a lower cost.

In the present study, an attempt has been made aiming to evaluate the efficacy of combined noninvasive markers including the score of MNi in BN lymphocytes in peripheral blood as well as digital EEG parameters for early diagnosis of AD.

All patients and controls were subjected to history taking, thorough clinical examination including neuropsychological examination, and MRI to exclude other causes of dementia. Screening for cognitive function has been done using MMSE. The mean score for MMSE was 18.07 ± 4.026 among the AD patients, while for controls the mean score was 28.73 ± 1.443. MMSE score was done for controls to exclude being in early undiagnosed stage of mild cognitive decline, to allow validation of both their EEG results and blood samples as control samples.

The use of EEG has grown prevalent for its capabilities in evaluating cerebral degenerative changes in dementia. Several studies have been directed to deal with EEG changes associated with dementia and to identify its degree of severity [20]. Furthermore, many studies praise

| Variable                        | Controls          | Patients          | P value  |
|---------------------------------|-------------------|-------------------|----------|
| Number of subjects              | 12                | 15                | ----     |
| MMSE                            | 28.73 ± 1.443     | 18.07 ± 4.026     | ----     |
| Mean value of alpha wave power  | 17.21 ± 0.5567    | 6.207 ± 0.3011    | P < 0.0001 |
| Mean score of MNi               | 3.167 ± 2.329     | 10.13±3.420       | P < 0.0001 |

MNI micronuclei, MMSE Mini-Mental State Examination
the role of EEG as a marker in the early detection of AD [21, 22].

By using conventional EEG in our study, we found normal background activity with no abnormal epileptogenic discharges in both the patient and the control groups. However, digital EEG revealed lower mean absolute power of alpha waves among the patients (6.207 ± 0.3011) compared to the controls (17.21 ± 0.5567). Statistical analysis revealed a highly significant difference between the two groups (P < 0.0001). Mean absolute power of other frequencies was within normal ranges for both patient and control groups. Our study was in agreement with the research study of De Waal et al. [23] which revealed more EEG abnormalities in AD patients compared to the controls (p < 0.001). Furthermore, Fauzan and Amran [24] in their study reported a significant reduction in rhythmic alpha frequencies among patients with mild cognitive impairment compared to the controls.

Moreover, Snyder et al. [13] stated that EEG may have an important role in detecting and classifying dementia regarding its significant influence in terms of rhythm activity that appears in patients with dementia. Micanovic and Pal [25] as well as Raymundo et al. [26] emphasized that visual analysis EEG can contribute in diagnosis of AD as it usually reveal power spectrum shifts from high-frequency components (alpha, beta, and gamma) toward low-frequency components (delta and theta).

In the current study, the correlations between cognitive state and the absolute power of alpha wave among the group of patients revealed a positive correlation (r = 0.2235). Although this correlation is considered relatively weak, we suggest that reduced alpha wave power could support the diagnosis when combined with another parameter.

We hypothesized in our study that depending on a single marker reduces the probability of reaching
appropriate diagnosis. So, we combined the neurophysiological markers with a genetic marker in a trial to reach reliable findings.

Bajic et al. [8] clarified that there is an increasing interest in the evaluation of DNA damage markers in individuals liable to develop AD. These biomarkers may identify individuals at early stages of neurodegeneration. This would be useful to allow for appropriate interventions prior to progression of the disease. Moreover, Zivković et al. [7] declared that genetic instability occurs a number of years prior to clinical diagnosis.

Additionally, Andreassi et al. [27] emphasized that scoring of the MNi is the most prevalent biomarker for assessing DNA damage in peripheral blood lymphocytes.

Regarding our study, the mean score of MNi among the patients was 10.13 ± 3.420, whereas among controls was 3.167 ± 2.329. Statistical analysis of data revealed a highly significant difference between the two groups (P < 0.0001). These results are consistent to the study done by Trippi et al. [28] which revealed an increase in the score of MNi in AD patients (P < 0.05), where the mean scores of the patient and control groups were 20.8 ± 9.2 and 9.0 ± 6.8, respectively. Additionally, Petrozzi et al. [29] revealed a compatible results to our study as they found a statistical significant difference between patients’ mean score of MNi (18.26) versus that of controls (8.56) (P< 0.05).

Our results are in contrast to the study done by Lee et al. [11] which showed a non-significant difference in the score of MNi among South Australian AD patients compared to controls (P < 0.18). They attributed these results to the environmental diversity between the study population in their research and the previous researches regarding dietary and lifestyle factors [30]. Additionally, the level of MNi in the controls may have already exceeded the threshold of spotting any significant differences compared to AD patients [11].

By linking scores of both cognitive screening using MMSE and MNi among the patients, our study found a significant negative correlation between the two scores (r = −0.6066). This result is in agreement with Lee et al. [11] who found a significant negative correlation of r = −0.4 between the two scores among the AD patients group.

Although increased score of MNi can be associated with different disease conditions and cannot be considered specific for AD [27]; however, the significant correlation between the scores of MNi and MMSE among the patients in our study can augment the prospect of using the MNi as a biomarker for the risk of cognitive decline and for early diagnosis of AD [11].

Zhang et al. [31] declared that increase frequency of MNi formation is associated with increased occurrence of chromatid. Chromatid occurs when a chromosome or a part of a chromosome experiences enormous shattering and consequent reunion of a single chromatid from a micronucleus which can result in accumulated affection of the DNA constitutes within the cells. This can result in a massive acquired genomic rearrangement in a single devastating event [32]. Consequently, this loss of genome integrity may be associated with increased risk for neurodegenerative disease [11]. So, we postulate that this mechanism may underlie the progressive course of AD and consecutive evaluation of the score of MNi for AD patient may help in follow-up. Continued research on micronucleated cells can unravel further unknown pathogenesis of different diseases including cancer and neurodegenerative diseases [33].

By comparing the association between cognitive state and both MNi scoring (r = −0.6066) and the absolute power of alpha wave (r = 0.2235) among the patients, we found that the MNi scoring is more correlated to the cognitive state which can reflect the distressing effect of genetic damage. This difference between the two correlations may be attributed to the early occurrence of genomic instability than the EEG changes, so at certain level of cognitive impairment genomic instability could be more obvious than the EEG changes. Also, the association between the cognitive state and the EEG changes may become more apparent with increasing the sample size, so subsequent studies with a larger number of AD patients are recommended.

The non-significant correlation between the value of alpha wave power and the MNi scoring among the patients group in our study (r = −0.06844) could also be rationalized by the difference in the course of the two parameters as the genomic instability is suggested to precede the EEG changes.

Conclusions
In the current study, we hypothesize that using combined markers including both cytogenetic and neurophysiological markers can augment the possibility of preclinical and early diagnosis of AD. The correlation between the scores of MMSE and MNi among the patients in our study is suggestive for the impact of genomic instability on the cognitive state of AD patients. Although cytogenetic and neurophysiological markers are defective in reaching a definitive diagnosis, they can increase the diagnostic sensitivity and specificity.

Abbreviations
AD: Alzheimer’s disease; BN: Binucleated cells; DNA: Deoxyribonucleic acid; EEG: Electroencephalogram; MMSE: Mini-Mental State Examination; MNi: Micronuclei; MRI: Magnetic resonance imaging

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Authors’ contributions
D.H. performed the cytogenetic study, wrote the manuscript and statistical analysis of the data. A.F. and M.M. performed the clinical examination and neurophysiological studies. S.H. supervised the study and shared in writing the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was conducted according to the guidelines of the Medical Research Ethics Committee of the National Research Centre based on the World Medical Association Declaration of Helsinki, and written informed consents have been taken from the patients or their guardians as well as from controls. The informed consents obtained were written consents.

Competing interests
The authors have no conflicts of interest to declare.

Author details
1 Department of Human Cytogenetics, Human Genetics and Genome Research Division, The National Research Centre, 33 El Buhouth Street, El Dokki, Cairo 12622, Egypt.
2 Department of Neurophysiology, Faculty of Medicine, Cairo University, Cairo, Egypt.
3 Department of Neurology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt.

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