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Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Haplotype analysis and linkage disequilibrium of *BRCA* genes in glioblastoma: Impact on treatment response

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Abstract

Glioblastoma (GBM) is an aggressive primary brain tumor prevalent in adults, characterized as a common malignant neoplasm of the human central nervous system with the worst survival rate among cancers. Treatment of GBM involves the addition of the alkylating agent temozolomide (TMZ) to radiotherapy, which improves overall survival by preventing replication through alkyl group-mediated DNA cross-linking. Genes related to homologous recombination (HR)-dependent DNA repair, such as the breast cancer susceptibility genes (*BRCA*), specifically *BRCA1* or *BRCA2*, contribute to cellular resistance to alkylating agents. We aimed to perform a haplotype-based study on the frequencies of *BRCA1* mutations in GBM patients compared to healthy individuals and investigate their linkage disequilibrium (LD) with the data population. Blood samples from GBM patients (n = 15) and healthy controls (n = 25) were sequenced using the Ion Torrent PGM platform to identify the *BRCA1* mutation. Subsequently, the reported variants were submitted to the LDlink tool for haplotype analysis, and their association with treatment response was assessed. Our results revealed that the *BRCA1* haplotype block consisted of seven SNPs, whose frequencies were reported with strong LD when compared to all available population data. This block was found to be represented by eight haplotypes. Five of these haplotypes were previously reported (four haplotypes were commonly reported, and one was rare), while the remaining three haplotypes were newly reported in this study. The relationship between newly reported haplotypes and response to treatment revealed that patients with these haplotypes responded to TMZ either as a complete or partial response. In addition, one haplotype in the heterozygote form was reported in the control case. In conclusion, haplotype analysis for *BRCA* genes in GBM cases can aid in predicting treatment responses and identifying cancer risk factors in individuals.

Keywords: Breast cancer susceptibility genes; Haplotype; Next-generation sequencing; Sequencing; Breast cancer

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1. Introduction

Glioblastoma (GBM) is the most prevalent primary brain tumor in adults. According to the World Health Organization, it is an aggressive, necrosis-prone, and mitotically vigorous grade IV malignant. It accounts for 45.2% of all malignant primary tumors of the brain and central nervous system (CNS). GBM is considered an inveterate disease with a 15-month median survival rate.[1] Only 5.5% of patients survive for 5 years after diagnosis.[2] GBMs are divided into primary and secondary subtypes that develop through various genetic routes, affecting individuals at different ages with varying outcomes.[3] Primary GBMs account for 80% of GBMs and are detected in people over the age of 62, while secondary GBMs occur in patients under the age of 45 and are triggered by low-grade astrocytomas or oligodendrogliomas. Secondary GBMs are more likely to occur in the frontal lobe, display less necrosis, and have a better prognosis than primary GBMs. At present, the detection of mutations in certain cancer cell genes has an extremely high predictive and prognostic value and enables the advancement of targeted therapies.

Existing treatment approaches for GBM are based on a combination of surgical methods along with radiotherapy and chemotherapy. Important variations in therapeutic techniques include the addition of temozolomide (TMZ) (alkylating chemotherapeutics) to the treatment regimen. Fractionated radiotherapy and six cycles of TMZ-based chemotherapy have been used in GBM patients.[4,5] Several clinical trials have demonstrated that long-term adjuvant TMZ chemotherapy improves progression-free survival (PFS) and 2-year survival rates in GBM patients and enhances their quality of life, but does not improve the overall survival (OS) significantly.[6] A recent randomized trial compared the results obtained from using TMZ in combination with interferon alpha and TMZ alone to evaluate the therapeutic efficacy and assess toxic consequences in high-grade glioma (grades 3 and 4). The study reported that the combination of TMZ with interferon alpha extended the survival time while maintaining tolerable toxicities compared to using TMZ alone.[7]

The breast cancer susceptibility genes (*BRCAs*) are a group of tumor suppressor genes, which includes *BRCA1 (*125,951bp) and *BRCA2 (*85,405bp).[6] *BRCA* genes are involved in cellular resistance to alkylating chemicals and are responsible for homologous recombination (HR) (a double-strand break repair process).[8-11] Mutations in these genes have been identified in several cancers, the most common of which are breast and ovarian cancer.[12] Although genomic instability may lead to a poor prognosis in GBM patients, the impaired DNA compromises pharmacological methodologies aiming to induce synthetic lethality that causes cancer cell death. This cell-killing procedure occurs only when two molecular pathways are concurrently lacking in a single cell, while an isolated defect is innocuous.[13] One category of drugs that applied in this cell-killing procedure includes inhibitors of poly (ADP-ribose) polymerase (PARP) enzyme, a significant DNA damage response constituent. PARP inhibitors (PARPis) may induce artificial lethality in tumor cells with pre-existent imperfections in the HR repair pathway, such as lethal mutations of the *BRCA1* and *BRCA2* suppressor genes.[14] Most GBM clinical experiments assessing PARPis do not assess the *BRCA* status to correlate outcomes among patient assemblies.[15] A plausible explanation for this omission could be the predictable low incidence of *BRCA* mutations in GBM compared to ovarian, breast, and pancreatic cancer.[16,17]

Germline mutations in several types of genes (tumor suppressors, oncogenes, and DNA repair genes) have been widely analyzed in genome-wide association studies.[18] The previous studies have reported that genomic variants linked to cancer often exhibit an ancestry-specific effect, termed "flip-flop," as the variant associated with cancer in an ancestral population might have no association or an opposite association as a result of linkage and epistatic effect.[19] There are few studies worldwide implicating local ancestry for cancer-causing alterations or recognizing novel ancestry-associated molecular characteristics.[20,21]

This research aims to investigate the frequency of *BRCA* mutations in GBM patients compared to healthy individuals, using sequencing technology to detect single nucleotide polymorphisms (SNPs) as well as insertions and deletions (InDels) that serve as the foundation for allele differentiation. Haplotype analysis, which contains a collection of linked SNPs, proves to be more informative than the analysis of single SNPs in determining associations with phenotypes.

2. Methods

2.1. Sample selection and inclusion criteria

On obtaining ethical approval from the Medical Ethical Committee of the National Research Centre (ID #17111) for this prospective study, 44 individuals were enrolled, divided into patients with GBM (n = 15) and healthy, normal individuals as controls. The inclusion criteria for the recruited groups were as follows: for GBM patients, they were above 18 years old, newly diagnosed using magnetic resonance imaging (MRI) and pathological examination, and their presentation was ≤2 according to the Ester Clinical Oncology Group, while for the normal

groups, they were of harmonized age and sexual category with malignancy findings. Individuals who did not meet the inclusion criteria were excluded from the study. The justification criteria for the selection of GBM patients were those who had not received any treatments (adjuvant therapy, including chemo- or radiotherapy), patients who were amenable to follow-up, and patients who had undergone surgery, and whose survival duration was above 1 month.

2.2. Medication strategy

All patients underwent MRI for analysis. Then, GBM patients were cured with clinical resection, near-total resection, or excisional biopsy. Thereafter, all patients were cured with regular therapy, which included radiotherapy (total dose of 60 Gy—administered in 30 parts over 42 days) with concomitant TMZ chemotherapy (100 mg/day for 45 days) followed by 6 cycles of TMZ treatment at a dose of 150 mg/m^2 of body surface area for days 1 – 5. All patients were followed up and assessed clinically and radiologically by MRI.

The tumor was accessed based on the radiological RANO response standards (2010)[22,23] as follows: Complete response (CR), defined as the disappearance of all identified brain injuries; partial response (PR), defined as a 50% or greater reduction of the assessable brain lesion or an objective progression of the assessable brain lesion; stable disease (SD), defined as an unchanged brain lesion (<50% reduction or <25% proliferation in the size of the assessable lesions); and progressive disease (PD), defined as ≥25% increase in the size of some or all brain injuries and/or the presence of new brain lesions.

2.3. DNA extraction

The QIAamp DNA mini blood kit (Cat. No. #51104, Qiagen, Germany) was used to extract DNA from ethylenediaminetetraacetic acid blood samples, according to the manufacturer's guidelines. The kit procedure was similar to the one established for the extraction of DNA using a spin column. For the extracted DNA, both purity and concentration were assessed using a nano-drop spectrophotometer (Quawell, Q-500, Scribner, USA) before further examination and then stored at –80°C until further estimates.

2.4. DNA library formulation and sanitization

Amplification of the target regions for *BRCA1* and *BRCA2* was carried out using the Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies Ltd., USA). This panel comprises three primer pools (167 amplicons) covering the entire coding region with 10 – 20 bp intronic neighboring sequences around the coding arrangement of the investigated genes. The amplification procedure involved a 20-µL scale using the Ion AmpliSeq Library Kit (Thermo Fisher Scientific, USA). The assay was performed using 4 µL of 5× Ion AmpliSeq HiFi Master Mix, 10 µL of 2× Ion AmpliSeq Primer Pool (every primer in a separate well for each sample), 10 ng of genomic DNA/sample (2 µL of 5 ng/µL from the test store), and lastly 4 µL of nuclease-free water. The applied thermal settings for the reaction were as follows: 99°C for 2 min, one round for triggering the enzyme activating by (99°C for 2 s, and 60°C for 4 min) for 16 cycles using a thermal cycler (SureCycler 8800, Agilent, USA). After enlargement, the primers were processed using the Fragmentase Universal Primer Assay (FuPa) Mixture and thermal conditions: 50°C for 10 min, 55°C for 10 min, and 60°C for 10 min, then ligated with two adaptors (IonCode™ Barcode Adapters, Life Technologies Ltd., USA) and the barcodes with the Ion Xpress Barcode Connectors-16 kit (Life Technologies Ltd., USA). Then, magnification was performed using the thermal conditions: 22°C for 30 min, 68°C for 5 min, and 72°C for 5 min, as indicated in the Ion AmpliSeq Library Kit user guide. Sanitization of the formulated libraries was carried out and measured by the Ion Library TaqMan Measurement Kit (Life Technologies Ltd., USA) by quantitative PCR using the real-time PCR technique (Stratagene Max3005P QPCR system, Agilent Biotechnology, USA). The QPCR was performed with the following thermal conditions: 50°C for 2 min, 95°C for 2 min, 40 rounds at 95°C for 15 s, and 60°C for 1 min. The amplified libraries were additionally subjected to template formulation.

2.5. Template formulation

The cleaned and measured libraries were assembled on molar comparable portions to achieve at least ×500 coverage for every sample. The collective collections were clonally magnified using the Ion PGM Hi-Q view OT2 kit (Life machineries, USA) on the Ion OneTouch 2 apparatus (Life Technologies Ltd., USA) according to the manufacturer's guidelines. Then, the template ion sphere particles ISP were improved using Ion PGM Enhancement Beads (Life Technologies Ltd., USA) on the Ion OneTouch ES system (Life Technologies Ltd., USA) based on the manufacturer's guidelines. Positive ISP excellence was analyzed on the Qubit 2.0 Fluorometer (Life Technologies Ltd., USA) and then sequencing was performed.

2.6. Sequencing with Ion Torrent PGM Proposal

Before starting sequencing using Ion PGM, calibration and pH amendments were carried out according to the user guide of the Ion PGM Hi-Q View Sequencing kit (Life

machineries, USA). Thereafter, using the Ion Torrent PGM platform (Ion Torrent PGM, Life Machineries, USA), all barcode-enriched samples were sequenced using the Ion 318 Chip Kit V2 BC (Life Technologies Ltd., USA). The depth of analysis was considered to be at least ×500.

2.7. Files examination

The annotations for the variations in the *BRCA1* and *BRCA2* genes were carried out using the cloud-constructed IonReporter Outfit software (version 5.4.0, Life machineries). The annotations were subsequently associated with human genome hg19 (Genome Reference Consortium GRCh37) through the Ion Torrent Server and different caller plugins, according to the criteria mentioned for the *BRCA1* and *BRCA2* panels. Investigation of alternatives was practical without prejudice, and the position arrangements for *BRCA1* and *BRCA2* were NM_007300.3 and NM_000059.3, respectively.

Haplotype analysis is the determination of the pattern of adjacent SNPs on the matching chromosome and heritability in a unit. Units may comprise a huge number of SNPs, but limited SNPs are sufficient to exclusively recognize the haplotypes in units. The HapMap is a chart of these haplotype units, and the exact SNPs that classify the haplotypes are called tag SNPs.

Linkage disequilibrium – the non-random connection of alleles at altered loci – is a sympathetic marker of the population-inherited forces that establish a genome. Due to the tremendous progress in approaches for measuring genetic differences at a fine scale, evolutionary biologists and human geneticists are progressively exploiting linkage disequilibrium to recognize past evolutionary and demographic measures, to map genes that are linked with measurable personalities and heritable illnesses, and to recognize the shared development of related groups of genes. LDlink (https://ldlink.nci.nih.gov/?tab=ldmatrix) is a set of web-based appliances to easily and proficiently interrogate LD in populations, with flexible query and communicative visualization of results.

The haplotype tables and communicating plots generated by LDlink are tailored for researchers interested in mapping common and rare disease susceptibility loci, with an emphasis on linking associated alleles and highlighting potentially functional variants.

In the present study, for the comprehensive investigation of LD and haplotype, the identified variations in both *BRCA1* and *BRCA2* were analyzed using the LDlink application. This is in contradiction to all population records, as there is currently no detailed Egyptian population database accessible.

3. Results

3.1. Clinical characteristics of the recruited individuals

A total of 44 individuals were sequenced for *BRCA* mutations, and they were categorized according to their diagnosis into 15 GBM patients and 29 healthy normal individuals as controls. Both the GBM and control groups were the same age, as there was no significant difference between their ages. In terms of gender status, there were ten females and five males in GBM, while there were 17 females and 12 males in controls.

According to the ECGO system, GBM patients were divided into two groups: those above two (9 cases, 60%) and those below or equal to two (6 cases, 40%). The sites of the primary lesion were frontal (n = 6), temporal (n = 5), and occipital (n = 4). Nine of the GBM patients who received surgical treatment underwent a biopsy, and the remaining six underwent either a total or sub-total excision. Then, ten GBM patients received radiotherapy/chemotherapy for six cycles, and three patients received the same treatment for three cycles, while the remaining patients received only radiotherapy. The GBM patients responded to the treatment strategies as follows: three GBM patients reported a CR, six GBM patients reported a PR, two showed SD, and the remaining four reported PD. The demographic and medical criteria are summarized in Table 1.

3.2. Haplotype analysis for BRCA gene mutations

In our previous study, six GBM patients were sequenced for the detection of *BRCA* gene mutations,[17] and the authors accordingly reported 13 single-nucleotide variations (SNVs) for the *BRCA1* gene using the LD matrix of the LDlink tool. Seven of these SNPs showed strong LD. Accordingly, these SNPs were separated into haplotype-like designs, and the LD matrix of the LDlink tool was used to investigate whether there was LD between the investigated SNPs compared to all data populations.

In the present study, we have sequenced *BRCA* genes in a total of 44 individuals, with 15 identified as GBM cases and the remaining as healthy controls. The primary objective was to investigate haplotype frequency in GBM cases compared to the recruited healthy individuals and to compare our findings with the data population. Accordingly, haplotype analysis and frequency assessments were carried out for the reported seven SNPs using the LDhap tool for all populations. Of these SNPs, five haplotypes were previously reported among the data population, four of them were frequently reported, one was rare, and three haplotypes were newly reported, as shown in Table 2.

Table 1. Demographic and clinical criteria for GBM (grade IV) patients

Patient ID	Gender	Age	Site of mass	ECGO	Type of surgery	Cyc.CT	Response	OS (month)
G1	Female	29	Rt. Frontal mass	2	Excision	6	CR	10
G2	Male	44	Rt. Frontal mass	1	Excision	6	PD	18
G3	Male	37	Rt. Frontal mass	1	Excision	6	PD	5
G4	Male	57	Rt. Partial mass Occipital	1	Gross total resection	6	PR	10
G5	Female	57	Rt. Frontal mass	2	Excision	3	CR	13
G6	Female	60	Rt. Frontoparietal	1	Excision	3	PD	3
G7	Female	60	Rt. Partial mass Occipital	2	Excision	6	PR	6
G8	Female	55	Rt. Partial mass Occipital	1	Excision	6	PR	7
G9	Female	38	Rt. Frontal mass Temporal	2	Partial resection	6	PR	7
G10	Female	59	Rt. Frontal mass	1	Total excision	6	PR	16
G11	Female	65	Rt. Tempropartial	2	Biopsy	2	PD	6
G12	Female	33	Lt. Frontal mass.	1	Total excision	6	CR	20
G13	Male	39	Rt. Frontal mass Temporal	1	Sub-total excision	none	SD	12
G14	Male	42	Rt. Partial mass Occipital	1	Sub-total excision	6	PR	14
G15	Female	78	Lt. Front temporal mas	2	Biopsy	none	SD	7

Abbreviations: CR: Complete response; CT: Chemotherapy; Cyc.: Cycles; Lt.: Left side; OS: Overall survival; PD: Progressive disease; PR: Partial response; Rt.: Right side; SD: Stable disease.

Table 2. Haplotype frequencies for the eight reported SNPs among enrolled individuals

SNPs Haplotypes	rs 1060915	rs 16042	rs 16941	rs 799917	rs 16940	rs 1699949	rs 3765640
Haplotype A	G	C	C	A	G	A	G
Haplotype B	A	T	T	G	A	G	A
Haplotype E	A	T	T	A	A	G	A
Haplotype F	A	C	T	A	A	A	G
Haplotype G	A	T	T	G	A	A	A
Haplotype C	G	T	C	A	G	A	G
Haplotype D	G	C	C	A	A	A	A
Haplotype H	G	C	C	G	G	A	G

Abbreviations: rs: Reference SNP cluster ID; SNPs: Single-nucleotide polymorphisms.

3.3. Correlation between haplotype analysis and clinical criteria in recruited individuals

The correlation between the detected haplotypes and the different clinical and demographic criteria for the recruited individuals (n = 44) revealed that haplotype [A] showed 1591 haplotypes in the entire data population, and its frequency was 0.3177. Among the studied groups, it was detected in 24% (7/29) of the control group, while it was detected in 13.3% (2/15) of the GBM cases. The GBM patients who were reported to possess homozygote haplotype (AA) showed PD. The haplotype [B] has a count of 955 and a frequency of 0.4525 in the data population. Among the controls, 37.9% (11/29) were homozygous for haplotype [B], and 0.68% (2/29) were heterozygous. Moreover, an individual with a family history of cancer was registered for each homo- or heterozygote haplotype. Among the GBM cases, homozygote haplotype was detected in 33.3% (5/15); four of them reported showing a PR towards TMZ, while one showed PD, while four out of 15 GBM (26.6%) were heterozygous, and their response to treatment varies from CR, PR, SD, and PD.

Haplotype [E] represented the highest haplotype count since it reported 2272 haplotypes with a frequency of 0.4537 and was, therefore, referred to as a wild haplotype across all populations. One control case was reported as homozygous (1/29), and two controls were heterozygous (2/29). Among the GBM cases, only one case was heterozygous with PD. The remaining haplotypes previously reported were [F] and [G], with a haplotype count and frequency of 89 and 0.0179, and 82 and 0.0164, respectively. Therefore,

haplotype G was considered the rare haplotype, as shown in Figure 1.

In this study, three haplotypes were detected that were newly reported and were designated as haplotypes [C], [D], and [H]; all were heterozygotes (Figure 1). GBM cases that reported heterozygotes in haplotypes [C] and [D] responded to TMZ treatment with either CR or PR. Haplotype [H] was only detected in control cases in a heterozygous form.

4. Discussion

The human genome consists of regions with strong LD and low historical recombination, termed haplotype blocks.[24] Regions of strong LD have been verified to have low haplotype multiplicity, with common haplotypes explaining the shared variation in different populations. Moreover, haplotypes can be anticipated using a subset of all existing (or known) markers (termed haplotype-tagging SNPs [htSNPs]). Hence, with the definitive haplotype block, the investigation of individual haplotypes and their familial associations could allow the indirect investigation of probable fundamental variants that may not have been genotyped. In this study, we investigated the haplotype analysis and LD among 16 GBM patients and compared them with 25 healthy controls as a first trial among Egyptian GBM patients. Moreover, these haplotypes were investigated with clinicopathological features and their response to treatment by adopting a haplotype-based method to investigate genetic discrepancies in *BRCA1*.

Previously, authors have analyzed *BRCA* gene mutations in GBM patients[25] and reported the potential role of *BRCA1* in cancer predisposition. In the present study, a total of 44 individuals were enrolled and categorized according to their clinical criteria into 15 GBM cases and 29 normal individuals as controls. The comparison of haplotype concepts between two groups can provide reliable information about the human genome[26] in GBM cases and normal individuals. At present, haplotype [A] is presented as homozygote (AA) in seven control individuals compared to two GBM cases. Although only a small number of cases were investigated in this study, reporting these haplotypes in both groups may require further investigation of a large number of GBM cases compared to normal controls, which is a study in progress. For the studied GBM cases reporting homozygous (AA) haplotypes, analyses and their association with treatment revealed their response to treatment. This may indicate the importance of using these haplotype sequences for early prediction of response to TMZ treatment among GBM patients. This finding is consistent with previously reported findings by Ding *et al.*, who reported that *BRCA1* is an effective candidate gene that induces artificial lethality with TMZ in GBM.[27] Recent strategies in the treatment landscape for tumors harboring defects in homologous repair (HR) genes (e.g., *BRCA1* and *BRCA2*) have been combined with poly (ADP-ribose) polymerase inhibitors

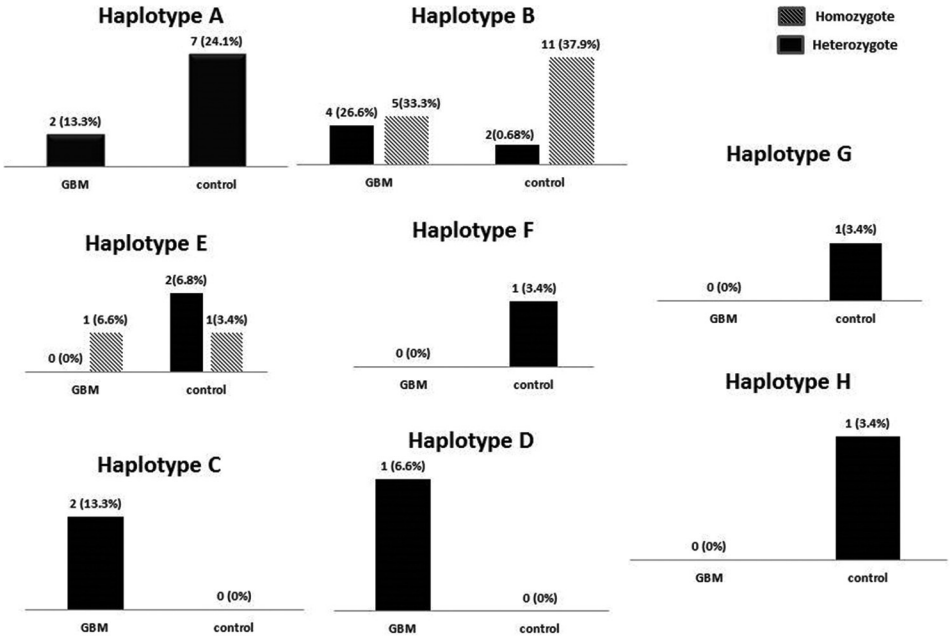


Figure 1. Frequencies and count for reported haplotypes.
Abbreviation: GBM: Glioblastoma.

(PRAPi) to improve efficacy through synergistic activity and potentially sensitize naturally PARPi-resistant tumors to PARPi. Initial combinations of PARPi and chemotherapy were delayed by major overlapping hematologic toxicity, but the latest combinations with lower toxicities and more targeted approaches are currently undergoing assessments.[28] In preclinical studies, the combination of TMZ with PRAPi has an enhanced cytotoxic effect, independent of HR,[29] as TMZ stimulates base damage, leading to the development of PARP-DNA complexes, which, in combination with effective PARP-trapping agents, enhance cytotoxicity. This has encouraged research into trials assessing this combination over different tumor histologies such as GBM,[30] colorectal cancer,[31] and breast cancer.[32] In general, the combination of TMZ and PARP inhibition was found to be well-tolerated, although efficacy was modest in most tumor subtypes.[33]

One of the assumed haplotypes includes haplotype [B], detected in homo- or heterozygous forms, which was associated with a familial history of breast cancer in the control individuals studied. This outcome was in agreement with the study of Tuazon *et al.*[34] Therefore, it will be important to carry out a future prospective study to confirm this finding in a large group of Egyptian individuals.

This paper is the first to report on the three identified haplotypes, termed haplotypes [C], [D], and [H], and all were in heterozygous form. GBM patients with haplotypes [C] and [D] responded to treatment with either CR or PR. Hence, it could be of great value to investigate the *BRCA1* haplotype among GBM patients before their treatment, as it was previously confirmed that BRCA1 protein expression may as well predict GBM survival.[35] Moreover, the newly reported haplotype [H] was only detected in healthy individuals (n = 25), which may indicate that this type of haplotype reports a lack of risk for cancer predisposition. A prospective study is currently underway to confirm this finding in a large Egyptian population.

Although GBM haplotype analysis is of great importance and the present study sheds light on this type of analysis in relation to GBM as one of the cancers worldwide, the small number of cases is one of the limitations of the study. This small number could be due to the fact that the incidence of cancer among Egyptians is not as high as breast or liver cancer. As this study is in the preliminary stage, the enrollment of a large number of cases will be considered in ongoing prospective research. Another limitation is the lack of some data for GBM patients, such as Isocitrate dehydrogenase1 (IDH1) and O6-methylguanine-DNA-methyltransferase (MGMT) status, so a comparison of haplotype variance regarding mutant IDH1 and MGMT methylation was not reported.

5. Conclusion

We build on our earlier finding that haplotype analysis among GBM patients can help predict patients who may benefit from TMZ treatment and that analyzing *BRCA1* haplotypes may identify those who are at low risk of developing cancer.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Ethical approval of the study was obtained from the Medical Ethical Committee of the National Research Center (ID#17111), Egypt, and all participants signed informed consent.

Consent for publication

Participants have signed their informed consent for publishing their data in this study.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

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