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Accumulation of Mitochondrial DNA Microsatellite Instability in Malaysian Patients with Primary Central Nervous System Tumors

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ABSTRACT

AIM: To determine the mitochondrial microsatellite instability (mtMSI) status in a series of Malaysian patients with brain tumors. Furthermore, we analyzed whether the mtMSI status is associated with the clinicopathological features of the patients.

MATERIAL and METHODS: Forty fresh frozen tumor tissues along with blood samples of brain tumor patients were analyzed for mtMSI by PCR amplification of genomic DNAs, and the amplicons were directly sequenced in both directions using Sanger sequencing.

RESULTS: Microsatellite analysis revealed that 20% (8 out of 40) of the tumors were mtMSI positive with a total of 8 mtMSI changes. All mtMSI markers were detected in D310 and D16184 of the D-loop region. Additionally, no significant association was observed between mtMSI status and clinicopathological features.

CONCLUSION: The variations, specifically the mtMSI, suggest that the mitochondrial DNA (mtDNA) can be targeted for genomic alteration in brain tumors. Therefore, the specific role of mtDNA alteration in brain tumor development and prognosis requires further investigation.

KEYWORDS: Mitochondrial microsatellite instability, D loop, brain tumor, Malaysian patients

INTRODUCTION

Central nervous system (CNS) tumors account for about 15% to 20% of all malignancies existing in childhood and adolescence. Approximately 90% of all CNS tumors are brain tumors, with the reminder occurring in the spinal cord and cranial nerves (21). However, the molecular mechanisms underlying brain tumorigenesis are still not completely defined or understood. It is believed that the accumulation of genetic alterations, such as mutations in proto-oncogenes and tumor suppressor genes, plays a vital role in the progression of brain tumors (7,45).

Genomic instability is considered to be responsible for an increased tendency of mutations and the promotion of further DNA alterations, thus being the hallmark of cancer initiation and progression. Elucidating the role of genomic instability in tumorigenesis is believed to pave the way for understanding cancer (42).

Microsatellites are well-recognized as short tandem repeats (STRs) or simple sequence repeats (SSRs) of DNA comprising 1-6 base pairs (24). These simple repetitive nucleotide motifs widely appear all over the genome and are susceptible to

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polymorphic changes among individuals (24). Accumulation of mutations are derived from either insertions or deletions (indels) in some repetitive sequences of microsatellites, which can cause a condition called microsatellite instability (MSI) (5). It has been shown that MSI can be triggered by a defective DNA mismatch repair system, which leads to an inability to fix errors in repetitive DNA sequences that occur during DNA replication (5,13). The first report of MSI contributing to human cancer was published in 1993, describing 28% of MSI in colon tumors examined (61). Since then, the role of MSI has been discovered in many different types of cancer (9,11,32,56,65). However, instability in mitochondrial DNA (mtDNA) is still insufficiently characterized and is not well studied. mtDNA is referred to as the second human genome, the alterations of which are believed to contribute to carcinogenesis in human.

Mitochondria are known for their pivotal roles in generating ATP via aerobic respiration, reactive oxygen species (ROS) production, fatty acid oxidation, and programmed cell death. Each mitochondrion contains its own 16,569 bp circular DNA, which encodes 37 genes for 13 subunits of electron transport chain complexes (I, III, IV, and V), 22 tRNAs and 2 rRNAs (1). mtDNA also comprises a non-coding region called the displacement loop (D-loop) that is responsible for the modulation of mitochondrial genome replication and transcription (2).

mtDNA alterations in the form of point mutations, deletions, insertions, copy number changes and MSI, are believed to be hotspots of cancer research. Such genetic alterations in mtDNA have been widely reported in various human cancers (22,35-37,47,62,63,67). Our previous studies have discovered a high frequency of somatic mtDNA mutations in brain tumor patients (46).

Although some studies have reported the occurrences of mitochondrial microsatellite instability (mtMSI) in multiple cancer types (8,10,16,33,38,64,68), thus far no published studies have directly investigated the role of mtMSI in brain tumorigenesis. Therefore, this study was designed to determine the role of mtMSI in brain tumors among Malaysian patients.

MATERIAL and METHODS

Patients and Tumor Samples

A total of 40 Malay patients with brain tumor cases, who diagnosed at the Department of Neurosciences, Universiti Sains Malaysia, Kelantan from October 2017 to February 2012, were included in this study. The study was approved by the research ethics committee of Universiti Sains Malaysia. Informed consent was obtained from each participating patient. All tumors were reviewed by at least two neuropathologists and confirmed to meet the World Health Organization (WHO) diagnostic criteria for the brain tumors classification (46). The tumor series included 4 astrocytomas WHO grade I (A I), 6 astrocytomas WHO grade II (A II), 4 anaplastic astrocytomas WHO grade III (AA III), 8 glioblastomas WHO grade IV (GBM), 16 meningiomas WHO grade I (M I) and 2 atypical meningiomas WHO grade II (M II). Twenty paraffin-embedded archival post-mortem human brain tissue from traffic accident victims with morphologically confirmed normal brain tissues served as controls. The DNAs were extracted from all samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

mtMSI Analysis

MSI in mtDNA was analyzed in genomic DNAs (isolated from tumor tissue and blood sample patients) that need amplification of the mtDNA fragments by PCR and then direct sequencing of amplicons using Sanger sequencing. Briefly, 12 sets of primers were used as described previously (68) to amplify the mtDNA fragments. PCR amplification was performed in the SureCycler 8800 Thermal Cycler (Agilent Technologies, Inc., Santa Clara, CA, USA) with PCR conditions as follows: initial denaturation at 98°C for 30 sec followed by 28 cycles of denaturation at 98°C for 10 sec, annealing at 58°C for 30 sec, elongation at 72°C for 1 min and a final elongation step at 72°C for 2 min. Purified PCR products were sequenced in both directions using the same primers used for PCR amplification. The sequencing was carried out using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3700 DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). Electropherogram results were aligned using BLAST software from the NCBI site (http://www.ncbi.nlm.nih.gov/blast) and then compared with the published revised Cambridge Reference Sequence (rCRS) of the human mtDNA (NC_012920) in the MITOMAP database (www.mitomap.org).

Statistical Analysis

Statistical data were performed using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). The relationship between mtMSI status and clinicopathological parameters was analyzed using Chi-square or Fisher Exact test. P-values of <0.05 were considered as statistically significant.

RESULTS

Clinicopathological characteristics and their association with mtMSI status are shown in Table I. There were 20 males (50%) and 20 females (50%) among 40 patients included in this study, with their age at diagnosis ranging from 5 to 73 (mean: 40.6) years. Based on histological grading, 8 cases (20%) were GBM, 6 cases (15%) A II, 4 cases (10%) A I, 4 cases (10%) AA III, 16 cases (40%) M I and 2 cases (5%) M II. mtMSI analysis revealed that 8 out of 40 patients (20%) had a total of 8 different alterations within two different loci of mtMSI markers.

Representative results of sequencing for the mtMSI analysis are shown in Figure 1A, B. Microsatellite marker alterations were observed within the D-loop region at the nucleotide positions 303-315 (D310) and 16184-16193 (D16184) (Table II). We identified one alteration, $C_5TC_4 > C_8TC$ at 16184-16193 region which has not been previously reported in the MITOMAP database. These mtMSI changes constituted the replacement of a single base T with C at position 16189 and a

Table I: Clinicopathological Parameters and mtMSI Status in Brain Tumor Patients

Parameter	Total no. of patients, n (%)	mtMSI status (%)		p
		mtMSI positive	mtMSI negative	
Gender				
Male	20 (50)	6 (30)	14 (70)	0.235
Female	20 (50)	2 (10)	18 (90)	
Age (years)				
<40	19 (47.5)	3 (15.8)	16 (84.2)	0.698
≥ 40	21 (52.5)	5 (23.8)	16 (76.2)	
Mean	40.6			
Range	5 - 73 mDNA Microsatellite Instability			
Tumor Type (grade)				
A I	4 (10)	1 (25)	3 (75)	0.709
A II	6 (15)	0 (0)	6 (100)	
AA III	4 (10)	1 (25)	3 (75)	
GBM	8 (20)	3 (37.5)	5 (62.5)	
M I	16 (40)	3 (18.8)	13 (81.2)	
M II	2 (5)	0 (0)	2 (100)	

A I: *Astrocytoma WHO grade I,* ***A II:*** *Astrocytoma WHO grade II,* ***AA III:*** *Anaplastic astrocytoma WHO grade III,* ***GBM:*** *Glioblastoma WHO grade IV,* ***M I:*** *Meningioma WHO grade I,* ***M II:*** *Atypical meningioma WHO grade II.*

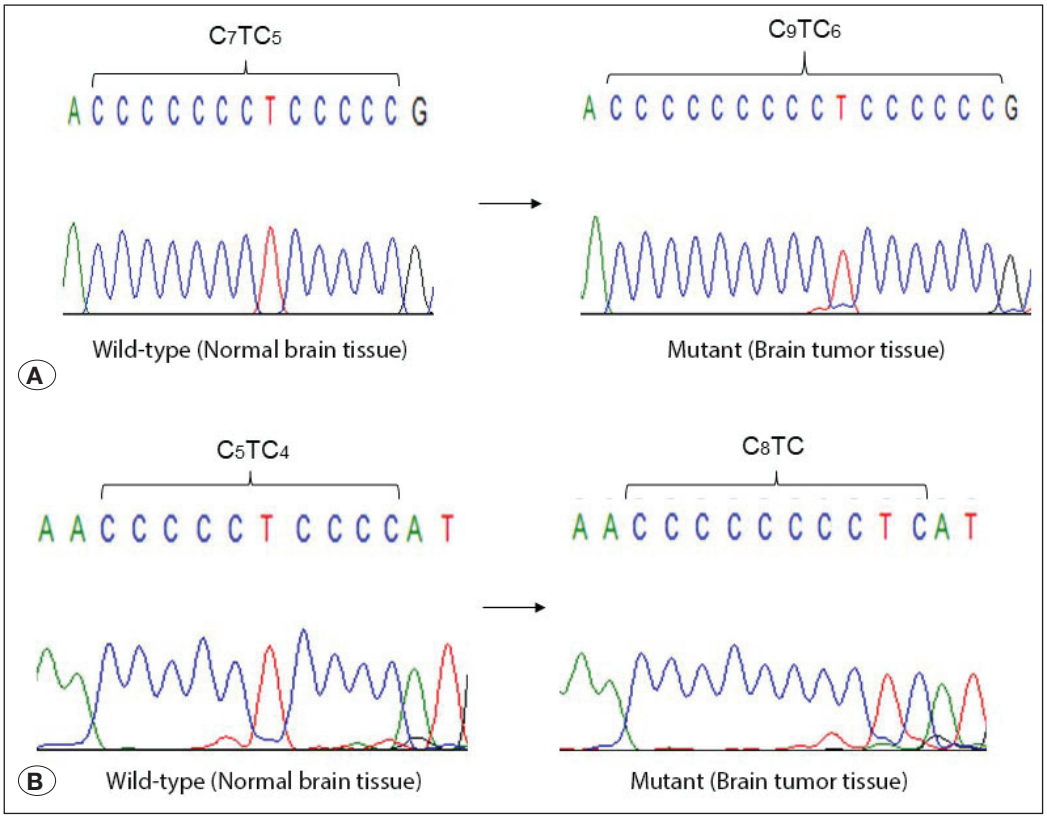

Figure 1: Representative sequencing results depicting examples of mtMSI changes. **A)** Electropherogram shows the insertions of poly C stretch at nucleotide position 303-315. **B)** mtMSI changes exhibit a single base T to C substitution at 16189 and C to T substitution at 16192, respectively (at 16184-16193 region).

Table II: Details of mtMSI Changes Detected in Brain Tumor Patients

Nucleotide changes	Region	Position	Occurrence in patients (n=40)	Histology	Novel/Reported	References
$C_7TC_5 > C_7TC_6$	D-loop	303-315	3	AA III, GBM	solid tumors, colorectal, ovarian	11, 33, 38
$C_7TC_5 > C_8TC_6$	D-loop	303-315	2	A I, M I	solid tumors, low grade astrocytomas, colorectal, ovarian	11, 25, 33, 38
$C_7TC_5 > C_9C_6$	D-loop	303-315	1	M I	colorectal, ovarian	33, 38
$C_5TC_4 > C_8TC$	D-loop	16184-16193	1	GBM	-	present study
$C_5TC_4 > C_{10}$	D-loop	16184-16193	1	GBM	Leukemia, low grade astrocytomas, endometrial female cancer	24, 25, 39, 64

A I: *Astrocytoma WHO grade I,* ***AA III:*** *Anaplastic astrocytoma WHO grade III,* ***GBM:*** *Glioblastoma WHO grade IV,* ***M I:*** *Meningioma WHO grade I*

conversion of C to T at position 16192. However, no instability was found in the remaining 10 microsatellite markers screened in the patients.

In this study, the association of mtMSI status with clinicopathological parameters in patients was further evaluated (Table I). mtMSI was observed much less frequently in females (n=2/20; 10%) than in males (n=6/20; 30%). However, there was no significant association between mtMSI status and sex (p=0.235).

In this study, a statistically insignificant association was found between mtMSI status with <40 and ≥ 40-year-old age groups (p=0.698). mtMSI was more prevalent in the ≥40-year-old age group (n=5/21; 23.8%) than in the <40-year-old age group (n=3/19; 15.8%).

Furthermore, there was no statistically significant difference between mtMSI status and histological types of brain tumors (p=0.709). mtMSI was mostly identified in GBM (n=6/16; 37.5%) followed by A I (n=1/4; 25%), A III (n=1/4; 25%), and M I (n=3/16;. 18.8%). No, instability was found in A II and M II samples.

DISCUSSION

Studies on mitochondrial instability have been described in a wide variety of cancers, including colorectal, gastric, lung, breast, cervical, ovarian, and endometrial cancers (17,32,44,53,68). In many cases, the incidence of mtMSI has been commonly reported in the hypervariable D-loop region, suggesting that this region may be considered a hotspot of genetic instability (6,12,19,30,44,52,53). Research efforts have recently focused on the occurrences of mtMSI in the non-coding D-loop region (3,12,31,40,52,57,61), with only a few studies examining the entire mitochondrial genome (38,69). As described by Habano et al., mtMSI of the poly C stretch has been detected mostly in the D-loop region in colorectal carcinoma patients (16). In 2003, a study of gliomatosis cerebri with mitochondrial genomic instability was performed with the outcome analyses uncovering that the poly-C tract in the hypervariable region is a clonal marker (27). Similarly, Wang et al. reported mtMSI at high frequencies in the D-loop region of four common types of cancer in females, including endometrial, breast, cervical, and ovarian cancers (68).

In our study, 20% (8 out of 40) of the CNS patients were discovered to harbor mtMSIs. The frequency of mtMSI observed in our series is similar to the one reported in colorectal cancer by Lim et al. (37), but is lower than the one reported by other studies on the breast, gastric and endometrial cancers, which reported that 29.4%, 38.2%, and 48.4%, respectively, carried mtMSI, (38,68). However, our frequency is higher than those reported by two previous studies by Lee et al. (32), and Fang et al. (10), in which squamous cell carcinomas of the lung (19%) and hepatocellular carcinomas (12.2%) were respectively investigated.

In our study, mtMSI was observed at only two loci, D310 and D16184 of the non-coding D-loop region. The D-loop region instabilities seem to be a frequent molecular abnormality detected in our group of brain tumor patients. In 2016, Yeung et al. examined the involvement of mitochondrial genome variants in a series of GBM cell lines using a combination of high-resolution melt analysis and next-generation sequencing. They revealed that the greatest frequency of mtDNA variants is in the D-loop and the origin of light strand replication in noncoding regions (70). Also, some researchers have reported a positive impact of the D-loop alterations in several other cancers. In the study of Venderbosch et al. in 182 metastatic colorectal cancer patients, 54.4% of the mtMSI were detected in the D-loop region (64). As previously stated, Wang et al. determined that mtMSI was frequently observed in the mitochondrial D-loop of endometrial (48.4%), breast (29.4%), cervical (25.4%), and ovarian cancers (21.9%) (68). Furthermore, Kleist et al. reported that the D-loop region had significantly more MSI in cases of the lymph node metastases (53.1%) (28).

The D-loop region comprises essential replication and transcription elements that are responsible for the expression

of the mitochondrial genome (6,50). Sequence variants in this region can potentially affect mitochondrial biogenesis. This region consists of a triple-stranded DNA structure, which also acts as the connection point of mtDNA to the mitochondrial inner membrane. Moreover, the structure is constantly exposed to a high oxidative stress environment as the ROS is generated during oxidative phosphorylation (53). Consequently, the D-loop region of mtDNA is extremely susceptible to mutations by ROS-triggered damage (50,53).

Insertions and deletions (indels) in the poly C stretch of the D-loop region were mostly present in this study. mtMSI (specifically in the D310 area) has now been targeted in several cancers (3,8,30,38,41,60,63,69) since this region was first recognized 19 years ago by Sanchez-Cespedes et al. as a mutational hotspot (55). The D310 is a highly polymorphic region, located within the hypervariable II (HV II) region. This region is required for the formation of a firm RNA-DNA hybrid or an R-loop to activate mtDNA replication. Therefore, it supports the view that this region contributes essentially to maintaining mitochondrial biogenesis and function (6). Additionally, the majority of indels related to the D310 region, which occur in some cancers, can lead to impaired of mitochondrial biogenesis (34). Since the first report published by Sanchez-Cespedes's group, several teams have investigated D310 instability in various cancer types. For instance, in a study of several different solid tumors conducted by Geurts-Giele et al., they reported a D310 mutation frequency of 32% in colorectal, 19% in lung, 18% in head and neck, 16% in skin, 13% in breast and 11% in gynecological tumor samples (12).

The instability in the nucleotide positions 16184-16193 is another remarkable mutational hotspot in the mitochondrial D-loop and has been extensively studied in several cancers (3,8,10,17,28,30,39,41). The 16184-16193 region is an unstable tract of poly cytosine residues and is prone to replication errors, most likely due to polymerase slippage. Instabilities in the nucleotides 16184-16193 are crucial to mtDNA due to their position on the 3'-end of a termination-associated sequence and at the 7S DNA binding site, which is pivotal to mtDNA synthesis modulation (39). Nevertheless, the elimination of the T residue producing a long repeat of poly C stretch tends to be polymorphic, possibly resulting in instability (12,15). These facts suggest that the sequence changes at this particular locus may contribute to the replication errors and mtDNA content alterations (39). A recent finding of Kim et al. showed a strikingly high frequency of mtMSI at D16184 in acute myeloid leukemia among Korean patients (70.3%) (25). As observed in another study, the presence of poly C stretch alterations in D16184 locus has also been detected among gastric carcinoma (16.1%) (17), and endometrial carcinoma patients (14.0%) (41).

To our knowledge, the mechanisms underlying the occurrence of mitochondrial instability are still largely unexplored. One of the potential reasons is the faulty mtDNA mismatch repair system. mtDNA polymerase γ (pol γ) with proofreading exonuclease activity plays a dominant role in the DNA mismatch repair system and replication (50,54). It was shown that mtDNA pol γ is vulnerable to slippage, resulting in reduced proofreading efficiency (23,43,49). mtDNA pol γ is also believed to be a suspected target of oxidative damage that may hinder the fidelity of mtDNA repair and replication (14). The previous study proposed that the high frequency of mtMSI might be caused by a high rate of mitochondrial exposure to oxidative damage, hence initiating slippage errors of mtDNA pol γ (54). The study by Habano et al. demonstrated that the mismatch repair system is crucial for mtDNA damage repair, thus, the malfunction of this system results in errors, which consequently increases the mutation rate (16).

Alterations in the mitochondrial genome may influence the mitochondrial function and regulation. Nonetheless, owing to the proximity of ROS source and the lack of protective histones, the accumulation of MSI may decrease the effectiveness of the mitochondrial respiratory chain, which is responsible for ROS production. Therefore, a defect in mitochondrial respiration might further increase ROS levels and cause an elevation in glucose-dependent metabolism (18,29,51). Notably, this condition may lead to a high rate of mutation, eventually resulting in mitochondrial abnormalities. Furthermore, various mtDNA alterations and the associated mitochondrial dysfunction that may contribute to cancer progression have been hypothesized (20,48,59).

It has been acknowledged that post-mitotic cells with a high demand for energy, especially muscle, heart, liver, and brain cells tend to have a greater number of mitochondria (39,47). Moreover, cancer cells also require sufficient energy to support their uncontrolled rapid proliferation. Therefore, it is relevant that mtDNA alterations are often found at their certain levels in post-mitotic tissues as the clonal expansion of aberrations occurs within these cells over time. Wallace demonstrated high levels of clonally expanded mtDNA mutations in the brain and skeletal muscles (66). In addition, Kirches et al. observed a high prevalence of mtMSI in glioblastoma samples (88%;15 out of 17) (26). These findings indicate that mutations in mtDNA might influence most areas, specifically the one comprising brain cells, which consequently give rise to mitochondrial diseases (47).

In our study, we concluded that there were no significant associations between MSI status and clinicopathological variables (age, sex, and histological type). Limited sample size and tumor types with very low heterogeneity included in this study may contribute to the lack of statistically significant different results. Of all brain tumors assessed, only 20% displayed MSI in our study. These results could be related to the sensitivity of the method used, which might influence the alteration rate detected. Perhaps the disadvantage of direct Sanger sequencing is its lower sensitivity than other methods. There is a need for further investigation using the so-called "gold standard" method or a combination of methods (such as capillary electrophoresis fragment analysis, next-generation sequencing) to enhance the limit of MSI detection in cancer (4).

The possible role of mtMSI in the process of tumorigenesis remains unclear, even though the hotspot areas for mtMSI have been widely recognized. However, it is believable that alterations in mtDNA might be selected during tumor

development. Considering that a number of our patients harbored the D-loop MSI, our findings may provide a clue that there is an essential role of mtDNA in MSI brain tumorigenesis. Moreover, this supports a hypothesis that alterations in mtDNA might be selected as a potential molecular biomarker for cancer detection (58).

CONCLUSION

The present preliminary results of this study propose a comparatively high frequency of mtMSI in brain tumors. Future larger sample size population-based studies are demanded to verify the findings and discover possible associations between the mtMSI and clinical parameters of patients with brain tumors. Therefore, the specific role and comprehensive molecular mechanisms of mtDNA alteration in brain tumor development and prognosis deserve further investigation.

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