Brain & Development 41 (2019) 245–249

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Oxidant and antioxidant levels and DNA damage in tuberous sclerosis

Mine Yuksel [a], Feyza Ustabas Kahraman [a,*], Sahbettin Selek [b], Omer Faruk Ozer [b] Akin Iscan [c]

[a] Department of Pediatrics, Bezmialem Vakif University Medical Faculty, Istanbul, Turkey
[b] Department of Biochemistry, Bezmialem Vakif University Medical Faculty, Istanbul, Turkey
[c] Department of Pediatric Neurology, Bezmialem Vakif University, Faculty of Medicine, Istanbul, Turkey

Received 26 May 2018; received in revised form 19 September 2018; accepted 29 October 2018

Abstract

Objective: The pathogenesis of inherited diseases is thought to involve oxidative stress and the associated DNA damage, which are also implicated in many other conditions including cancer. Tuberous sclerosis is a genetic disease with autosomal dominant inheritance pattern that is characterized by the development of hamartomas in multiple organ systems. Oxidative stress and the related DNA damage are also likely to play a significant role in the pathogenesis of this condition. Thus, our study aimed to assess total oxidant-antioxidant level, oxidative stress index and DNA damage in patients diagnosed with tuberous sclerosis.

Methods: The study included 30 patients with tuberous sclerosis between the ages of 0 and 16 years. The control group consisted of 29 age-matched healthy children. Blood samples obtained from each subject were centrifuged to separate the sera. The Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) were measured in serum samples with a Thermo Scientific Multiscan plate reader (FC, 2011-06, USA) at wavelengths of 240 nm and 520 nm, respectively. The measured TAS and TOS values were used to calculate the Oxidative Stress Index (OSI). In addition, the Comet Assay Method was used to determine DNA damage in the samples. Data were analyzed using SPSS software.

Results: Patients with tuberous sclerosis complex (TSC) and controls were compared with respect to TAS, TOS, and OSI. TAS was significantly lower ($p < 0.01$), while TOS and OSI were significantly higher ($p < 0.01$, for both) in patients as compared to controls. In addition, patients had significantly higher DNA damage as shown by the Comet Assay ($p < 0.01$).

Conclusions: Increased oxidative stress and DNA damage may contribute to the pathogenesis of tuberous sclerosis.

Keywords: Tuberous sclerosis; Oxidant level; Antioxidant level; DNA damage

1. Introduction

Tuberous sclerosis (TS) is a genetic disease with autosomal inheritance pattern characterized by hamartomas in multiple organ systems, with a reported incidence of one in about 5000–10,000 births [1,2]. Most common sites of involvement include the brain, skin, heart, kidney, lung, and eyes [2–5]. The manifestations of tuberous sclerosis complex (TSC) include refractory epilepsy, cognitive disorders, angiofibromas, hypomelanotic macules, fibrous plaques, cardiac rhabdomyomas (RM), renal angiomyolipomas, and retinal hamartomas due to glioneuronal hamartomas.

* Corresponding author at: Karayollari Mah., Abdi Ipekci Cad., TEM Avrupa Konutlari, 33/5 Gaziosmanpasa, 34255 Istanbul, Turkey. Tel.: 05327713714.
E-mail address: fkahraman@bezmialem.edu.tr (F. Ustabas Kahraman).

https://doi.org/10.1016/j.braindev.2018.10.014

Two genes, namely the tuberous sclerosis complex gene 1 (TSC1) and tuberous sclerosis complex gene 2 (TSC2), are responsible for the etiopathogenesis of the disease. The former of these two genes encodes the hamartin protein, while the latter encodes the tuberin protein. These proteins regulate the mammalian target of rapamycin (mTOR) complex activity, which controls numerous vital functions such as cell growth, proliferation, and protein synthesis. Mutations in these genes lead to excessive activity of the mTOR complex activity, with the subsequent development of TS with hamartomas in multiple organs characterized by abnormal cell growth and proliferation [6,7].

The mechanisms through which free radicals cause oxidative damage in biomolecules include the initiation of potent reactions in the organism [8,9]. Normal physiological conditions are characterized by an equilibrium between the production of free oxygen radicals and antioxidant defense systems [8]. On the other hand, in certain states, a shift in the direction of oxidative stress is observed as a consequence of increased oxidant and/or decreased antioxidant production [8,9]. The damage associated with oxidative stress may take the form of base and sugar modifications in the deoxyribonucleic acid (DNA) that occur through variable mechanisms such as single and double chain fractures, and DNA-protein cross-linking, etc. [10].

2. Materials and methods

A total of 30 patients with tuberous sclerosis between ages of 0 and 16 years followed-up at the Pediatric Neurology Outpatient Unit, Bezmialem Vakif University Hospital and 29 healthy children were included in this study. The study was conducted between January 1, 2015 and June 30, 2017. Tuberous sclerosis complex diagnostic criteria recommended by the International Tuberous Sclerosis Complex Consensus Group (2012 update) were used for the clinical diagnosis of tuberous sclerosis [11]. Four milliliters of whole blood sample was obtained from each subject (2 ml in EDTA and 2 ml in heparinized tube) for TAS, TOS, OSI, and DNA damage assessments, which were performed at Medical Biochemistry/Biology Laboratory, Bezmialem Vakif University in accordance with the procedures described below. The study protocol was approved by Bezmialem Vakif University Clinical Research Ethics Committee (Date, 25 December 2014, No, 1564) and the study was conducted in accordance with Declaration of Helsinki. Informed consent was obtained from the parents of all subjects prior to study entry.

3. Blood analyses

For biochemical analyses, blood samples were centrifuged and stored at −80 °C until the day of analysis. Recently reported methods by Erel were utilized for total antioxidant status (TAS) and total oxidant status (TOS) measurements [12,13]. TAS and TOS were measured in serum samples at wavelengths of 240 nm and 520 nm, respectively, using a plate reader (Thermo Scientific Multiskan FC, 2011-06, USA). For TAS measurements, Trolox, a water-soluble compound of vitamin E was used as a calibrator and the results were expressed as mmol.Trolox equivalent/L. For TOS measurements, H2O2 was used as the standard, and the results were expressed as μmol·H2O2 equivalent/L. The measured TAS and TOS values were used for the calculation of the Oxidative Stress Index (OSI) using the following formula: oxidative stress index (OSI) is equal to TOS (mmol·H2O2. equivalent /L) × 100 divided by TAS (mmol.Trolox. equivalent /L) [14]. DNA examinations with Comet Assay Method was done according to the principles described by Singh and colleagues [15]. Whole blood samples were taken to heparinized biochemical tubes for DNA damage analysis and lymphocyte separation was performed immediately.

4. Statistical analyses

SPSS 20 program was used for statistical analysis. Data were expressed as mean, standard deviation, median, frequency, rate, minimum, maximum, where appropriate. Student's t test and Mann Whitney U test were used for the comparisons of variables with or without normal distribution, respectively. Statistical significance was assessed at a p level of <0.05.

5. Results

Thirty cases with tuberous sclerosis and 29 controls were included. Demographic characteristics of the cases are presented in Table 1. The study was conducted between January 1, 2015 and June 30, 2016 at the Department of Pediatric Neurology, Faculty of Medicine, Bezmialem Vakıf University. A history of first- or second-degree consanguineous marriage was present in seven cases (15.3%). Mother and/or father was found to have been diagnosed with tuberous sclerosis in four patients (13.3%).

The age at diagnosis ranged between 0 months (intrauterine) and 108 months (mean: 30.6 ± 38.5 months) (Table 2). In four cases (13.3%) family

Table 1
Demographic characteristics of tuberous sclerosis cases and healthy controls.

	Tuberous sclerosis Group	Control Group
Age (min–max)	24–192 (month)	18–186 (month)
Gender (boy/girl)	16/14	16/13
Consanguineous marriage (±)	7/23	2/27
Total	30	29

Table 2
Diagnostic characteristics of tuberous sclerosis cases.

	Min-Max	Mean ± SD
Age at diagnosis (month)	0–108	30.6 ± 38.5
Complaint/sign leading to diagnosis	Number	%
Family history of tuberous sclerosis	4	13.3
Hypopigmented Macule	1	3.3
Seizure	22	73.3
Cardiac mass lesion (Rhabdomyoma)	3	10
Total	30	100

Table 3
Anti-epileptic drug distribution among patients.

Anti-Epileptic Drugs	Number	%
Sodium Valproate	5	16.7
Carbamazepine	1	3.3
Vigabatrin	1	3.3
Levetiracetam	2	6.7
Sodium Valproate + Topiramate	1	3.3
Sodium Valproate + Carbamazepine + Clonazepam	2	6.7
Sodium Valproate + Carbamazepine + Vigabatrin	1	3.3
Carbamazepine + Vigabatrin	4	13.3
Carbamazepine + Levetiracetam	1	3.3
Carbamazepine + Clobazam + Lamotrigine	1	3.3
Vigabatrin + Levetiracetam + Topiramate	1	3.3
No anti-epileptic medication	10	33.3
Total	30	100

Table 4
Skin, renal and brain involvement in tuberous sclerosis cases (n = 30).

Involvement	n (%)
Skin	
Hypopigmented macules	29 (96.7%)
Facial angiofibroma	14 (46.7%)
Shagreen patches	2 (6.7%)
Hyperpigmented macules	2 (6.7%)
Renal	
Renal cyst	6 (20.0%)
Renal angiomyolipoma	6 (20.0%)
No renal finding	18 (60.0%)
Brain	
Cortical/subcortical tuber + subependymal nodule	28 (93.3%)
Giant cell astrocytoma	2 (6.7%)

history leaded to the diagnosis of tuberous sclerosis, whereas hypopigmented macula, seizures, and murmur or cardiac mass lesion was the complaint/sign leading to the diagnosis in 1 (3.3%), 22 (73.3%), and 3 (10%) cases, respectively (Table 2).

At time of initial diagnosis, 26 tuberous sclerosis cases had a history of seizures and 20 had a history of epileptic use. Table 3 shows the details of antiepileptic use in cases. On the other hand, EEG examinations performed at the time of study period showed the presence of focal epileptic disorder in 5 cases (16.7%) and multifocal epileptic disorder in 8 cases (26.7%), while EEG was normal in 17 (56.7%).

The dermatological findings included hypopigmented macules in 29 (96.7%) patients, facial angio-fibroma in 14 (46.7%), shagreen patches in 2 (6.7%), and hyperpigmented macules in 2 (6.7%) (Table 4). Retinal hamartoma was present in 2 (6.7%) patients, while renal cyst was present in 6 (20%) cases and renal angiomyolipoma in 6 (20%). Eighteen patients (60%) had no renal findings (Table 4). In addition, there were 28 subjects (93.3%) with cortical/subcortical tuber and subependymal nodules, and 2 with giant cell astrocytoma with a subependymal nodule (Table 4).

Eleven patients (36.7%) had mental retardation requiring special educational support, and 6 (20%) had severe mental retardation requiring rehabilitation.

6. TAS, TOS, OSI and DNA damage in the groups

Table 5 shows the results of TAS, TOS and DNA damage assessments in the study groups. TAS was significantly lower ($p < 0.01$), while TOS and OSI were significantly higher ($p = 0.001$ and $p < 0.01$, respectively) in patients as compared to controls (Table 5). Also, DNA damage assay showed significantly more marked damage in patients than in controls ($p < 0.01$, Table 5). Spearman's Correlation Analysis found no significant correlations between TAS, TOS and DNA damage in study groups ($p > 0.05$). In addition, sub-group analyses

Table 5
Comparison of groups in terms of TAS, TOS, OSI and DNA damage.

		Group		*p*
		Patients (n = 30)	Controls (n = 29)	
TAS	*Min-Max*	0.61–0.99	0.66–1.97	***<0.01***[a]
	Mean ± SD	0.83 ± 0.09	1.42 ± 0.42	
TOS	*Min-Max*	8.54–13.98	5.66–10.06	***<0.01***[b]
	Mean ± SD	11.44 ± 1.26	7.82 ± 0.98	
OSI	*Min-Max*	10.81–20.16	3.78–12.41	***<0.01***[a]
	Mean ± SD	13.91 ± 1.97	6.09 ± 2.26	
Comet (%TALE)	*Min-Max*	36.76–69.76	16.26–33	***<0.01***[a]
	Mean ± SD	58.90 ± 6.91	26.69 ± 3.42	

Abbreviations: TAS, Total Antioxidant Status; *TOS,* Total Oxidant Status; *OSI,* Oxidative Stress Index.
[a] Mann Whitney U Test.
[b] Student-t Test.

showed that TAS, TOS and DNA damage was similar across patients with vs. without renal involvement, facial angiofibroma, or history of seizure at the time of initial diagnosis ($p > 0.05$ for all comparisons).

7. Discussion

Tuberous sclerosis is a genetic disorder affecting many organs and systems. The mean age of the patients who participated in the study was 30.6 ± 38.5 months, and convulsions represented the initial manifestation of the disease in 73.3% of the cases, consistent with previous reports [16].

EEG abnormalities have been reported in 50–75% of patients with tuberous sclerosis [17,18], while this figure was 43.3% in our study.

Skin findings are also common in tuberous sclerosis, and the most common skin finding is hypopigmented lesions. These are oval or leaf-shaped lesions varying in size, most commonly located at the trunk and limbs. In general, lesions are present at birth and/or become apparent during the first years of life [2,19]. Osbourne et al. reported hypomelanic macules in all patients under the age of five years, whereas they were present in 78% of the cases aged over 5 years [2]. This study included children aged both below and over 5 years and the most common skin finding was hypopigmented macule with 96.7% frequency, which is in line with those previous findings.

In a previous study, Rowley and colleagues [20] detected retinal hamartoma in 44 of 100 cases (44%) in their study, while two (6.7%) patients had retinal hamartoma in the present study. The median age of the patients was 27 (range 2–76 years) in the study by Rowley, et al. It is far higher than the age of the patients in this study, which may account for the major variation in retinal hamartoma frequencies across the two studies.

In patients with tuberous sclerosis, the most common renal lesions include angiomyolipomas and renal cysts. However, renal carcinomas may also occur. Angiomyolipomas can be identified in approximately 50–80% of patients. On the other hand, the exact incidence of renal cysts is unknown [21]. The frequency of angiomyolipomas increases with age, while no such age relationship exits for renal cysts [6,22]. In our study, 6 (20%) patients had renal cysts and 6 (20%) patients had renal angiomyolipomas. The lower incidence of retinal hamartomas and renal angiomyolipomas in our study could be due to the relatively younger age of our study participants.

Subependymal nodules and corticosubcortical tubers represent important brain-imaging findings in the diagnosis of tuberous sclerosis. Previous studies have reported the presence of subependymal nodules and cortical-subcortical tubers in 50%–100% of the patients with this condition [23–25], while 93.3% of our study subjects were found to have subependymal nodules together with cortical/subcortical tubers.

As compared with previous reports suggesting that 17–20% of patients may also have giant cell astrocytomas [23,24], only 6.7% of our patients had this type of lesions. The most common cardiac manifestation of tuberous sclerosis involves cardiac rhabdomyomas, that have been reported to occur in 50–80% of the patients [23,26]. In our study, 10% of patients had cardiac rhabdomyomas (Table 2).

In tuberous sclerosis, mental functions are commonly affected and mental retardation has been reported in up to 50% of the patients [1,23]. In addition, patients with mental retardation are more likely to suffer from convulsions. Furthermore, in patients with early onset seizures, seizure control rates are generally poor and the disease course is more severe [27]. Six of our patients (20%) required intensive rehabilitation and special training, and these patients were receiving treatment with multiple antiepileptic drugs due to early-onset seizures, consistent with the literature.

mTOR repressors represent a novel therapeutic option for SEGAs, cardiac rhabdomyomas, or angiomyolipomas in selected cases of TSC [28]. Two of our patients were receiving treatment with rapamycin, one with resistant epilepsy, and another with renal angiomyolipoma.

Reactive oxygen species that arise as a result of an increase in oxidative stress attack the double bonds of the intracellular lipid and protein structures and the double bonds of the bases in the DNA, resulting in damage to the macromolecules such as intracellular lipids, proteins and DNA, leading to cell damage or cell death. [29].

In a previous study, Ozcan and colleagues showed increased endoplasmic reticulum stress and abnormal protein production in cells with TSC1 and TSC2 defects, and thus mTOR activity was adversely affected [30]. Similarly, Di Nardo and colleagues showed that oxidative stress and number of ER were increased in rat brain neurons with TSC2 deficiency, with a consequent limitation of mTOR activity [31]. With regard to oxidative stress, TOS and OSI were significantly increased in our patient group, consistent with the above-mentioned findings. In the study conducted by Reiling and colleagues, the increase in mTOR activity has been shown to induce stress response [32]. These authors also showed that stress was not a cause for the increase in mTOR activity, but rather the result of increased mTOR activity. The direction of the causal relationship between oxidative stress and mTOR activity remains to be elucidated.

One potential explanation for the increased oxidative stress and decreased antioxidant activity in tuberous sclerosis may be the clinical manifestations themselves. However, subgroups analyses for clinical manifestations did not identify a relation between specific system

involvements and oxidant/antioxidant activity in this study. However, it is of note to emphasize that the study was not powered to detect such differences since the sample size is too low; thus, such a potential relation remains to be clarified in future studies.

In terms of the DNA damage associated with oxidative stress, previous studies on renal cells found increased oxidative DNA damage in cells with tuberin defects [33]. In that same study, tuberin was also reported to play an important role in the formation and purification of oxygen radicals and, if defective, increased oxidative stress and the associated DNA damage. In a similar study by the same research team, the tuberin gene defect was shown to reduce the production of OGG1, a DNA repair enzyme, and to increase oxidative DNA damage [34]. Similarly, in our study, patients with tuberous sclerosis had a significant increase in the extent of DNA damage as compared to controls, as documented by the Comet Assay. The dysregulation of oxidative/antioxidative balance may play an important role in the pathogenesis of tuberous sclerosis.

Funding

This study was funded by Bezmialem Vakif University BAP Unit.

References

[1] Fryer AE, Osborne JP. Tuberous sclerosis a clinical appraisal. Paediatric Rev Commun 1987;1:239–55.
[2] Osborne JP, Fryer A, Webb D. Epidemiology of tuberous sclerosis. Ann N Y Acad Sci 1991;615:125–7.
[3] Crino PB, Nathanson KL, Henske EP. The tuberous sclerosis complex. N Engl J Med 2006;355:1345–56.
[4] Schwartz RA, Fernandez G, Kotulska K, Jozwiak S. Tuberous sclerosis complex: advances in diagnosis, genetics, and management. J Am Acad Dermatol 2007;57:189–202.
[5] Curatolo P, Bombardieri R, Jozwiak S. Tuberous sclerosis. Lancet 2008;372:657–68.
[6] Narla LD, Slovis TL, Watts FB, Nigro M. The renal lesions of tuberosclerosis (cysts and angiomyolipoma)–screening with sonography and computerized tomography. Pediatr Radiol 1988;18:205–9.
[7] Halpenny D, Snow A, McNeill G, Torreggiani WC. The radiological diagnosis and treatment of renal angiomyolipoma-current status. Clin Radiol 2010;65:99–108.
[8] Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 1991;91:14S–22S.
[9] Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381–478.
[10] Williams GM, Jeffrey AM. Oxidative DNA damage: endogenous and chemically induced. Regul Toxicol Pharmacol 2000;32:283–92.
[11] Northrup H, Krueger DA, International Tuberous Sclerosis Complex Consensus G. Tuberous sclerosis complex diagnostic criteria update: recommendations of the. Iinternational Tuberous Sclerosis Complex Consensus Conference. Pediatr Neurol 2012;2013(49):243–54.
[12] Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004;37:277–85.
[13] Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38:1103–11.
[14] Kosecik M, Erel O, Sevinc E, Selek S. Increased oxidative stress in children exposed to passive smoking. Int J Cardiol 2005;100:61–4.
[15] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.
[16] Pampiglione G, Moynahan EJ. The tuberous sclerosis syndrome: clinical and EEG studies in 100 children. J Neurol Neurosurg Psychiatry 1976;39:666–73.
[17] Chu-Shore CJ, Major P, Camposano S, Muzykewicz D, Thiele EA. The natural history of epilepsy in tuberous sclerosis complex. Epilepsia 2010;51:1236–41.
[18] Westmoreland BF. The electroencephalogram in tuberous sclerosis. In: Gomez MR, Sampson JR, Whittemore VH, editors. Tuberous sclerosis complex: developmental perspectives in psychiatry. New York: Oxford University Press; 1999. p. 63.
[19] Fitzpatrick TB. History and significance of white macules, earliest visible sign of tuberous sclerosis. Ann N Y Acad Sci 1991;615:26–35.
[20] Rowley SA, O'Callaghan FJ, Osborne JP. Ophthalmic manifestations of tuberous sclerosis: a population based study. Br J Ophthalmol 2001;85:420–3.
[21] Bernstein J. Renal cystic disease in the tuberous sclerosis complex. Pediatr Nephrol 1993;7:490–5.
[22] Stillwell TJ, Gomez MR, Kelalis PP. Renal lesions in tuberous sclerosis. J Urol 1987;138:477-81.
[23] Baron Y, Barkovich AJ. MR imaging of tuberous sclerosis in neonates and young infants. Am J Neuroradiol 1999;20:907–16.
[24] Braffman BH, Bilaniuk LT, Naidich TP, Altman NR, Post MJ, Quencer RM, et al. MR imaging of tuberous sclerosis: pathogenesis of this phakomatosis, use of gadopentetate dimeglumine, and literature review. Radiology 1992;183:227–38.
[25] Roach ES, Williams DP, Laster DW. Magnetic resonance imaging in tuberous sclerosis. Arch Neurol 1987;44:301–3.
[26] Quek SC, Yip W, Quek ST, Chang SK, Wong ML, Low PS. Cardiac manifestations in tuberous sclerosis: a 10-year review. J Paediatr Child Health 1998;34:283–7.
[27] Shepherd CW, Houser OW, Gomez MR. MR findings in tuberous sclerosis complex and correlation with seizure development and mental impairment. AJNR Am J Neuroradiol 1995;16:149–55.
[28] Kohrman MH. Emerging treatments in the management of tuberous sclerosis complex. Pediatr Neurol 2012;46:267–75.
[29] Ozcan O, Erdal H, Cakirca G, Yonden Z. Oxidative stress and its impacts on intracellular lipids, proteins and DNA. Journal of Clinical and Experimental Investigations 2015;6:331–6.
[30] Ozcan U, Ozcan L, Yilmaz E, Duvel K, Sahin M, Manning BD, et al. Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis. Mol Cell 2008;29:541–51.
[31] Di Nardo A, Kramvis I, Cho N, Sadowski A, Meikle L, Kwiatkowski DJ, et al. Tuberous sclerosis complex activity is required to control neuronal stress responses in an mTOR-dependent manner. J Neurosci 2009;29:5926–37.
[32] Reiling JH, Sabatini DM. Increased mTORC1 signaling upregulates stress. Molecular Cell Previews 2008;29:533–5.
[33] Habib SL, Abboud HE. Tuberin regulates reactive oxygen species in renal proximal cells, kidney from rodents, and kidney from patients with tuberous sclerosis complex. Cancer Sci 2016;107:1092–100.
[34] Habib SL, Liang S. Hyperactivation of Akt/mTOR and deficiency in tuberin increased the oxidative DNA damage in kidney cancer patients with diabetes. Oncotarget 2014;5:2542–50.