

Mutational profile of *TP53* in esophageal squamous cell carcinoma associated with chagasic megaesophagus

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SUMMARY. Chaga's disease is an important communicable neglected disease that is gaining wider attention due to its increasing incidence worldwide. Achalasia due to chagasic megaesophagus (CM), a complication of this disease, is a known—yet, poorly understood—etiological factor for esophageal squamous cell carcinoma (ESCC) development. In this study, we aimed to perform the analysis of *TP53* mutations in a series of Brazilian patients with ESCC that developed in the context CM (ESCC/CM), and to compare with the *TP53* mutation profile of patients with benign CM and patients with nonchagasic ESCC. Additionally, we intended to correlate the *TP53* mutation results with patient's clinical pathological features. By polymerase chain reaction (PCR) followed by direct sequencing of the hotspot regions of *TP53* (exon 5 to 8), we found that *TP53* mutations were present in 40.6% (13/32) of the ESCC/CM group, 45% (18/40) of the nonchagasic ESCC group, and in only 3% (1/33) of the benign CM group. Missense mutations were the most common in the three groups, yet, the type and mutated exon mutation varied significantly among the groups. Clinically, the groups exhibited distinct features, with both cancer groups (ESCC and ESCC/CM) been significantly associated higher consumption of alcohol and tobacco, older age, worse Karnofsky performance status, poor outcome than the patients with benign CM. No significant association was found between *TP53* mutation profile and clinical–pathological features in any of the three groups. We describe first the time the analysis of *TP53* mutations in ESCC that developed in the context of CM, and the observed high frequency of mutations, suggest that *TP53* also plays an important role in the tumorigenic process of this unexplored etiological condition.

KEY WORDS: esophageal squamous cell carcinoma (ESCC), megaesophagus, mutation, *TP53*.

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INTRODUCTION

Esophageal cancer is the eighth most common cause of cancer worldwide, the sixth most frequent cause of cancer death.¹ In Brazil, according to estimates

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from the Brazilian National Cancer Institute (INCA) for 2016, esophageal cancer appears as the 10th most common cause of cancer, with an estimated 10,810 new cases for the year 2016.²

Esophageal cancer has two main histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EADC).³ These two histological types differ in both incidence trends and etiological factors. ESCC is the most frequent histological subtype in less developed countries, being tobacco use and alcohol consumption, followed by drinking hot beverage and nutritional deficiencies, the major risk factors.^{1,3–5} In contrast, EADC represents up to 50% of esophageal cancer cases in Western countries and its incidence has been rapidly increasing, mostly explained by increasing in prevalence of obesity and other lifestyle-associated factors.^{1,3} In Brazil, ESCC is the most frequent histological subtype, due to the abovementioned etiological agents.^{6,7} Interestingly, in the southeast region of Brazil, the high prevalence of megaesophagus and achalasia due to Chagas disease has been also described as another predisposition factor for ESCC development.^{8–10}

Achalasia is an esophageal motility disorder characterized by aperistalsis of the esophageal body and failure of lower esophageal sphincter relaxation, which lead to esophageal dilatation or megaesophagus and progressive dysphagia in affected patients.¹¹ The pathogenesis of achalasia is poorly understood and Chagas disease is the only proven etiological factor for achalasia.^{12–14}

Chagas disease, which is triggered by *Trypanosoma cruzi* infection, affects approximately 16 million people in Latin America and Caribbean.¹⁵ Today, Chagas disease also affects other nonendemic regions of the world, due to migration, with several infected cases notified in countries such as Spain, the United States, Canada, Australia, and Japan.¹⁵ It is estimated that in Brazil, between 3 and 4 million people are infected, with some endemic regions, mainly in rural areas with 3,6 deaths per 100.000 inhabitants/year in the last 30 years.^{16,17}

The initial association of megaesophagus with ESCC risk factor was reported by Fagge in the XIX century.¹⁸ Following this observation, several studies showed that patients with megaesophagus exhibited an increasing risk of 3.9%–10% of ESCC development, when compared to the normal population.^{8,19,20} The pathophysiological mechanism of development of ESCC in patients with CM is poorly understood. It is believed to be due the parasitic destruction of neurons intramural myenteric, which leads to hypertrophy of the smooth muscle cells and increased connective tissue and thickening of the esophagus wall (megaesophagus), with consequent food stasis and inflammation, acanthosis, hyperkeratosis, and leucoplakia, some of them considered as

probably premalignant conditions for ESCC.^{8,19–21} The studies evaluating the molecular mechanisms underlying this carcinogenic process are scarce.²²

TP53 tumor suppressor gene exhibited a major role in the tumorigenesis of ESCC.²³ *TP53* is, by far, the highest mutated gene, with frequencies varying between studies and population, and recently, whole genome sequencing analysis, confirm the high frequency ($\approx 60\%$) of *TP53* mutations.²⁴ In Brazil, it is been reported that approximately 35% of ESCC exhibited *TP53* mutations.²⁵

In the present study, we aimed to interrogate the *TP53* mutation status in a series of ESCC that develops in patients with chagasic megaesophagus (CM). We further intended to compare with the *TP53* mutation profile of patients with benign CM and with patients with nonchagasic ESCC. Furthermore, we will correlate the *TP53* mutation profile with patient's clinical pathological features.

MATERIALS AND METHODS

Study population

In this retrospective study, we analyzed 126 patients treated between 1990 and 2012 at three Brazilian hospitals from the southeast of Brazil: Hospital de Câncer de Barretos, Barretos, São Paulo State; Universidade Estadual Paulista (UNESP), Botucatu, São Paulo State; and Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, Minas Gerais State. A comprehensive clinical-pathological questioner, including risk factors for esophageal cancer, Chagas serology of all patients was performed and data were retrospectively collected from medical records of the respective hospitals medical.

Of the 126 patients, we were not able to perform the *TP53* mutation analysis in 21 patients, due to absence of available tumor tissue or poor quality of the obtained DNA. The remaining 105 patients were divided in three groups: (i) 32 patients with ESCC associated with chagasic megaesophagus confirmed by histopathologic and radiologic reports, and positive serology for Chagas disease (denominated ESCC/CM); (ii) 33 patients with radiological and histopathological confirmation of chagasic megaesophagus without ESCC (benign chagasic megaesophagus) and positive serology for Chagas disease (denominated CM); and (iii) 40 patients with ESCC without the presence of chagasic megaesophagus and negative serology for Chagas disease (denominated ESCC). Patients previously treated with chemotherapy or radiotherapy and without Chagas serology or tomography were not included in the study. This study was approved by the local ethic committees (409/2010).

Table 1 The clinical-pathological features of the three groups of patients

Variable	Category	Groups (N = 105 cases)			P-value
		ESCC (N = 40)	CM (N = 33)	ESCC/CM (N = 32)	
Gender	Male	33 (82.5%)	29 (87.9%)	28 (87.5%)	0.828*
	Female	7 (17.5%)	4 (12.1%)	4 (12.5%)	
Age (years)	Mean (SD)	57.63 (9.2)	43.43 (12.3)	60.16 (10.6)	<0.001**
	Min-max	36-75	24-60	37-76	
Karnofsky	>70	38 (95%)	33 (100.0%)	22 (73.3%)	0.001*
	≤70	2 (5%)	0 (0.0%)	8 (26.7%)	
Tobacco	Never	6 (15.8%)	17 (51.5%)	7 (24.1%)	0.003***
	Ever	32 (84.2%)	16 (48.5%)	22 (75.9%)	
Alcoholism	Never	10 (26.3%)	23 (69.7%)	6 (20.7%)	<0.001***
	Ever	28 (73.7%)	10 (30.3%)	23 (79.3%)	
Megaesophagus	Grade I and II	NA	4 (12.1%)	6 (27.3%)	0.175*
	Grade III and IV	NA	29 (87.9%)	16 (72.7%)	
Differentiation	Grade I	3 (20.0%)	NA	3 (33.3%)	0.828*
	Grade II	10 (66.7%)	NA	5 (55.6%)	
	Grade III	2 (13.3%)	NA	1 (11.1%)	
TNM staging	I and II	15 (37.5%)	NA	7 (23.3%)	0.299***
	III and IV	25 (62.5%)	NA	23 (76.7%)	
Current status	Alive without disease	9 (22.5%)	27 (81.8%)	2 (6.7%)	<0.001***
	Alive with disease	14 (35.0%)	0 (0.0%)	9 (30.0%)	
	Death by disease	16 (40.0%)	0 (0.0%)	19 (63.3%)	
	Death by other causes	1 (2.5%)	6 (18.2%)	0 (0.0%)	

*Fisher's exact test.

**Analysis of variance.

***Chi-square test.

CM, chagasic megaesophagus; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus; N, number of cases, NA, not applicable; SD, standard deviation.

DNA isolation

Formalin-fixed paraffin-embedded (FFPE) tumors and tissue representative of the lesions were retrieved from the Department of Pathology of the respective hospitals, and all cases were histopathologically reclassified. DNA was obtained from FFPE sections as previously described, with some modifications.²⁶ Briefly, serially 10 μm thick unstained sections of paraffin blocks were sectioned, with one H&E section for identification and selection of the selected portion (ESCC/CM—tumor tissue; CM—megaesophagus tissue; and ESCC—tumor tissue). Selected areas were macrodissected into a microfuge tube using a sterile needle (Neolus, 25 G -0.5 mm). The macrodissected tissue was deparaffinized by a serial extraction with xylol and ethanol (100%–70%–50%) and allowed to air dry. DNA was extracted using Qiagen's QIAamp DNA Micro Kit following manufacture instructions and quantified by NanoDrop[®] 2000 (Thermo Scientific). DNA samples were stored at -20°C until further genetic analysis.

TP53 mutation analysis

Analysis of hotspot mutations of *TP53* (exons 5 to 8) was performed by polymerase chain reaction (PCR) followed by direct sequencing, as previously described by our group with some modifications.²⁷ The PCR

reaction was carried out in a total volume of 15 μL , comprising 1 μL of DNA, 1 \times buffer solution, 2 mM MgCl_2 , 200 μM of each dNTPs, 0.3 μM of each primer (sense and antisense) and 0.5 U Taq DNA polymerase (Invitrogen). The primers sequenced used were previously described.²⁷ The PCR reaction was performed in a Veriti 96-Well Thermal Cycler with an initial denaturation at 95°C for 10 min, amplified for 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C (exons 6 and 8) 57°C (exon 5 and 7) for 45 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 10 minutes. The PCR products were applied in 2.0% agarose gel with GelRed (Biotium).

The PCR product was then submitted to direct sequencing using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed an Genetic Analyzer ABI PRISM 3500 (Applied Biosystems). The chromatograms were compared to a *TP53* reference sequence (Ensembl—ENSG00000141510). All samples that present any alterations were confirmed with at least two new PCR amplifications and sequencing.

Statistical analysis

All statistical analyses were performed using the SPSS software (Statistical Package for Social Science,

Table 2 *TP53* mutation profile in the three groups of patients

Groups	DNA ID	Exon	Codon	Mutation	Mutation type	Amino acid change	Nature of mutation	
ESCC	2	8	273	CGT→CTT	G:C→T:A (CpG)	Arg→Leu	Missense	
	3	8	282	CGG→TGG	G:C→A:T (CpG)	Arg→Trp	Missense	
	7	6	196	CGA→TGA	G:C→A:T (CpG)	Arg→Stop	Nonsense	
	9	5	151	CCC→TCC	G:C→A:T	Pro→Ser	Missense	
	11	6	220	TAT→TGT	A:T→G:C	Tyr→Cys	Missense	
	14	5	151	CCC→CAC	G:C→T:A	Pro→His	Missense	
	16	8	285	GAG→AAG	G:C→A:T	Glu→Lys	Missense	
	17	8	275	TGT→TTT	G:C→T:A	Cys→Phe	Missense	
	18	8	275	TGT→TTT	G:C→T:A	Cys→Phe	Missense	
	22	8	275	TGT→TTT	G:C→T:A	Cys→Phe	Missense	
	24	8	273	CGT→TGT	G:C→A:T (CpG)	Arg→Cys	Missense	
	26	8	286	GAA→GTA	A:T→T:A	Glu→Val	Missense	
	27	8	280	AGA→AGC	A:T→C:G	Arg→Ser	Missense	
	32	8	298	GAG→TAG	G:C→T:A	Glu→Stop	Nonsense	
	34	7	248	CGG→CAG	G:C→A:T (CpG)	Arg→Gln	Missense	
	37	5	179	CAT→TAT	G:C→A:T	His→Tyr	Missense	
38	7	248	CGG→TGG	G:C→A:T (CpG)	Arg→Trp	Missense		
43	5	167	Del 1bp	Del	Gln→NA	Frameshift		
CM	74	6	204	GAG→AAG	G:C→A:T	Glu→Lys	Missense	
	40	8	282	CGG→TGG	G:C→A:T (CpG)	Arg→Trp	Missense	
	108	7	250	CCC→CTC	C:G→T:A	Pro→Leu	Missense	
ESCC/CM	114	5	141	TGC→TAC	G:C→T:A	Cys→Tyr	Missense	
	120	6	196	Del 1 bp	Del	Arg→NA	Frameshift	
	121	5	145	CTG→CAG	A:T→T:A	Leu→Glu	Missense	
	122	6	195	ATC→TTC	A:T→T:A	Ile→Phe	Missense	
	123	5	157	GTC→TTC	G:C→T:A	Val→Phe	Missense	
	124	8	306	CGA→TGA	G:C→A:T (CpG)	Arg→Stop	Nonsense	
	125	6	214	Del 2 bp	Del	His→NA	Frameshift	
	128	8	272	GTG→GAG	A:T→T:A	Val→Glu	Missense	
	129	5	132	AAG→AGG	A:T→G:C	Lys→Arg	Missense	
	ESCC/CM	130	8	267	Del 1 bp	Del	Arg→NA	Frameshift
			5	172	GTT→TTT	G:C→T:A	Val→Phe	Missense
		8	285	GAG→TAG	G:C→T:A	Glu→Stop	Nonsense	
		136	7	237	ATG→AAG	A:T→T:A	Met→Lys	Missense

A, adenine; bp, base pairs; C, cytosine; CM, chagasic megaesophagus; CpG, CpG dinucleotide; Del, deletion; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus; G, guanine; NA, not applicable; T, thymine.

v19.0). The characterization of the study population, including the results clinical-pathological, follow-up and molecular data were performed using descriptive statistics (for continuous variables: mean and standard deviation; for categorical variables: frequencies and percentages), presented in the form of tables. The associations of qualitative variables were performed using tests of association by the chi-square or Fisher's exact test. In the analysis of quantitative variables were used analysis of variance (when comparing three groups) and Student's t test (when comparing 2 independent groups), assuming a significance level of 5%. Overall survival of the three groups of patients was calculated employing the product limit estimator of the Kaplan–Meier method using the log-rank test to compare survival curves.

RESULTS

The major clinical-pathological features of the three groups of patients are summarized on Table 1. Some characteristics were significantly different between all

groups: patients with ESCC/CM and ESCC are older, exhibited a worse Karnofsky performance status, and showed a poor outcome than the patients with only CM (Table 1). We also observed a significant higher consumption of alcohol and tobacco in both cancer groups (ESCC and ESCC/CM), than in CM patients without cancer (Table 1).

Molecularly, the presence of nonsynonymous (silent mutations excluded) *TP53* mutations was observed in 32 patients (Table 2 and Fig. 1). We found *TP53* mutations in 13/32 (40.6%) ESCC/CM patients and in 18/40 (45%) ESCC patients. In the ESCC/CM group, two patients (ID129 and ID130) exhibited two distinct mutations (Table 2). Only one (3%, 1/33) CM patient showed the presence of *TP53* mutation (Table 2).

The *TP53* mutation frequency was statistically significantly different between the three groups ($P < 0.001$) (Table 3), mainly due to the low prevalence of *TP53* mutation observed in the CM group, when compared to the ESCC/CM and ESCC (Table 3). We further compared the distribution of *TP53* mutations by different exons in the three groups (Table 3). In the two

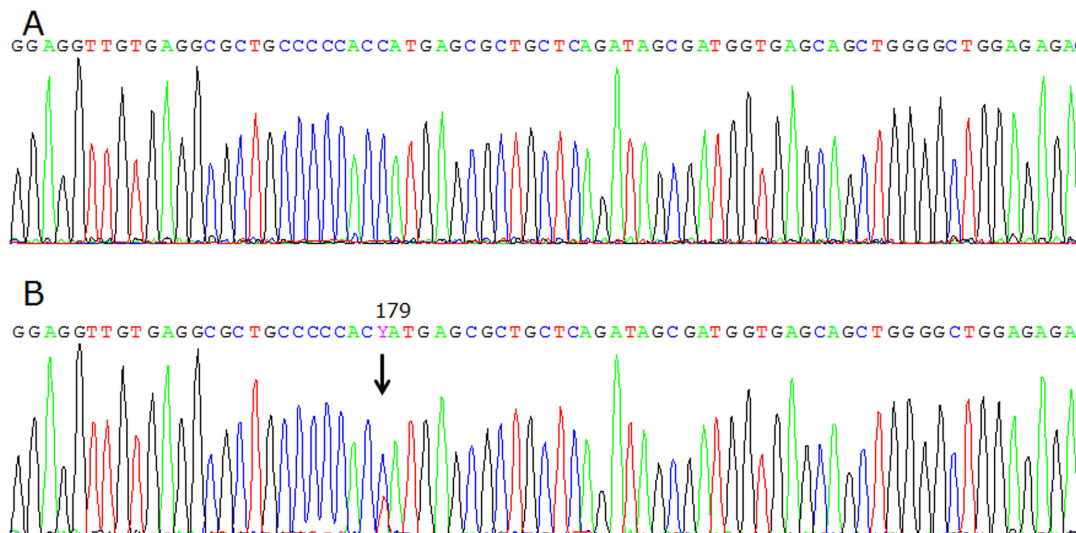


Fig. 1 Electropherogram of *TP53* gene. (A) *TP53* wild-type sequence. (B) *TP53* mutant (His179Tyr) sequence. A, adenine; C, cytosine; G, guanine; His, histidine; T, thymine, Tyr, tyrosine; Y, C and T.

Table 3 Association between frequency, exon distribution, nature and type of *TP53* mutations in the three groups of patients

			Groups (N = 105 cases)			P-value		
			ESCC (N = 40)	CM (N = 33)	ESCC/CM (N = 32)			
Frequency	<i>TP53</i>	Wild-type	22 (55.0%)	32 (97.0%)	19 (59.4%)	<0.001*		
		Mutant	18 (45.0%)	1 (3.0%)	13 (40.6%)			
Exons	5	Wild-type	36 (90.0%)	33 (100.0%)	27 (84.4%)	0.037**		
		Mutant	4 (10.0%)	0 (0.0%)	5 (15.6%)			
	6	Wild-type	38 (95.0%)	32 (97.0%)	29 (90.6%)	0.584**		
		Mutant	2 (5.0%)	1 (3.0%)	3 (9.4%)			
	7	Wild-type	38 (95.0%)	33 (100.0%)	30 (93.8%)	0.463**		
		Mutant	2 (5.0%)	0 (0.0%)	2 (6.2%)			
	8	Wild-type	30 (75.0%)	33 (100.0%)	27 (84.4%)	0.003**		
		Mutant	10 (25.0%)	0 (0.0%)	5 (15.6%)			
	Nature of mutations	Missense	Absent	25 (60.0%)	32 (97.0%)	22 (65.6%)	0.001*	
			Present	15 (40.0%)	1 (3.0%)	10 (34.4%)		
		Nonsense	Absent	38 (97.5%)	33 (100.0%)	30 (96.9%)		0.758**
			Present	2 (2.5%)	0 (0.0%)	2 (3.1%)		
Frameshift	Absent	39 (97.5%)	33 (100.0%)	29 (90.6%)	0.147**			
	Present	1 (2.5%)	0 (0.0%)	3 (9.4%)				

*Chi-square test.

**Fisher's exact test.

CM, chagasic megaesophagus; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus; N, number of cases.

groups of patients suffering from cancer (ESCC/CM and ESCC), mutations occurred in all 4 exons and in the CM group, the only mutation observed was on exon 5 (Table 3). In the ESCC/CM group and ESCC exons 5 and 8 were equally affected (15.6% and 10% respectively). Regarding the nature of the mutations, missense mutations were the most prevalent in all three groups (Table 3).

Moreover, the correlation of *TP53* mutation profile with patients clinical-pathological features indicates the absence of any significant association (Table 4). We also compared the overall survival curves between

TP53 mutated (red colour) and wild-type (blue colour) patients in each group and no significant differences ($P = 0.175$) were reported (Fig. 2).

In order to evaluate the potential carcinogenic fingerprinting of each patient group, we considered all nonsynonymous and synonymous (silent) mutations (Table 5 and Fig. 3). We observed that in the ESCC group, the most frequent type of mutation was G:C→A:T in the CpG (31.8%), followed by G:C→A:T non CpG (27.4%) and G:C→T:A (22.3%). In group ESCC/CM, the most frequent mutation was A:T→T:A (25%) and G:C→T:A (25%), followed by

Table 4 Association between *TP53* mutation status with clinical-pathological features in the three groups (*n* = 105)

Variable	Category	ESCC		CM		ESCC/CM		P-value
		<i>TP53</i> WT	<i>TP53</i> Mut	<i>TP53</i> WT	<i>TP53</i> Mut	<i>TP53</i> WT	<i>TP53</i> Mut	
Gender	Male	18 (81.8%)	15 (83.3%)	29 (90.6%)	0 (0.0%)	17 (89.5%)	11 (84.6%)	1.000*
	Female	4 (18.2%)	3 (16.7%)	3 (9.4%)	1 (100.0%)	2 (10.5%)	2 (15.4%)	
Age (years)	Mean (SD)	56.4 (8.3)	58.5 (10.2)	52.5 (10.0)	49.0 (10.0)	58.6 (10.1)	59.9 (9.9)	0.909**
	>70	20 (90.3%)	18 (100.0%)	32 (100.0%)	1 (100.0%)	13 (76.5%)	9 (69.2%)	0.698*
Karnofsky	≤70	2 (9.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (23.5%)	4 (30.8%)	0.452*
	Never	4 (18.2%)	2 (11.8%)	13 (43.3%)	1 (100.0%)	3 (17.6%)	4 (30.8%)	
Tobacco	Ever	18 (81.8%)	16 (88.9%)	17 (56.7%)	0 (0.0%)	14 (82.4%)	9 (69.2%)	1.000*
	Never	5 (22.7%)	4 (22.2%)	18 (62.1%)	1 (100.0%)	3 (17.6%)	2 (15.4%)	
Alcoholism	Ever	17 (77.3%)	14 (77.8%)	11 (37.9%)	0 (0.0%)	14 (82.4%)	11 (84.6%)	1.000*
	Grade I and II	NA	NA	4 (12.5%)	0 (0.0%)	3 (25.0%)	3 (30.0%)	
Megaesophagus	Grade III and IV	NA	NA	28 (87.5%)	1 (100.0%)	9 (75.0%)	7 (70.0%)	1.000*
	Grade I	2 (33.3%)	1 (11.1%)	NA	NA	1 (25.0%)	2 (40.0%)	
Differentiation	Grade II	3 (50.0%)	7 (77.8%)	NA	NA	2 (50.0%)	3 (60.0%)	1.000*
	Grade III	1 (16.7%)	1 (11.1%)	NA	NA	1 (25.0%)	0 (0.0%)	
TNM staging	I and II	11 (50.0%)	4 (22.2%)	NA	NA	6 (35.3%)	1 (7.7%)	0.104*
	III and IV	11 (50.0%)	14 (77.8%)	NA	NA	11 (64.7%)	12 (92.3%)	
Current Status	Alive without disease	7 (31.8%)	2 (11.1%)	26 (81.2%)	1 (100.0%)	1 (5.9%)	1 (7.7%)	1.000*
	Live with disease	7 (31.8%)	7 (38.9%)	0 (0.0%)	0 (0.0%)	5 (29.4%)	4 (30.8%)	
	Death by disease	7 (31.8%)	9 (50.0%)	0 (0.0%)	0 (0.0%)	11 (64.7%)	8 (61.5%)	
	Death by other causes	1 (4.5%)	0 (0.0%)	6 (18.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	

* Fisher's exact test.

** Analysis of variance.

CM, chagasic megaesophagus; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus; Mut, mutant; NA, not applicable; SD, standard deviation; WT, wild-type.

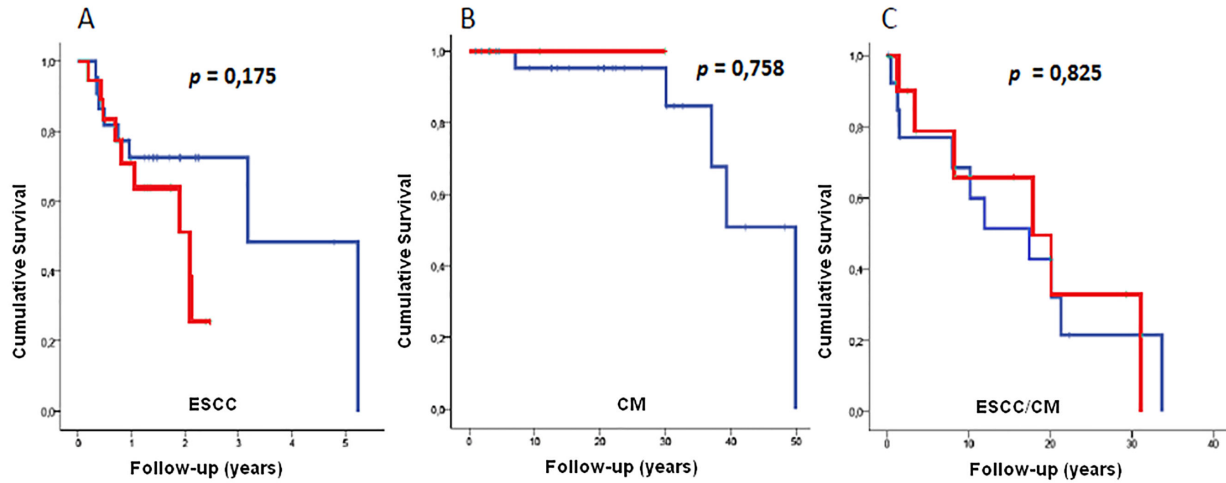


Fig. 2 Cumulative survival associated with the *TP53* gene status. (A) ESCC, squamous cell carcinoma of the esophagus; (B) CM, chagasic megaesophagus; (C) ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus.

Table 5 Frequency of the mutation type of *TP53* in exons 5, 6, 7, and 8 (nonsynonymous and synonymous included)

Mutation type	Groups (N = 105 cases)		
	ESCC (N = 40) N mut = 22	CM (N = 33) N mut = 6	ESCC/CM (N = 32) N mut = 16
A:T→C:G	0 (0.0%)	0 (0.0%)	0 (0.0%)
A:T→G:C	2 (9.1%)	0 (0.0%)	1 (6.2%)
A:T→T:A	1 (4.5%)	0 (0.0%)	4 (25.0%)
G:C→A:T	6 (27.4%)	6 (100.0%)	2 (12.5%)
G:C→A:T (CpG)	7 (31.8%)	0 (0.0%)	2 (12.5%)
G:C→C:G	0 (0.0%)	0 (0.0%)	0 (0.0%)
G:C→T:A	5 (22.3%)	0 (0.0%)	4 (25.0%)
Deletion	1 (4.5%)	0 (0.0%)	3 (18.8%)

CM, chagasic megaesophagus; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus, Mut, mutant; N, number of cases.

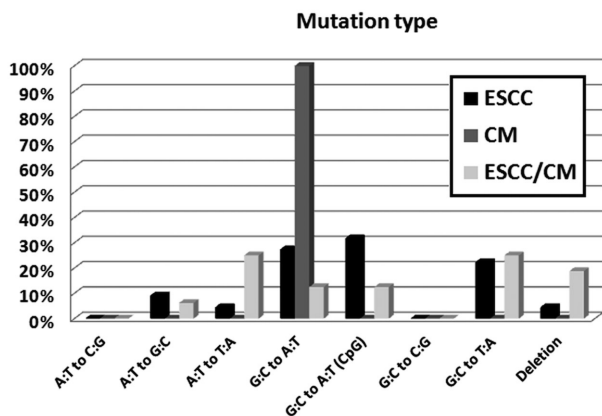


Fig. 3 Frequency distribution of fingerprinting profile. A, adenine; C, cytosine; CM, chagasic megaesophagus; ESCC, squamous cell carcinoma of the esophagus; ESCC/CM, squamous cell carcinoma of the esophagus associated with chagasic megaesophagus; G, guanine, T, thymine.

deletions (18.8%). In the CM group, all six mutations identified (1 nonsynonymous and 5 synonymous) were G:C→A:T non CpG (100%) (Table 5 and Fig. 3).

DISCUSSION

Chagasic megaesophagus (CM), a complication of Chagas disease, is considered a predisposition condition for the development of ESCC.^{8,19,20} Despite this known association, the molecular mechanisms underlying ESCC development in patients with CM are unexplored. Such studies are of particular relevance for Latin America and Brazil, where the Chagas disease is still endemic in some regions and represents an important health problem.¹⁵

Herein, we investigate for the first time the profile of *TP53* mutation in ESCC associated with CM (ESCC/CM) and compare it with the molecular profile of ESCC not associated with megaesophagus and with the benign CM. We showed that 40.6% of the ESCC that develop in the context of CM harbor *TP53* mutations, which was similar to the ESCC group (45%) and statistically superior to CM group (3%). The high frequency of *TP53* mutations in the ESCC/CM group suggests that *TP53* is also a key player in tumorigenesis of ESCC in the context of Chagas disease, as it has been widely accepted for ESCC.²⁴ The presence of *TP53* mutation in CM

patients, albeit at a significant lower frequency, may mean that the *TP53* mutation occurs already in pre-malignant lesions. Interestingly, a previous study analyzed a series of CM, also from the same region of the present study, for the presence of genomic imbalances in genes relevant in esophageal carcinogenesis, and any significant event was observed.²² So, *TP53* mutations in the conjugation other etiological factors may contribute to cancer development. In fact, our observation of a higher consumption of alcohol and tobacco observed in the ESCC/CM group in comparison with the CM group, supported this hypothesis. Nevertheless, further studies, using prospective epidemiological and case-control designed approaches and involving a higher number of patients are warranted to clarify this issue. We observed slightly higher frequency (40.6%) of *TP53* mutations in the ESCC without CM group, than previous published for Southern (34.8%) and Southeast (34.5%) Brazilian population,^{25,28} and relatively lower than what reported for countries with high incidence of ESCC, such as China (42%–77%) and Iran (50%–90%).^{29,30} These differences in ESCC *TP53* mutation frequencies observed worldwide may be associated with multiple factors, including different exposure agents, different population and different methodologies issues.

The *TP53* gene is frequently mutated in human tumors.²³ The majority of these mutations are missense and occur between exons 5–8 leading to a functional inactivation of cellular protein.²³ According to the IARC *TP53* mutation database, the hotspots *TP53* codons in ESCC of the esophagus are 170, 175, 179, 193, 220, 245, 248, 273, and 282.³¹ Most mutations identified in our cases were in codons 151 and 275 (with three mutations each codon), followed by codons 195, 196, 248, 273, 282, and 285 (with two mutations each codon). Although is not usually considered to be a hotspot codon, mutation at position 151 leads to a structural alteration, which results in significant functional changes in the p53 protein that impact tumor progression.³² Although codon 275 is not common referred to as hotspot mutations, the Cys275Phe found in our cases is reported as a deleterious somatic mutation that leads to loss of protein function.³¹

Besides the biological consequence, the analysis of *TP53* mutations allows interrogating about the potential carcinogenic agent who operated in each case, and therefore to understand the biological mechanisms that drive in malignant transformation. In other words, the mutation spectrum analysis can function as a molecular fingerprint of the agent that causes DNA damage.³³ The results of this study showed that the most frequent type of mutation in the ESCC group was G:C→A:T (CpG) (31.8%), followed by G:C→A:T at non CpG sites (27.4%) and G:C→T:A (22,3%). The transversion G:C→T:A mutations have

been frequently detected in lung tumors from heavy smokers and experimentally associated with DNA adduct formation by B[a]P metabolites.^{34,35} The G:C→A:T transitions at non CpG sites are associated with both acetaldehyde and nitrosamines,³⁴ so there is a concordance between the identified risk factors (alcohol and tobacco). This type of mutations is the most frequently described in CE.³⁶ In CM group, mutation type observed in all cases was a G:C→A:T, as described above associated with acetaldehyde and nitrosamines.³⁴ These latter agents are important compounds derived from food stasis observed inside the megaesophagus.³⁷ Finally, the most prevalent mutations in the group ESCC/CM are G:C→T:A and A:T→T:A, being the mutation G:C→T:A associated with benzopyrene (tobacco)^{34,35} and the A:T→T:A mutation are associated with acetaldehyde.³⁸ The high prevalence rates of these mutations in ESCC/CM patients, further suggests that smoking and alcohol are important cofactors for the development of ESCC in patients with CM.

In conclusion, we observed a high frequency of *TP53* mutation in ESCC/CM, suggesting the central role of *TP53* the development of this unknown and neglected tumor. Future studies will be important to validate and extend these important findings that may contribute to the elucidation of the carcinogenic pathway of ESCC associated with chagasic megaesophagus.

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