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PIK3CA mutations are frequent in esophageal squamous cell carcinoma associated with chagasic megaesophagus and are associated with a worse patient outcome

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Abstract

Background: Chronic diseases such as chagasic megaesophagus (secondary to Chagas' disease) have been suggested as etiological factors for esophageal squamous cell carcinoma; however, the molecular mechanisms involved are poorly understood.

Objective: We analyzed hotspot *PIK3CA* gene mutations in a series of esophageal squamous cell carcinomas associated or not with chagasic megaesophagus, as well as, in chagasic megaesophagus biopsies. We also checked for correlations between the presence of *PIK3CA* mutations with patients' clinical and pathological features.

Methods: The study included three different groups of patients: i) 23 patients with chagasic megaesophagus associated with esophageal squamous cell carcinoma (CM/ESCC); ii) 38 patients with esophageal squamous cell carcinoma not associated with chagasic megaesophagus (ESCC); and iii) 28 patients with chagasic megaesophagus without esophageal squamous cell carcinoma (CM). *PIK3CA* hotspot mutations in exons 9 and 20 were evaluated by PCR followed by direct sequencing technique.

Results: *PIK3CA* mutations were identified in 21.7% (5 out of 23) of CM/ESCC cases, in 10.5% (4 out of 38) of ESCC and in only 3.6% (1 case out of 28) of CM cases. In the CM/ESCC group, *PIK3CA* mutations were significantly associated with lower survival (mean 5 months), when compared to wild-type patients (mean 2.0 years). No other significant associations were observed between *PIK3CA* mutations and patients' clinical features or *TP53* mutation profile.

Conclusion: This is the first report on the presence of *PIK3CA* mutations in esophageal cancer associated with chagasic megaesophagus. The detection of *PIK3CA* mutations in benign chagasic megaesophagus lesions suggests their putative role in esophageal squamous cell carcinoma development and opens new opportunities for targeted-therapies for these diseases.

Keywords: Esophageal cancer, *Trypanosoma cruzi*, Achalasia, Esophageal squamous cell carcinoma, Chagasic megaesophagus, *PIK3CA*, Mutation

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Introduction

Esophageal cancer is the eighth most frequent type of cancer in the world and the sixth most lethal, occurring mainly in developing countries such as Brazil [1]. The most frequent histological subtype is esophageal squamous cell carcinoma (ESCC), accounting for 90% of cases, especially in high-risk areas [2]. The main risk factors for ESCC are alcohol consumption, tobacco (mainly in association) and hot-beverage consumption [3]. It is also reported that chronic diseases, such as the chagasic megaesophagus, can be associated with ESCC development [4].

Chagasic megaesophagus is the late manifestation of Chagas' disease (caused by the protozoan *Trypanosoma cruzi*) [5]. Direct infection of *Trypanosoma cruzi* will lead to destruction of the intramural myenteric neurons in the esophagus, causing inflammation and production of neurotoxins. This will result in uncoordinated contractions and reduction of peristalsis of the organ, altering the functioning of the lower esophageal sphincter and progressive dilation of the esophagus (megaesophagus) [6]. In Brazil, one of the endemic regions of Chagas' disease, approximately 4 million people are infected with the parasite and about 6–7% of these patients will develop chagasic megaesophagus [5]. Patients affected with this lesion are more likely to develop ESCC (3–10%) when compared to the general population [4].

The carcinogenic mechanisms of ESCC development in the context of chagasic megaesophagus have been little explored. Recently, our group showed the high frequency (13/32, 40.6%) of *TP53* mutations in ESCC associated with chagasic megaesophagus [7]. Moreover, we also reported the presence of microsatellite instability (MSI) in a small fraction (1/19, 5.3%) of cases [8]. However, many other genes are known to be involved in ESCC carcinogenesis as demonstrated by the TCGA consortium [9].

One of these genes is the *PI3KCA*, which encodes the protein phosphatidylinositol 3-kinase (PI3K), that belongs to a family of lipid kinases that encodes the p110 α catalytic subunit [10]. PI3K is a quite complex signaling pathway since it regulates cell growth, proliferation, cell motility, the production of new proteins, apoptosis and cell survival [10]. Therefore, its activation will lead to many downstream pathways that regulate several cellular functions, including those involved in the development of cancer [10, 11]. Recurrent *PI3KCA* oncogenic mutations were identified in several types of tumors, including colorectal, breast, ovary, gastric, and recently in ESCC [12]. The mutations occur mainly in exons 9 (E542K and E545K) and 20 (H1047R) [12]. Recently, it was shown that *PIK3CA* mutations, namely H1047R, also disrupt cellular genetic stability, increasing the frequency of chromosomal errors and leading to

tetraploidy [13]. Importantly, therapeutic strategies targeting the PIK3/Akt signaling pathway have been developed and could constitute effective treatment options for patients harboring *PI3KCA* mutations [14].

Therefore, in the current study we performed the mutation analysis of *PIK3CA* gene in patients with ESCC and chagasic megaesophagus associated or not with ESCC, and searched for associations between the mutation status and patients' clinical and pathological features.

Materials and methods

Study population

In this retrospective study, we analyzed 89 formalin-fixed paraffin-embedded (FFPE) tissues of three groups of patients: i) 23 patients with chagasic megaesophagus associated with esophageal squamous cell carcinoma (CM/ESCC); ii) 38 patients with esophageal squamous cell carcinoma without chagasic megaesophagus (ESCC); and iii) 28 patients with chagasic megaesophagus without esophageal squamous cell carcinoma (CM). All chagasic megaesophagus patients were serologic positive for Chagas' disease and/or had exams (imaging and histopathology) that confirmed the presence of megaesophagus. The patients with esophageal squamous cell carcinoma without chagasic megaesophagus were all serologic negative for Chagas' disease and had exams (imaging and histopathology) that confirmed malignant disease. These patients were previously described for their clinical-pathological and molecular *TP53* and MSI features [7, 8].

The samples were obtained from patients treated between 1990 and 2016 in three different institutions from the Southeast of Brazil, namely: Barretos Cancer Hospital, Barretos, São Paulo State; Federal University of Triângulo Mineiro (UFTM), Uberaba, Minas Gerais State and São Paulo State University (UNESP), Botucatu, São Paulo State. All clinical and pathological information was obtained through medical record review.

DNA isolation

Following tissue macro-dissection, DNA was isolated from FFPE tissue representative of the tumor lesions in ESCC and CM/ESCC groups and esophageal tissues in CM group, as previously described [7]. Briefly, the tumor area was delineated in a hematoxylin-eosin stained (HE, Merck KGaA, GE) section by a pathologist, and the marked area was scraped by scalp from 3 to 5 10 μ m unstained slides into a 1,5 ml tube. Afterwards, the tissue was subjected to the dewaxing step by heating (80 °C – 20 min), followed by sequential washing in xylol (5 min) and decreasing concentrations of ethanol (1 min - 100, 70 and 50%) and nuclease-free water (1 min). DNA isolation was performed using the QIAamp DNA Micro Kit (Qiagen) following the manufacture's protocol.

PIK3CA mutation analysis

Polymerase chain reaction (PCR) followed by direct sequencing (Sanger method) was performed for the analysis of hotspot mutations (exons 9 and 20) of the *PIK3CA* gene as previously described [15]. The PCR was performed on the 89 samples under the following conditions: 5X Flexi Buffer (pH 8.5) and 25 mM MgCl₂ (Promega, USA), 200 μM dNTPmix (Invitrogen, USA), 200 nM primers exon 9 (forward 5'-CTGTGAATCCAGAGGGGA AA-3' and reverse 5'-ACATGCTGAGATCAGCCAA AT-3') and exon 20 (forward 5'-ATGATGCTTGGCTC TGGAAT-3' and reverse 5'-GGTCTTTGCCTGCT GAGAGT-3'), 1.25 U GoTaq®Hot Start Polymerase (Promega, USA), and nuclease-free water (Gibco, BRL, Life Technologies, USA) in a final volume of 25 μl and 5 μl of DNA at 50 ng/μl from each patient were added [15]. Amplification was performed in a thermocycler according to the protocol: 96 °C for 15 min, followed by 40 cycles at 96 °C for 45 s, 55.5 °C for 45 s and 72 °C for 45 s and final extension of 72 °C for 10 min, followed by a hold at 4 °C. PCR products were subjected to 1.5% agarose gel electrophoresis with Gel Red (Biotium, Hayward, CA) to evaluate the amplification of the gene of interest.

After agarose gel validation, we purified the preparation using the enzyme ExoSap-IT (GE Technology, Cleveland, USA), followed by the sequencing reaction using BigDye Terminator v3.1 (Applied Biosystems, USA) and 3.2 μM of specific primers and re-purified with xTerminator (Life Technology). The products were sequenced using the 3500 series Genetic Analyzer Capillary Sequencer (Applied Biosystems, USA). All the cases that showed mutations were confirmed with a new PCR reaction and direct sequencing.

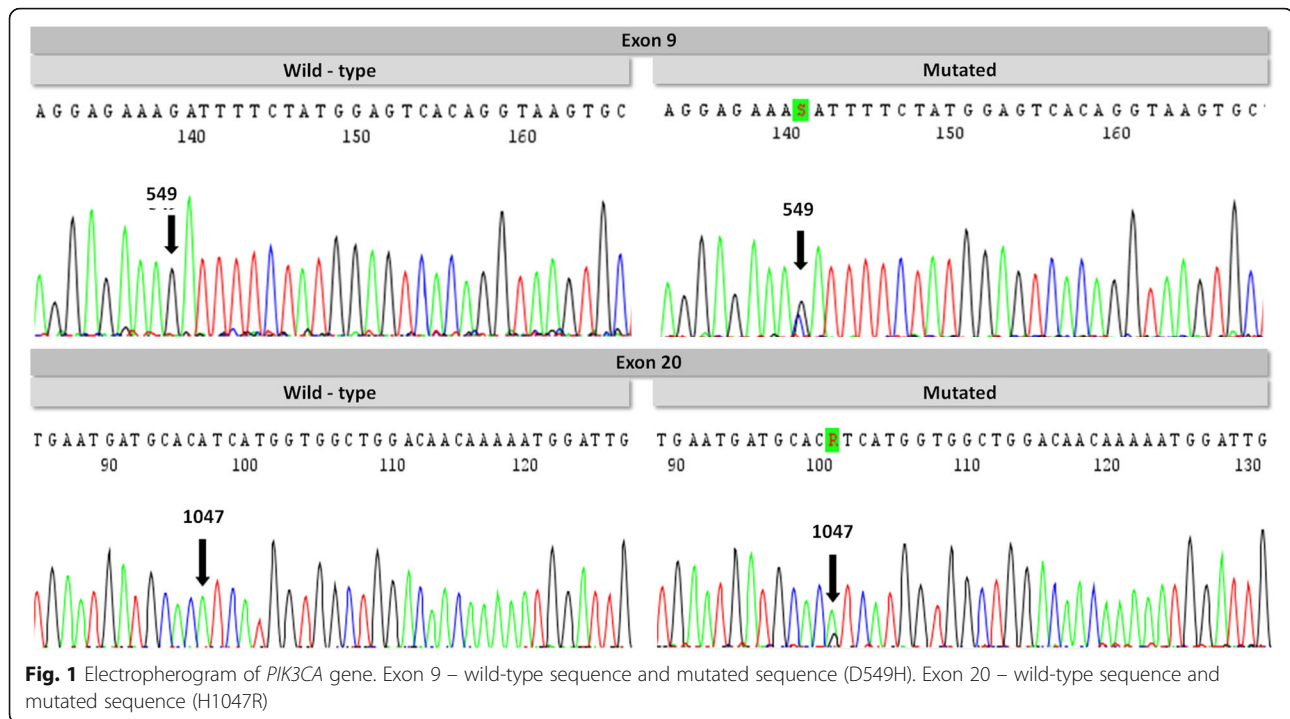
Statistical analysis

Characterization of the study population was performed through frequency tables for qualitative variables, and measures of central tendency and dispersion (mean, standard deviation, minimum and maximum) for the quantitative variables, comparing the different groups. To verify the association between *PIK3CA* mutation status and clinical groups, pathological and molecular features, Chi-square or Fisher's exact tests were applied. We performed an overall survival analysis using the Kaplan-Meier limit estimator and the Log-rank test to compare the survival curves between the groups.

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

Variable	Category	Groups (n = 89)			p-value
		CM/ESCC (n = 23)	ESCC (n = 38)	CM (n = 28)	
Gender	Female	4 (17.4%)	7 (18.4%)	4 (14.3%)	0.937**
	Male	19 (82.6%)	31 (81.6%)	24 (85.7%)	
Age (years)	Mean (SD)	59 (11)	57 (9)	59 (11)	0.039***
	Min - Max	37–76	36–75	37–76	
Alcohol consumption	No	7 (31.8%)	8 (21.1%)	20 (71.4%)	< 0.001*
	Yes	15 (68.2%)	30 (78.9%)	8 (28.6%)	
	Missing	11	0	0	
Tobacco consumption	No	4 (18.2%)	6 (15.8%)	16 (57.1%)	< 0.001*
	Yes	18 (81.8%)	32 (84.2%)	12 (42.9%)	
	Missing	1	0	0	
Tumor differentiation	Well differentiated	5 (22.7%)	7 (19.4%)	NA	0.639**
	Moderate differentiated	16 (72.7%)	24 (66.7%)	NA	
	Poorly differentiated	1 (4.5%)	5 (13.9%)	NA	
	Missing	1	2	NA	
TNM Staging	I/II	4 (23.5%)	16 (42.1%)	NA	0.235*
	III/IV	13 (76.5%)	22 (57.9%)	NA	
	Missing	6	0	NA	
Megaesophagus grades	GI/GII	10 (45.5%)	NA	4 (14.3%)	0.025*
	GIII/GIV	12 (54.5%)	NA	24 (85.7%)	
	Missing	1	NA	0	

*Chi-square association test; **Fisher's exact test; ***Analysis of variance. CM/ESCC – chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – esophageal squamous cell carcinoma without chagasic megaesophagus; CM – chagasic megaesophagus without esophageal squamous cell carcinoma; SD - standard deviation; NA – not applicable; Bold numbers - statistical significance



The level of significance adopted was 5% ($p \leq 0.05$). Statistical analyzes were in SPSS software v.21.0.

Results

Characterization of the population

The clinical-pathological characteristics of the patients in the three groups are described in Table 1. The mean age of the patients was higher in the chagasic groups (Table 1). As already reported in our previous studies [7], concerning risk factors for esophageal cancer, the ESCC and CM/ESCC groups were statistically associated with higher tobacco and alcohol consumption (Table 1).

Mutation analysis of *PIK3CA* gene

The *PIK3CA* mutation analysis showed the presence of mutations in 21.7% of patients in CM/ESCC group, followed by 10.5% in ESCC group and 3.6% in CM group (Fig. 1 and Table 2). The frequency of mutations was similar in exons 9 and 20 (Table 3). With the exception of three

variants (A1027D, K1030R and T1053K), all the other mutations have already been reported in the Catalogue of Somatic Mutations in Cancer database – COSMIC (<http://cancer.sanger.ac.uk/cosmic>) (Fig. 2 and Table 3).

No significant associations were observed between the *PIK3CA* mutation status and patients pathological features (Table 4). Furthermore, we assessed the role of *PIK3CA* mutations on patients' overall survival in both groups affected by cancer (CM/ESCC and ESCC) (Fig. 3). In CM/ESCC group, we observed that the presence of *PIK3CA* mutations was significantly associated with a lower survival rate from the diagnosis of cancer compared to wild-type patients (Fig. 3a). The mean patients' overall survival in cases from the CM/ESCC group mutated for the *PIK3CA* was 5 months, in comparison with 2.0 years for wild-type *PIK3CA* patients (Log-rank, $p < 0.001$) (Table 5).

Additionally, we evaluated the association of *PIK3CA* and *TP53* mutation status, and no association was found (Table 4).

Table 2 Frequency of *PIK3CA* mutations in the three study groups

Variable	Category	Groups (n = 89)			p-value
		CM/ESCC (n = 23)	ESCC (n = 38)	CM (n = 28)	
<i>PIK3CA</i> gene	WT	18 (78.3%)	34 (89.5%)	27 (96.4%)	0.132**
	MUT	5 (21.7%)	4 (10.5%)	1 (3.6%)	

**Fisher's exact test. CM/ESCC – chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – esophageal squamous cell carcinoma without chagasic megaesophagus; CM – chagasic megaesophagus without esophageal squamous cell carcinoma. WT – wild-type; MUT – mutant; N – number of cases

Table 3 Profile of oncogenic *PIK3CA* mutations in the three study groups

Group	Sample ID	Exon	Codon	Codon (WT – MUT)	Type of mutation	Amino acids change	Nature of mutation	COSMIC ID
CM/ESCC	111 T	9	545	GAG – GCA	A → C	E545A	Missense	COSM12458
	122 T	9	549	GAT – CAT	G → C	D549H	Missense	COSM219119
	114 T	20	1027	GCC – GAC	C → A	A1027D	Missense	Not reported
	119 T	20	1047	CAT – CTT	A → T	H1047L	Missense	COSM776
	124 T	20	1047	CAT – CGT	A → G	H1047R	Missense	COSM775
ESCC	26 T	9	545	GAG – GCG	A → C	E545A	Missense	COSM12458
	120 T	9	555	AGG – AAG	G → A	R555K	Missense	COSM1716158
	36 T	9	524	AGG – AAG	G → A	R524K	Missense	COSM53245
	4 T	20	1030	AAA – AGA	A → G	K1030R	Missense	Not reported
CME	101 M	20	1053	ACA – AAA	C → A	T1053K	Missense	Not reported

CME/ESCC – chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – esophageal squamous cell carcinoma without chagasic megaesophagus; CME – chagasic megaesophagus without esophageal squamous cell carcinoma. A – adenine; C – cytosine; G – guanine; T – thymine. WT – wild-type; MUT – mutant

Discussion

Among the several risk factors for the development of ESCC, the chagasic megaesophagus (late complication of Chagas' disease) has been a minor etiological factor and little explored [4]. Nevertheless, Chagas' disease is still an important public health problem, particularly in Latin-America, where approximately 20 million people are infected with Chagas' disease and approximately 6–7% of these people will develop chagasic megaesophagus [5, 16].

In the present study, we investigated the frequency of *PIK3CA* mutations in regions of hotspot (exons 9 and 20) in patients with chagasic megaesophagus associated with esophageal squamous cell carcinoma (CM/ESCC) and compared with patients with esophageal squamous cell carcinoma without chagasic megaesophagus (ESCC) and patients with chagasic megaesophagus without esophageal squamous cell carcinoma (CM). We observed that patients in the CM/ESCC group had a higher frequency of mutations (5/23, 21.7%) followed by patients in the ESCC group (4/38, 10.5%), and in the CM group (1/28, 3.6%). This is the first report of *PIK3CA*

mutation in ESCC that developed in the context of chagasic megaesophagus and the significant frequency of mutations (~ 22%) suggest that *PIK3CA* plays an important role in the carcinogenesis of CM/ESCC patients. Moreover, the presence of *PIK3CA* mutation in a benign lesion further supports the putative role of chagasic megaesophagus as an ESCC-related condition. The frequency of mutations identified in our study is in line with that reported in the literature for ESCC patients, with frequencies varying from 2.2 to 32.8% (Table 6) [9, 17–33]. This variation can be due to several factors, such as type of tissue (frozen vs FFPE), distinct methodologies for mutation screening and distinct ethnic groups of patients. (Table 6).

The *PIK3CA* gene is often mutated in several tumors types and most of its mutations occur in hotspot regions, such as E542K and E545A located in the helical domain (exon 9), and H1047R and H1047L in the kinase domain (exon 20) [11]. These mutations lead to the activation of the PIK3 pathway and have a great potential in oncogenic activities [11]. Interestingly, most of these mutations (E545A, H1047R and H1047L) occurred in

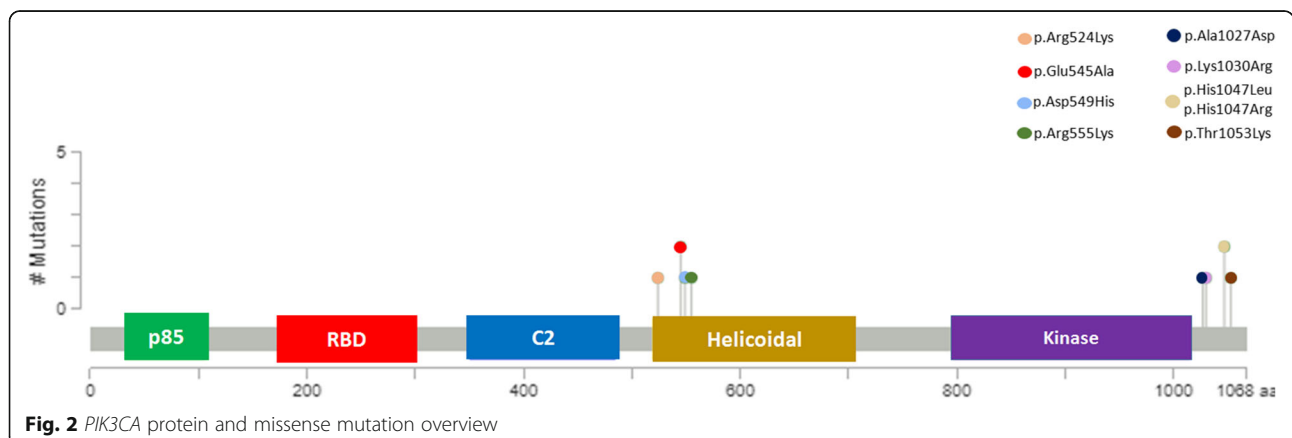


Table 4 Association between *PIK3CA* mutation status with main clinical-pathological features in the three groups

Variable	Category	<i>PIK3CA</i> gene								
		CM/ESCC group			ESCC group			CM group		
		WT	MUT	p-value	WT	MUT	p-value	WT	MUT	p-value
Alcohol consumption	No	6 (35.3%)	1 (20%)	1.000**	7 (21.2%)	1 (20%)	1.000**	19 (70.4%)	1 (100%)	1.000**
	Yes	11 (64.7%)	4 (80%)		26 (78.8%)	4 (80%)		8 (29.6%)	0 (0%)	
Tobacco consumption	No	4 (23.5%)	0 (0%)	0.535**	5 (15.2%)	1 (20%)	1.000**	15 (100%)	1 (100%)	1.000**
	Yes	13 (76.5%)	5 (100%)		28 (84.8%)	4 (80%)		12 (44.4%)	0 (0%)	
Tumor differentiation	Well	4 (22.2%)	1 (25%)	1.000**	7 (22.6%)	0 (0%)	0.171**	NA	NA	NA
	Moderate	13 (72.2%)	3 (75%)		21 (67.7%)	3 (60%)		NA	NA	
	Poorly	1 (5.6%)	0 (0%)		3 (9.7%)	2 (40%)		NA	NA	
TNM Staging	I e II	4 (28.6%)	0 (0%)	0.541**	15 (46.9%)	1 (20%)	0.421**	NA	NA	NA
	III e IV	10 (71.4%)	3 (100%)		17 (53.1%)	4 (80%)		NA	NA	
Megaesophagus degree	GI/GII	7 (41.2%)	3 (60%)	0.406**	NA	NA	NA	3 (11.1%)	1 (100%)	0.143**
	GIII/GIV	10 (58.8%)	2 (40%)		NA	NA		24 (88.9%)	0 (0%)	
<i>TP53</i> gene [7]	WT	9 (50%)	2 (40%)	1.000**	20 (60.6%)	2 (40%)	0.632**	26 (96.3%)	1 (100%)	1.000**
	MUT	9 (50%)	3 (60%)		13 (39.4%)	3 (60%)		1 (3.7%)	0 (0%)	

Fisher’s exact test; *Analysis of variance. CME/ESCC – chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – esophageal squamous cell carcinoma without chagasic megaesophagus; CME – chagasic megaesophagus without esophageal squamous cell carcinoma. N – number of cases; SD – standard deviation; NA – not applicable; WT – wild-type; MUT – mutated

patients in the CM/ESCC group and only one (E545A) in one patient in the ESCC group. We also identified other previously described important mutations (Table 3), the D549H mutation observed in the CM/ESCC group was reported in vulva and hepatocellular cancer [34]; R524K mutation found in the ESCC group was reported in colorectal cancer [35]; and the R555K mutation was reported in ovary cancer [36]. Interestingly, it is important to note that we identified three mutations in exon 20 that have

not yet been reported (A1027D and K1030R in CM/ESCC group; T1053K in CM group). All these mutations occurred in patients with chagasic megaesophagus whose mutational profile of *PIK3CA* was never reported.

Importantly, we observed that CM/ESCC patients harboring *PIK3CA* mutations were associated with lower overall survival, suggesting its role as a prognostic biomarker in this group of patients. Interestingly, the results of our analyzes of the survival of the mutated

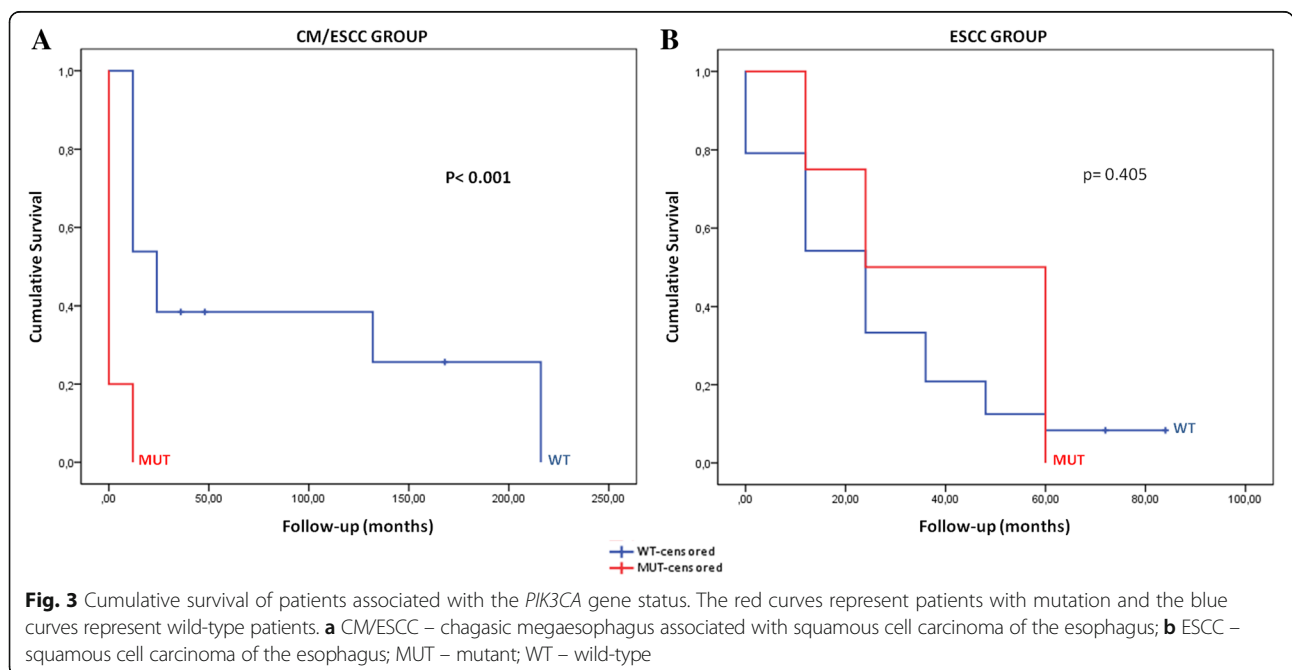


Table 5 The time and average of patients' overall survival according to *PIK3CA* mutation status

Groups	Variable	Total	N events	Time				Median survival	p-value
				6 months	1 year	3 years	5 years		
CM/ESCC	WT	13	10	79.4%	72.2%	43.3%	28.9%	2 years	< 0.001*
	MUT	5	5	20.0%	0.0%	0.0%	0.0%	5 months	
ESCC	WT	24	22	82.6%	64.7%	32.6%	14.5%	2 years	0.405
	MUT	4	4	80.0%	80.0%	60.0%	0.0%	2.5 years	

*Log-rank test. CM/ESCC – chagasic megaesophagus associated with esophageal squamous cell carcinoma; ESCC – esophageal squamous cell carcinoma without chagasic megaesophagus; WT – wild-type; MUT – mutated; Bold numbers - statistical significance

patients differ from those reported by others studies, especially in regions of some risk such as Asia, in which patients with ESCC with mutations of the *PIK3CA* gene had a favorable overall survival compared to patients wild-type [37].

Notably, inhibitors of the PIK3-Akt-mTOR pathway have been developed as cancer target therapy alternatives, and patients harboring *PIK3CA* gene mutations could be potential candidates for such therapeutic approach [14]. Interestingly, phase I and II clinical trials using pan-*PIK3CA* agents (PIK3-class I), such as buparlisib (BKM120), an oral agent that affects α , β , γ and δ isoforms of PI3K [38], showed efficacy in several solid tumors, including head and neck cancer [39]. Copanlisib (BAY80–6946), an intravenous agent that affects α and δ isoforms of PI3K, also showed promising results in non-Hodgkin's lymphomas [40]; as well as pictilisib

(GDC-0941), an oral agent that affects γ and δ isoforms of PI3K, where a good response was reported in breast, colorectal, ovarian and non-small cell lung cancer [41]. Therefore, we can hypothesize that a subset of ESCC and CM/ESCC patients with *PIK3CA* mutations may benefit from these targeted-therapies and consequently improve their dismal survival.

In conclusion, this is the first study that analyzed and identified *PIK3CA* activating mutations in patients with esophageal squamous cell carcinomas associated with chagasic megaesophagus (CM/ESCC), which were associated with a worse outcome. Moreover, the identification of mutations in benign chagasic megaesophagus suggests their putative role in the etiology of esophageal squamous cell carcinoma and opens new opportunities for the treatment of these neglected patients with targeted-therapies.

Table 6 Frequency of *PIK3CA* mutations identified in patients with esophageal squamous cell carcinoma worldwide

References	Year	Country (Region)	Patients of study	<i>PIK3CA</i> mutated (%)	Type of sample	Techniques
Mori et al. [24]	2008	Japan	88	2.2%	FT	Direct Sequencing
Wang et al. [30]	2013	China	76	3.9%	FT	Direct Sequencing
Akagi et al. [17]	2009	Japan	52	7.7%	FFPE	Direct Sequencing
Kim et al. [21]	2016	Korea	534	10.5%	FFPE	Direct Sequencing
Phillips et al. [25]	2006	Australia	35	11.8%	FFPE	Direct Sequencing
Zheng et al. [33]	2016	China	79	19.7%	FT	Pyrosequencing
Shigaki et al. [27]	2013	China	219	21.0%	FT	Pyrosequencing
Liu et al. [23]	2017	China	210	22.9%	FFPE	Pyrosequencing
Baba et al. [18]	2015	Japan	440	23.0%	FFPE	Pyrosequencing
Yang et al. [31]	2017	China	24	4.2%	FFPE	NGS
Song et al. [28]	2014	China	158	4.5%	FT	NGS
Lin et al. [22]	2014	China	139	7.0%	FFPE	NGS
Gao et al. [19]	2014	Japan	133	9.0%	FT	NGS
Sawada et al. [26]	2016	Japan	144	10.4%	FT	NGS
TCGA et al. [9]	2017	Western and Eastern	90	13.0%	FT	NGS
Yokota et al. [37]	2018	Japan	126	13.5%	FFPE	NGS
Zhang et al. [32]	2015	China	90	17.0%	FT	NGS
Wang et al. [29]	2015	USA	71	24.0%	FFPE	NGS

NGS – next generation sequencing; FFPE – formalin-fixed paraffin-embedded tissue; FT – fresh frozen tissue

Abbreviations

CM: Chagasic megaesophagus; ESCC: Esophageal squamous cell carcinoma; FFPE: Formalin-fixed paraffin-embedded; MSI: Microsatellite instability; PCR: Polymerase chain reaction

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FFM participated in the conception of the study, data collection, analysis and interpretation of the results, and draft of the manuscript. ACC, CFL, ATTO, CSN, SRMS, EC, SJA, MAMR and MACAH participated in the acquisition and quality assessment of the data collection and results interpretation. ALF and DPG participated in the, data collection, analysis and interpretation and draft of the manuscript. RMR participated in the designed, supervision, data interpretation and drafting and final revision of the manuscript. All authors gave final approval of the manuscript.

Ethics approval and consent to participate

The local ethic committees approved the study (number 1010/2015).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests for this present study.

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