Autophagy and nuclear morphometry are associated with histopathologic features in esophageal squamous cell carcinoma

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Research Article

Keywords: Esophageal Squamous Cell Carcinoma (ESCC), autophagy, nuclear morphometry, histopathology, prognosis

Posted Date: April 27th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2821223/v1

Version of Record: A version of this preprint was published at Journal of Molecular Medicine on October 25th, 2023. See the published version at https://doi.org/10.1007/s00109-023-02387-4.

Abstract

Less than 15% of patients with esophageal squamous cell carcinoma (ESCC) survive five years after the diagnosis. A better understanding of the biology of these tumors and the development of clinical biomarkers is necessary. Autophagy is a physiological mechanism involved in the turnover of cellular components, playing critical roles in cancer. In this study, we evaluated the differential levels of three major autophagy regulators (SQSTM1, MAP1LC3B, and BECN1) in ESCC patients. We associated autophagy with histopathologic features, including the differentiation grade, mitotic rate, inflammation score, and the intensity of tumor-infiltrating lymphocytes. We also assessed the nuclear morphometry of the tumor parenchyma and associated it with autophagy and histopathology. The three markers were significantly increased in ESCC in comparison to control. Based on the mean expression of each protein in the control group, 57% of ESCC patients showed high levels of the three markers, compared to 14% in controls. The most frequent profiles found in ESCC were $BECN^{high}$/$MAP1LC3^{high}$ and $BECN^{high}$/$SQSTM1^{high}$. Using the TCGA database, we found that the autophagy is upregulated in ESCC. Furthermore, high levels of autophagy markers were associated with poor prognosis. Considering the nuclear morphometry, ESCC samples showed a significant reduction in nuclear area, which strongly correlated negatively with autophagy. Finally, the percentage of normal nuclei was associated with tumor differentiation, while lower levels of SQSTM1 were observed in poorly differentiated tumors. We found that the ESCC progression may involve an increase of autophagy and alterations in the nuclear structure, associated with clinically relevant histopathological features.

1. Introduction

Esophageal cancer (EC) accounts for more than 460.000 new cases annually, being the sixth leading cause of cancer worldwide[1]. Less than 15% of patients survive 5 years free of disease. EC has two main histological subtypes, the esophageal squamous-cell carcinoma (ESCC) and the esophageal adenocarcinoma (EAC). Eighty percent of the ECs occur in less developed countries, where the ESCC represents 90% of the cases[2]. ESCC is related to tobacco, alcohol, hot beverages and low vitamins consumption [3]. ESCC evolves from the normal mucosa to dysplasia, then carcinoma *in situ* and invasive carcinoma. Its identification normally occurs late and therapeutic options are very limited. In the last decades, few advances have been achieved in the diagnosis and treatment of ESCC, while its carcinogenic process remains poorly understood, as does the availability of diagnostic and prognostic biomarkers [4].

A process that has gained attention in the last decade in cancer is autophagy, a catabolic mechanism involved in the degradation of old, dysfunctional, or excessive cellular components [5]. Autophagy is a physiological mechanism that contributes to cellular homeostasis and adaptation, increasing in response to nutrient stress and damage to organelles or DNA [6]. Molecularly, autophagy is controlled by the ATG proteins, which mediate the formation of a double-membrane transient organelle called autophagosome [7, 8]. This step is called early autophagy. Two major players of this stage are BECN1, which participates in the initiation of autophagosome formation [9, 10]; and MAP1LC3B (the mammalian homologue of the yeast autophagy-related gene 8, ATG8), which interacts with the cargo and mediates the closure of autophagosome [11, 12]. The autophagosome englobes cellular components that were marked to degradation by molecular adaptors, especially the protein Sequestosome 1 (SQSTM1 or p62) [13]. Then, the autophagosome merges with lysosomes to form the autolysosome, where cell components are degraded by lysosomal hydrolases. Reduced levels of SQSTM1 are commonly used as a marker of acute autophagy induction, while long term stresses course with the restoration of SQSTM1 levels [14]. At the end, the products of degradation are released through lysosomal permeases back to the cytoplasm. Through this, basal autophagy contributes to the turnover of cellular components and cell homeostasis, while its increase contributes to the adaptation of cells to stressful conditions [15, 16].

In the carcinogenic process, autophagy contributes to the maintenance of homeostasis in healthy cells, preventing tumor initiation. The mechanisms underlying this tumor suppressive role involve the maintenance of DNA integrity and organelles turnover, normal cell metabolism, among others [5, 17]. Indeed, loss of full autophagy capacity is associated with tumor initiation in several cancer types, as shown in animal models deficient to *ATG5*, *ATG7*, *BECN1*, and others [18]. On the other hand, the reactivation of autophagy is crucial for tumor progression, allowing tumor cells to adapt to the metabolic stress associated with tumor growth [5, 19, 20]. Autophagy also play non-autonomous roles, participating on the organization and composition of tumor microenvironment [21, 22]. Autophagy has also been associated with therapy resistance in major types of human cancers [23], including esophageal cancer cells [24, 25]. As a consequence, autophagy inhibition increases the toxicity of anticancer drugs [26], what has been explored in dozens of clinical trials [27].

The role of autophagy in the biology and the prognosis of ESCC has been under investigated, especially considering molecular data in association with histopathological and clinical features[28]. Here, we evaluated the levels of three main autophagy markers (SQSTM1, MAP1LC3B and BECN-1) in ESCC primary samples in comparison to non-neoplastic esophageal mucosa. We defined the autophagy

status of single individuals and associated it with clinical and histopathologic measurements. We also assessed nuclear morphometry and associated it with autophagy and tumor histopathology. At the end, we complemented our primary data with a survey of the landscape of ATG somatic mutations in ESCC and the influence of expression levels of main autophagy genes in the prognosis of ESCC in the The Cancer Genome Atlas (TCGA) cohort.

2. Materials And Methods

2.1. Samples recruitment

Control and ESCC patients were recruited from the archive of endoscopic biopsies of the Pathology Service of Hospital de Clínicas de Porto Alegre (HCPA), Brazil, from 2013 to 2020. The inclusion criterion for the ESCC group was the diagnosis of ESCC, which was confirmed by two blinded pathologists. The control group consisted of endoscopic biopsies of non-neoplastic origin, including: nonspecific findings, esophagitis without squamous epithelial dysplasia or metaplasia related to gastroesophageal reflux disease. We excluded patients with neoplastic lesions and pre-neoplastic lesions such as Barrett's esophagus, squamous epithelium dysplasia and HPV infection.

2.2. Immunohistochemistry

Immunohistochemistry was done for SQSTM1, MAP1LC3B and BECN1. To this, 3µm sections were dewaxed and rehydrated. The antigens retrieval was done by water bath treatment of the cuts in 50 mM TrisEDTA (pH 9.0) for 20 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 minutes at room temperature. Non-specific binding was blocked adding a 5% solution of nonfat dry milk for 15 minutes. The slides were incubated at 4°C for 12 h with the anti-BECN1 1:750 (ab55878), anti-SQSTM1 1:200 (ab56416) and anti-MAP1LC3B 1:1000 (ab128025) antibodies, all from Abcam (Cambridge, MA, US). The sections were then incubated with a econdary antibody at 25^{o}C for 1 hour. The chromatographic reaction was performed on 3,3'-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate Chromogen System Code K3468, Dako, Carpinteria, CA, USA) for 5 min. After the immunohistochemical reaction cuts were stained with Harri´s hematoxylin.

Then, we acquired at least 10 fields from each sample, and the intensity of DAB staining was quantified in the ImageJ through the color deconvolution method. We also calculated an 'Autophagic Index' (AutoIndex) through the sum of the intensity of the three autophagy markers to each sample.

2.3. Nuclear Morphometric Analysis in Tissue (tNMA)

The Nuclear Morphometric Analysis in tissue (tNMA) allows the objective assessment of size and shape of cell nucleus from hematoxylin and eosin (HE) images [29]. To this, at least 10 fields from HE slides were imaged to each sample in an Olympus BX51 microscope. Using the magic wand tool of the Image Pro Plus 6.0 software, nuclear segmentation was performed, followed by the acquisition of 5 variables related to nuclear size and shape from at least 100 nuclei to each participant. In the tNMA, *Area* is the variable acquired for nuclear size, while the variables for nuclear shape are *AreaBox, Roundness, RadiusRatio and Aspect.* Shape variables were used to calculate the Nuclear Irregularity Index (NII). The plot of Area *versus* NII separate different nuclear populations based on their morphometry. Data were analyzed in an Excel datasheet available at www.ufrgs.br/labsinal.

From these primary variables (*i.e.*, Area and NII) we calculated secondary variables associated with nuclear pleomorphism, called Diff Av-Med Area (the arithmetic difference between the average and the median, a measure of nuclear heterogeneity) and Var Area (variance of the nuclear area).

2.4. Histopathological features

We measured the association between autophagy or nuclear morphometry with the following histopathological features:

Mitotic rate

defined as the average of mitotic figures in five fields (×400 overall magnification) from HE slides using bright-field microscopy. After assessing the median distribution (1), values < 1 were classified as low and values above 1 were classified as high.

Tumor histologic grade: the tumor samples were classified at differentiation stage according to WHO parameters [30]: well differentiated, moderately differentiated, poorly differentiated. For this purpose, we assessed the differentiation of tumor cells, cellular and nuclear pleomorphism, keratin formation, and tumor cells cohesion.

Inflammation score

the intensity of inflammation was classified as no inflammation, mild inflammation, moderate inflammation, or high inflammation according to the number of inflammatory cells within tumor stroma.

Stromal Tumor-infiltrating lymphocytes (sTILs)

the percentage of stroma infiltrated by lymphocytes was obtained [30], and after calculating the median equal to 40, values between 0 and 30 were classified as low and values between 50 and 90, classified as high.

2.5. Bioinformatics

To complement our primary data, we assessed: a) the differential expression of crucial autophagy-related genes in ESCC samples in comparison to the normal esophagus by using the University of Alabama Cancer Database (UALCAN) Browser [31]; b) the association between autophagy-related genes and the survival of ESCC patients by using the KMPlot [32]; c) the mutational landscape of autophagy-related genes by assessing the Catalog of Somatic Mutations in Cancer (COSMIC) [33]. We assessed three autophagy databases to select the autophagy genes: *autophagyregulation.org/; autophagy.lu/index.html; tanpaku.org/autophagy/*. Only genes whose encoded protein participate directly in the autophagic process and appeared in the three databases were selected.

2.6. Statistical Analysis

Statistical analysis was conducted in SPSS 18.0. For comparison between the levels of autophagy markers, AutoIndex and nuclear measurements between control and ESCC groups we performed a non-parametric Mann-Whitney test. Histopathological features, expression of autophagy markers and nuclear morphometric variables were tested with Independent-Samples Kruskal Wallis test, and Mann-Whitney. We also performed a non-parametric Spearman correlation test to match autophagy with nuclear morphometry. P values under 0.05 were considered as significant.

3. Results

3.1. Population characteristics

Our study included 32 controls and 53 ESCC (Table 1). The mean age for control and ESCC groups were, respectively, 45 ± 27 and 64 ± 10. We found a prevalence of male in the ESCC group (67%), which agrees with the incidence rate worldwide, while in the control the distribution was similar (53% male / 47% female). The percentages of smokers were 22% and 83% in control and ESCC, respectively.

Table 1
– Epidemiological data of the study

	Controls	ESCC	p-value
N of patients	32	53	-
Gender % (M/F)	53/47	67/33	*0.093*
Age (average ± SD)	44.6 ± 26.8	64 ± 10.1	*0.178*
Smokers (%)	22%	83%	*0.003*

Table 2. Histopathological findings and expression of autophagy markers in ESCC.

	SQSTM1			MAP1LC3B			BECN1			Autophagy index		
	Median	IQR p25-p75	p	Median	IQR p25-p75	p	Median	IQR p25-p75	p	Median	IQR p25-p75	p
Grade of differentiation												
Well differentiated	68.2[ab]	49.0–83.3	**0.015**	54.1	43.3–58.2	0.313	151.0	140.0–164.8	0.236	270.4	232.8–292.7	0.075
Moderately differentiated	81.3[a]	60.0–93.3		68.2	48.0–82.9		161.2	143.7–187.0		299.7	260.6–328.3	
Poorly differentiated	45.9[b]	34.0–63.3		70.2	19.9–76.7		165.1	133.6–185.7		271.9	241.4–296.7	
Inflammation score												
Mild	82.3[a]	66.0–103.5	**0.039**	54.1	31.4–74.2	0.141	167.5	155.3–182.6	0.124	292.7	265.5–321.1	0.093
Moderate	59.7 [ab]	46.4–84.6		65.5	42.9–74.8		161.3	140.6–187.0		290.2	256.5–314.4	
High	59.8 [b]	35.2–76.1		73.4	55.1–81.7		148.2	119.0–172.5		267.5	238.5–291.4	
Mitotic count												
Mitotic low	65.5	33.4–87.7	0.967	68.2	38.4–79.7	0.848	155.3	125.7–174.3	0.305	279.3	233.5–312.6	0.859
Mitotic high	61.0	46.3–72.1		66.3	45.3–75.4		175.4	140.6–185.4		274.5	239.1–317.5	
Score Stromal Tumor-infiltrating lymphocyte (sTILs)												
sTILs low	69.8	59.4–89.7	0.143	54.1	37.7–74.2	0.209	164.8	151.4–182.5	0.062	290.5	265.5–317.5	0.110
sTILs high	56.6	45.3–80.2		72.7	48.0–79.5		150.6	117.2–170.5		264.1	244.2–294.3	

Different letters indicate significant differences between groups ($p < 0.05$). Values represent median level with interquartile range.

Abbreviations: IQR (Interquartile range).

3.2. Autophagy is increased in ESCC in relation to non-neoplastic esophageal tissue

We firstly measured the immunocontent of SQSTM1, MAP1LC3B and BECN1. We found an increase in the levels of these proteins in ESCC in relation to control **(**Fig. 1A**)**. Then we quantified the intensity of proteins' levels in each individual. The mean level to each marker in controls **(**Fig. 1B **- red line)** was used to set the percentage of patients with low **(**Fig. 1B **– purple area)** or high levels **(**Fig. 1B **– blue area)** of each marker. The percentage of ESCC with high levels of SQSTM1, MAP1LC3B and BECN1 was 91%, 81% and 77% respectively **(**Fig. 1B **– blue area)**. We also determined the percentage of 'very low' and 'very high' samples, corresponding to samples with expression levels that were lower or higher than the average of control ± 1 standard deviation. The percentage of 'very low' cases was around 2 to 4% in the ESCC group **(Fig. S1A – orange area)**. The percentages of samples with very high levels, on the other hand, were 81% (BECN1), 57% (MAP1LC3) and 38% (SQSTM1) **(Fig. S1A – light pink area).**

Finally, considering that autophagy is coordinated by the integrative activity of ATG proteins - rather than by individual players - and keeping in mind the intertumoral heterogeneity, we then integrated results from protein expression in each individual. Firstly, we determined the autophagic status of each sample through the generation of the 'AutoIndex'. Compared to single autophagy markers, the

difference of AutoIndex was even higher between ESCC and controls (Fig. 1C **and Fig. S1B)**. Altogether, our data show that autophagy is increased in ESCC in relation to non-neoplastic esophagus.

3.3. Profiles of expression of SQSTM1, MAP1LC3 and BECN1 in single samples

We next determined the profile of expression of the three autophagy proteins in each sample based on the classification of 'low' and 'high'. We found that the percentage of samples classified as 'high' for all the three autophagy markers was 19% for controls and 53% for ESCC. Corroborating this data, 22% of controls were classified as low to the three autophagy markers, while no ESCC sample had this profile (Fig. 2A**)**. Furthermore, 41% controls had at least 2 autophagy markers considered as low, while this percentage was 6% in ESCC. On the other hand, 94% of ESCC showed at least 2 autophagy markers as high, while in controls this percentage was 59% (Fig. 2B**)**. Despite the above mentioned differences, both groups showed the same profiles as the most prevalent: 'BECN1 high + SQSTM1 high' and 'BECN1 high + MAP1LC3 high' (Fig. 2B **– top)**.

3.4. Association between histopathological features and autophagy markers in ESCC

In the analysis of the association of histological characteristics of ESCC and autophagy-related proteins (**Table 2**), the values of each protein alone (SQSTM1, LC3B and BECN1) and also the AutoIndex were considered. Lower levels of SQSTM1 were observed in samples classified as poorly differentiated (p = 0.015). Regarding the inflammation score, we observed an increase in SQSTM1 in samples classified as mild inflammation (0.039). There were no significant associations between autophagy markers and mitosis count and sTIL analysis.

3.5. Levels of autophagy markers are not different considering age, gender or smoking

The association between clinical characteristics and autophagy is usually neglected in the literature. Here, we did not find differences between male and female **(Fig. S2A)** neither between 'young' and 'old' individuals (based on the median of each group) **(Fig. S2B)**. Finally, we also did not find differences in the level of autophagy between smokers and non-smokers **(Fig. S2C)**.

3.6. Autophagy network is upregulated and associated with poor prognosis in ESCC in the TCGA cohort

We then explored the TCGA database for ESCC. Firstly, we compared the mRNA levels of BECN1, MAP1LC3B and SQSTM1 in control versus ESCC samples. SQSTM1 was higher in ESCC in comparison to control, while BECN1 and LC3B levels did not differ significantly, probably due to intertumoral heterogeneity (Fig. 3A**)**. We also assessed the differential expression of others 22 autophagy-related genes. Except for ULK1, which was downregulated in ESCC in comparison to control, and ATG16L1, BECN1, BIF1 and NBR1, which did not differ from normal esophagus, 83% of autophagy genes were overexpressed in tumor samples, suggesting that the autophagy network is upregulated in ESCC **(Fig. S3A and 3B)**. These results were similar in esophageal adenocarcinoma (EAC), in which the autophagy network is also upregulated in relation to normal tissue, even at higher levels than ESCC (**Fig. S3A and 3B**). Importantly, we also assessed whether these alterations occur early or late considering the carcinogenic process in the esophagus, separating tumor samples according to tumor stage. We observed that the stage of esophageal carcinogenesis in which there is greater activation of the autophagic pathway is from normal tissue to stage I, suggesting that autophagy contributes to the early promotion of esophageal neoplasms **(Fig. S3C)**.

We then assessed whether the expression levels of BECN1, MAP1LC3B or SQSTM1 were associated with the median overall survival (mOS) of ESCC patients. High levels of the three genes were associated with poorer survival (Fig. 3B**)**. From them, LC3B levels had the strongest prognostic effect (p = 0.0003). Considering the landscape of somatic mutations assessed through the Catalog of Somatic Mutations in Cancer (COSMIC), none of the genes of interest showed a significant percentage of mutations in ESCC samples. In fact, the frequency of mutations for all major autophagy-related genes was low in ESC samples (Fig. 3C**).**

3.7. Autophagy and nuclear morphometry are strongly correlated in the esophageal tissue

It has been suggested that epigenetic events and chromatin remodeling, which affect nuclear morphometry, are key regulators of autophagy genes expression [34–36]. Furthermore, nuclear alterations play a key role in tumor progression [37–39], while providing clinically

relevant information about prognosis and diagnosis [40–42]. With this in mind, we assessed nuclear morphometry and its correlation with autophagy markers in ESCC.

Firstly, we evaluated nuclear size, shape, and homogeneity through the tNMA, a technique that allows the objective assessment of these features from HE slides. We observed that nuclei from ESCCs were smaller and more regular (*i.e.* round) than control nuclei (**Fig. 4AB and C** – top). This reduction was concomitant with the increase in the percentage of 'Small Regular' (SR) nuclei (Fig. 4B **– SR population**, Fig. 4C – middle). Importantly, the reduction of nuclear area was not due to a mechanical pressure caused by an increase in the number of cells since the number of epithelial nuclei per area was similar between controls and ESCC (data not shown). Considering its relation with histopathological characteristics (**Table 3**), we observed that well-differentiated tumors had a greater number of normal nuclei (p = 0.02) and higher median nuclear area (0.032). Tumors with high inflammation had a higher percentage of normal nuclei (p = 0.016), higher median nuclear area (p = 0.046) and lower DiffA-M (p = 0.039). A higher percentage of sTILs was associated with lower NII (p = 0.04) and lower DiffA-M (p = 0.032).

We then searched for correlations between autophagy and nuclear morphometry. We did not find significant correlations between single autophagy markers and nuclear variables, as exemplified in **Fig S4**. However, we found that the smaller the nucleus, the higher the AutoIndex, which led to strong correlation between AutoIndex and Average Nuclear Area **(**Fig. 4D**)**, Median Nuclear Area **(**Fig. 4E**)** or Average Area of normal Nuclei **(**Fig. 4F**)**. It is important to note that we can observe a 'gray zone' between the transition from control to ESCC, in which some controls showed a phenotype of autophagy nuclear morphometry that resemble ESCC, while some ESCC cases resemble control features (see Fig. 4D to 4F**)**. This observation can contribute to the understanding of the crosstalk between autophagy and nuclear architecture during the carcinogenesis of ESCC.

4. Discussion

In less than two decades, advances in the basic understanding of the role of autophagy in cancer enabled the first clinical studies using autophagy modulators. Thus, it is necessary to characterize the status of autophagy markers, especially considering integrated analysis, in the main types of human cancers, as well as their association with other clinically relevant markers and potential mechanisms involved in their modulation [5, 27]. Here, we found elevated levels of three autophagy proteins (BECN1, MAP1LC3B e SQSTM1) in ESCC primary samples. We also determined the individual expression profile for each individual, and we observed that more than 90% of the patients had at least two markers with high expression, the profiles $BECN^{high}$+$MAP1LC3^{high}$ and $BECN^{high}$+$SQSTM1^{high}$ being the most frequent. Additionally, 53% of patients had all the three markers increased. We proposed an association of these markers as an AutoIndex, which showed even higher differences comparing the two groups. Likewise, strong overexpression of autophagy related genes was found in ESCC samples from the TCGA cohort, while high expression of BECN1, MAP1LC3B, and SQSTM1 were associated with poorer prognosis. In parallel, we found alterations in nuclear morphometry in ESCC, which strongly correlated with autophagy levels in single patients.

BECN1, MAP1LC3B, and SQSTM1 play critical roles in different stages of autophagy and have shown clinical relevance in cancers other than ESCC. BECN-1 is responsible for the initiation of autophagosome formation. We found higher levels of BECN-1 in the ESCC compared to the non-neoplastic samples, in agreement to data from gastric, colorectal, thyroid, oral, and ovarian carcinoma [43–46]. Other tumors, however, have lower levels of BECN-1 compared to non-neoplastic tissue, such as esophageal adenocarcinoma, hepatocellular carcinoma, cervical and gastric adenocarcinomas [47–49]. Furthermore, in contrast to our data and data from TCGA, two recent studies found lower levels of BECN-1 in ESCC compared to normal adjacent tissue [50]. These studies suggest that alterations in BECN-1 levels in cancer may depend not only on the cell and organ of origin but also on genetic or environmental factors associated with the population of interest. In lining epithelia, which are more exposed to external stressing agents as the esophagus, Roesly and colleagues found increased levels of BECN-1, while the opposite was observed in the glandular epithelia. Lining epithelial cells may have high autophagic capacity to adapt to environmental stress [51]. Considering its role in patients survival, corroborating the results obtained here in the TCGA cohort, high levels of BECN-1 were associated with poor prognosis in oral squamous carcinomas [46], endometrial adenocarcinoma [52] and hypopharyngeal squamous cell carcinoma [53]. In opposite, in hepatocarcinoma and cervical cancer, lower levels of BECN-1 were associated with advanced staging, lymph nodes metastasis, and poor differentiation [47, 49]. Similar findings were observed in ovarian carcinoma, where a decrease in BECN1 levels compared to normal tissue is observed [54]. Therefore, it is possible to propose that the greater the difference in BECN-1 level between tumor and non-neoplastic tissue, the poorer the prognosis, regardless of whether the alteration is for more or less. Corroborating this hypothesis, the greater the tumor grade, the lower the levels of BECN1 in ovarian carcinoma [55, 56]. This hypothesis is also valid to MAP1LC3B, as discussed below.

The MAP1LC3B protein participates in autophagosome formation, and its levels correlate with the number of autophagosomes formed [12]. We found higher levels of MAP1LC3B in ESCC samples than in non-neoplastic tissue, in agreement to oral squamous cell carcinoma [57]. Another study with ESCC found moderate to high levels of MAP1LC3 in 71% of the tumor samples [58]. Differently, MAP1LC3B and BECN1 levels were lower in squamous cell carcinoma of hypopharyngeal compared to non-neoplastic tissue. In this cancer type, lower levels of MAP1LC3B associate with neoplastic invasion, lymph node metastasis, and poor prognosis [53]. In the TCGA cohort, we found an association of high MAP1LC3B levels and a worse prognosis in ESCC, similar to astrocytoma [59]. On the opposite, in hepatocellular carcinoma a reduction of MAP1LC3 levels compared to non-neoplastic tissue was observed. Furthermore, the lower the levels of this protein the shorter the survival time [60], reinforcing our hypothesis raised to the prognostic role of BECN1.

SQSTM1 marks defective cellular components holding the cell trash into the autophagosome. Therefore, the SQSTM1 is also catabolized by autolysosome, and its level tends to reduce during acute stress. In chronic conditions like carcinogenesis, however, the expression of SQSTM1 is re-increased, even at levels above baseline, as an adaptive system to guarantee the total autophagic capacity of cells [14]. In this context, the progression of the dysplastic oral epithelium to a neoplastic condition was associated with an increase of SQSTM1 [61, 62]. This model reinforces the hypothesis suggesting that autophagy favors the progression of pre-malignant lesions to malignant tumors [5]. Indeed, we found a substantial increase in SQSTM1 in the transition from normal tissue to stage I tumors from the TCGA cohort. Furthermore, we found higher levels of SQSTM1 in ESCC than in non-neoplastic esophageal tissue, which is consonant to data from TCGA and data from squamous cell carcinomas of head and neck [57, 61, 62]. Considering its role in cancer prognosis, SQSTM1 was associated to poorer prognosis in squamous cell oral carcinomas [61], non-small cell lung cancer [63], gastric and colorectal adenocarcinomas [64]. Our analysis of the TCGA cohort corroborates these findings. On the opposite, our histopathological analysis in primary samples showed that poorly differentiated tumors have lower levels of SQSTM1, which could be related to the acute activity of autophagy in these tumors. Molecularly, recently Shi *et al* showed that SQSTM1 protects ESCC cells from apoptosis by stabilizing SKP2 protein [65]. Similar mechanisms may also affect the response to therapy, since high SQSTM1 levels have been associated to low sensitivity to radio and chemotherapy in head and neck squamous cell carcinomas [61, 62] and ovarian carcinomas [56].

Recently, the impact of autophagy genes in the outcome of esophageal cancer was investigated. The authors provided a suggestive prognostic signature of four genes (DNAJB1, BNIP1, VAMP7, and TBK1), which could predict patients' survival [50, 66, 67]. Indeed, we believe that the combination of markers is more reliable to analyze autophagy than single markers since it is a dynamic process involving a sequence of events mediated by various proteins. This observation justifies the proteins we chose to study since each participates in a specific stage of autophagy. Here, we purpose the integration of single gene expressions in an autophagic index (AutoIndex) to better represent the autophagic status. AutoIndex showed even more significant differences between control and ESCC groups. The examination of any of these markers individually may not have the same biological information as the AutoIndex. Nevertheless, these data are clinically relevant since 94% of our ESCC samples presented high levels of at least two autophagy markers. It is therapeutically important considering that the inhibition of autophagy sensitizes cancer cells and increases the efficacy of radio- and chemotherapy, including in esophageal cancer [24, 68]. Thus, the determination of AutoIndex could more accurately select patients who could benefit from autophagy inhibitors combined with chemotherapy or radiotherapy.

Alterations in nuclear morphometry are typical in cancer and can influence the epigenetic control of gene expression [69, 70], in crosstalk that may be involved in several aspects of the carcinogenesis [38]. Here, we found a strong reduction in the nuclear area in ESCC, associated with histopathological characteristics of greater tumor aggressiveness. Corroborating this, the smaller the nucleus the worst is the prognosis in breast cancer [41, 71], colorectal adenocarcinoma [42, 72], and lung adenocarcinoma [73]. Nuclear morphometry can also predict tumor recurrence [72]. Finally, we search for correlations between autophagy and nuclear morphometry since epigenetics is involved in autophagy "turn on, turn off" along the carcinogenesis [35, 74]. We observed a strong negative correlation between the AutoIndex and the nuclear size reduction, suggesting that this crosstalk could participate in tumor progression.

In conclusion, our data suggest that autophagy is increased in ESCC and may be involved in the progression from the normal esophagus to ESCC, especially in the early stage of carcinogenesis. Our data also shed some novelty on the association between autophagy markers, which may be relevant both in the carcinogenic process and in the prognosis. Furthermore, autophagy strongly correlated with nuclear alterations and clinically relevant histopathological features in primary samples. The pro-survival context conferred by autophagy to tumor cells could support the design of schedules combining autophagy inhibitors with chemotherapy in the management of ESCC.

Abbreviations

AutoIndex – Autophagic Index; **ATG or Atg** – Autophagy-Related Genes or Proteins; **BECN1** Beclin-1; **ESCC** – *Esophageal Squamous Cell Carcinoma;* **EC –***Esophageal Carcinoma;* **MAP1LC3A/B/C (LC3A/B/C)** Microtubule-Associated Protein 1 Light Chain 3 (isoforms A, B and C); **NMA –** Nuclear Morphometric Analysis; **tNMA** – Nuclear Morphometric Analysis in Tissue; **NII** – Nuclear Irregularity Index; **N nuclei** – Normal Nuclei; **OST** – Overall Survival Time**;SQSTM1 –** Sequestosome 1; **SR nuclei** – Small and Regular Nuclei**; TCGA –** The Cancer Genome Atlas

Declarations

Statements and Declarations

Ethical Approval and Consent to Participate

The project was evaluated by the Ethical Committee of the Hospital de Clinicas de Porto Alegre and received the approval Certificate of Presentation for Ethical Consideration (CAAE): 57102216.0.0000.5327. The risk of confidentiality break was controlled by the signature of Responsibility Term for use of biological material, where researchers compromise not to use references that identify the patient in the study.

Funding

This work was supported by the Research and Event Incentive Fund (FIPE) of the Hospital de Clínicas of Porto Alegre (HCPA), project number: 16-0233.

Acknowledgements

We thank the Research and Event Incentive Fund (FIPE) of the Hospital de Clínicas of Porto Alegre (HCPA) for funding. We would also like to thank the statistical analysis service at the Hospital de Clínicas de Porto Alegre for assisting in developing the project.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Ricardo Iserhard, Emilly Ferreira Salles Pilar e Eduardo Cremonese Filippi-Chiela contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ricardo Iserhard, Paula Ferst, Patricia Luciana da Costa Lopez and Sidia Maria Callegari-Jacques. Histopathological analysis were performed by Francine Hehn and Fernanda Visioli. The first draft of the manuscript was written by Ricardo Iserhard and Eduardo Cremonese Filippi-Chiela, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The immunohistochemistry generated during the current study are not publicly available due to ethical privacy but are available from the corresponding author on reasonable request.

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Tables

Table 3 is available in the Supplementary Files section.

Figures

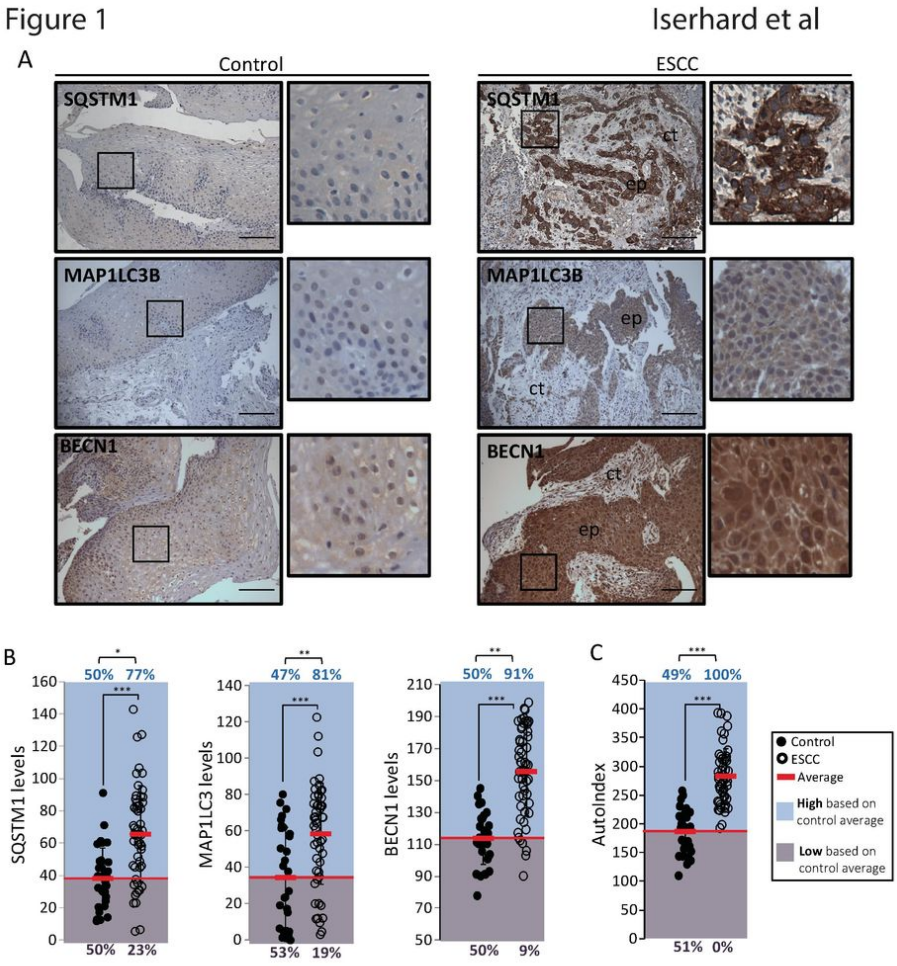

Figure 1

Immunohistochemistry for SQSTM1, MAP1LC3B and BECN1 in ESCC and control groups. (A) Representative images of autophagy markers in control and ESCC. Ep – epithelium; ct – connective tissue. Inserts are shown in detail. Images were acquired at 200x. (B) Quantification SQSTM1, MAP1LC3B and BECN1 levels in each patient (black circles – control; unfilled circles – ESCC). Red markers represent the average of each group; continuous red line represents the average of control group. Gray and blue areas correspond to patients classified as low or high, respectively, in comparison to the average of control group. The percentage of individuals classified as low (bottom, in purple) or high (top, in blue) is shown. (C) Autophagic Index; similar to (B), each individual was classified as low or high. *p<0.05, **p<0.01, ***p<0.001 in relation to control.

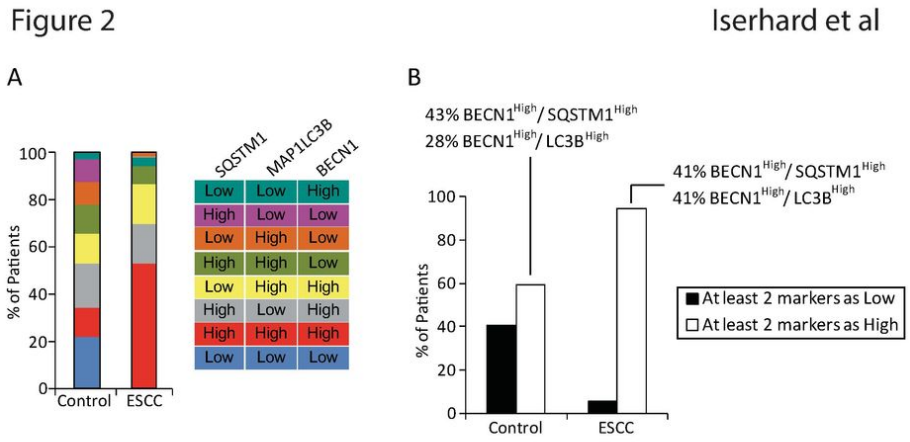

Figure 2

Profiles of expression of SQSTM1, MAP1LC3B and BECN1 in each individual. (A) Classification of patients according to the protein expression levels. Each color represents a specific profile considering the autophagy markers. (B) Percentage of patients showing at least 2 markers as low or as high. On the top is shown the most prevalent profiles among patients with at least two 'high' markers.

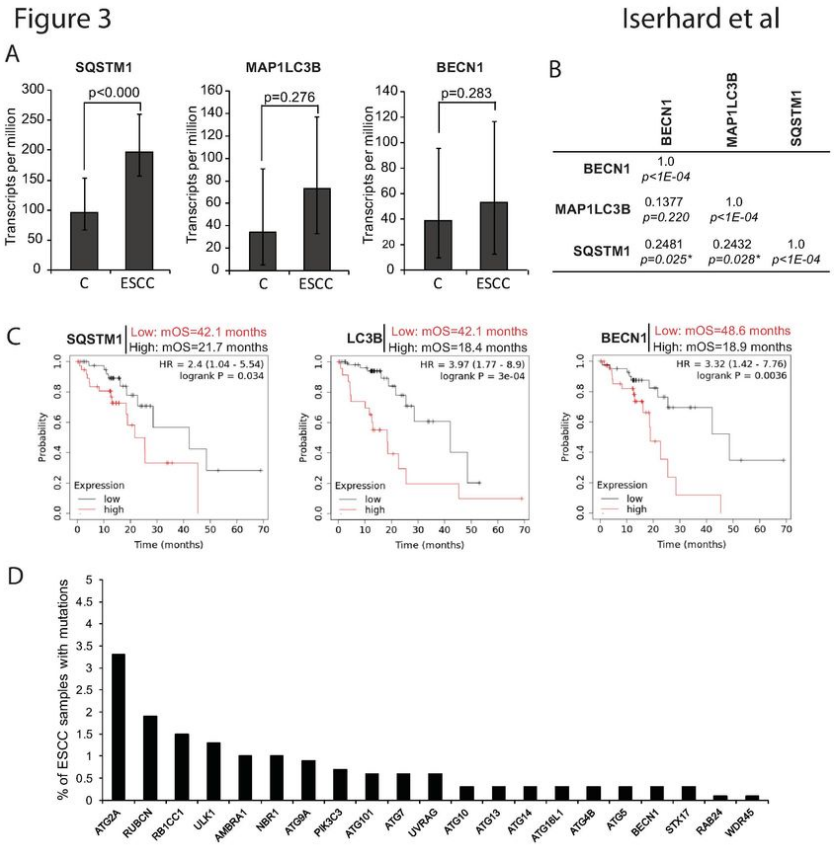

Figure 3

Levels of autophagy markers in control and ESCC group, and its association with median overall survival time. (A) Expression levels (mRNA). (B) Pearson correlation to expression levels. (C) Kaplan-Meier curves according to the expression levels of SQSTM1, MAP1LC3B and BECN1. The median Overall Survival Time (OST) is shown to each condition. (D) Frequency of somatic mutations in autophagy genes in ESCC.

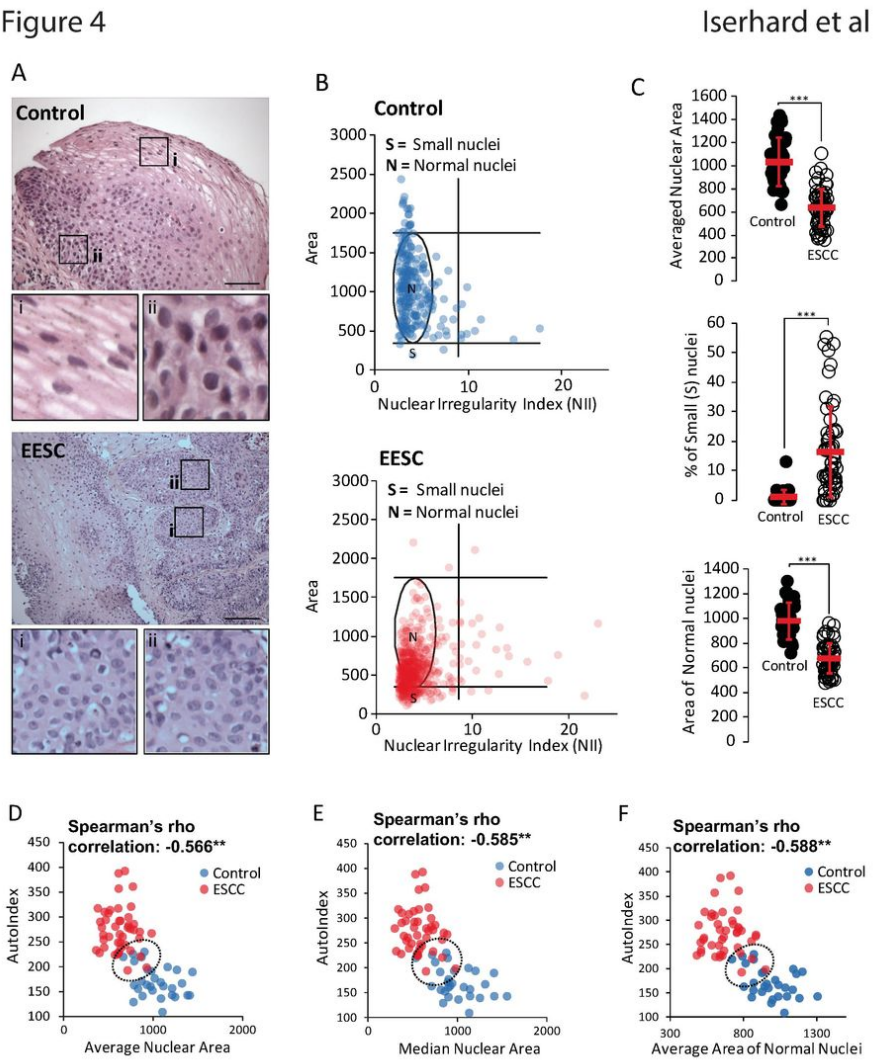

Figure 4

Nuclear area from ESCC is lower than nuclear area of control and negatively correlates with autophagy. From HE images we evaluated nuclear morphometry. (A) Representative images from controls (top) and ESCC (bottom). Inserts are shown in detail. (B) Nuclear Morphometric Analysis in tissue (tNMA) for control (top) and ESCC (bottom). Area x Nuclear Irregularity Index (NII) plot. SR – quadrant of Small Regular nuclei; N – quadrant of normal nuclei. (C) Data from the average of nuclear area, the percentage of small nuclei and the area of normal nuclei. (D) Correlation of AutoIndex (y axis) with variables of nuclear area (x axis). The spearman coefficients are also shown on the top of graphs; **p>0.01.

Supplementary Files

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