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Tumor autophagy is associated with survival outcomes in patients with resected non-small cell lung cancer

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ABSTRACT

Objectives: LC3A protein is associated with autophagosomes, and LC3A immunohistochemistry (IHC) is used for the detection of autophagy activity. The aim of this study was to assess the prognostic value of LC3A expression in patients with resected non-small cell lung cancer (NSCLC).

Materials and methods: We used tissue microarrays (TMAs) constructed from 116 resected stage IB-III NSCLC patients. Standard immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections using antibody against LC3A autophagic potein. Stained slides were scanned by Olympus dotSlide Digital Virtual Microscopy System (Japan) and the LC3A staining was evaluated digitally. Groups were compared using the Mann Whitney *U* test, and correlations were assessed using Spearman's rank test. Survival was calculated using Kaplan-Meier analysis. Primary study endpoint was overall survival (OS), secondardy study endpoint disease-free survival (DFS). Cut-off optimization for LC3A prognostic value was performed using the "cut-off finder' 'software (Charite, Berlin, Germany). In addition, the Kaplan Meier plotter (KmPlot) was used to assess the relationship between LC3A mRNA expression and clinical outcome (OS and DFS) in patients with NSCLC.

Results: From 116 patients, 88 tissue samples were available for final examination. No significant association was found between LC3A staining and other clinicopathological variables, including tumor grade, stage and histological subtype. A higher number of LC3A stone-like structures (SLSs) (> 20), was significanly associated with poor OS (HR = 2.27, p = 0.011) and DFS (HR = 2.27, p = 0.003). A significant association between high LC3A mRNA and both a worse OS and worse DFS was found by KMPlot analysis in patients with stage I-III NSCLC.

Conslusion: This retrospective study suggests that SLSs as assessed by LC3A IHC as well as LC3A mRNA expression has a clinically relevant negative prognostic value in patients with resected NSCLC, and should be further investigated.

1. Introduction

Cancer cells survive the adverse conditions of the extracellular milieu (i. e. hypoxia, nutrient deprivation, and reduced growth factors) through angiogenesis and anaerobic glycolysis [1]. Autophagy is a self-degradative process, which is characterized by enclosure of cytoplasmic material inside autophagic vacuoles containing lysosomal enzymes [2]. Enzymatic degradation of engulfed material results in cell death. In oxygen and glucose

Abbreviations: NSCLC, non small cell lung cancer; LC3A, microtubule associated protein 1 chain 3; Atg8, autophagy-related protein 8; SLSs, stone like structures; HCC, hepatocellular carcinoma; WHO, World Health Organization; OS, overas survival; PFS, progression-free survival; SCC, squamous cell carcinoma; LCC, large cell carcinoma; ER, endoplasmatic reticulum

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depleted states, autophagy also activates an alternative metabolic pathways, providing tumor cells with additional energy [2]. Hence, autophagy represents a dual mechanism for cell survival and death. However, the role of autophagy in cancer prognosis is incompletely understood.

Electron microscopy is considered as a gold standard for measuring autophagy activity in clinical and research specimens [3]. However, this method lacks of specificity. In particular, sometimes it is difficult to distinguish autophagic vacuoles from other structures and it is not possible to separate late stage autophagic vacuoles from heterophagic vacuoles [4]. Antibodies against LC3A have been introduced into laboratory practice to specifically detect increased autophagic activity under standard light microscopy [3].

LC3A (microtubule associated protein 1 chain 3 alpha) is an important component of the autophagosomes. It is an autophagosomal orthologue of yeast Atg8, with approximately 30% amino acid homology with Atg8 [5]. LC3A exists in two forms, LC3A-I (cytosolic) and LC3A-I I (membrane bound) [6]. LC3A-I I is a component of pre-autophagosomal and autophagosomal membranes and has been shown to be a valid surrogate for autophagy activity [6]. Three intracellular patterns of LC3A expression are observed: perinuclear and cytoplasmic pattern as well as „stone-like‘ ‘structures (SLSs). These different patterns are thought to have a different meaning and prognostic significance. Strong LC3A cytoplasmic staining indicates an increased production of the soluble form of LC3A-I that represents an early event in autophagosome formation. After autophagosome formation, autophagic cargo is delivered to the lysosome-rich perinuclear area and, thereby, perinuclear accumulation of LC3A might be a marker of functional autophagy machinery [7,8]. The presence of LC3A stained SLSs are considered as a marker of accelerated and abberant autophagy activity [9]. SLSs are dense, rounded, amorphous, LC3A positive material, enclosed within cytoplasmic vacuoles [9]. SLSs marked by LC3A are frequently found in malignant cells, and increased autophagy activity detected by LC3A SLSs has been shown to occur in a majority of aggressive cancer subtypes and is associated with poor prognosis [7,9–11]. Based on LC3 A immunohistochemistry (IHC), it has been shown that autophagy is differentially regulated in breast [12], endometrial [11], cololectal [13], cutaneous squamous-cell [10], gastric [14], non-small cell lung [15,16] and hepatocellular carcinomas (HCC) [17], as well as in hematological malignances [18] and human glioblastomas (GBM) [13]. Sivridis et al. described the juxta-nuclear pattern of autophagy as a common finding in both neoplastic and non-neoplastic breast tissue, where it was correlated with more favorable outcome [12]. On the other hand, SLSs pattern detected by LC3A IHC in the same tumors, was associated with less favorable prognosis [12]. Basal levels of autophagy activity as assessed by diffuse cytoplasmic and cytoplasmic/perinuclear patterns of LC3A staining had no prognostic impact in patients with endometrial carcinoma [11], whilst perinuclear accumulation of LC3 A protein in colorectal cancer was associated with improved prognosis [7]. On the other hand, abnormal or excessive autophagy activity as detected by high SLSs was associated with a poor prognosis in the same group of colorectal cancer patients [7]. Karpathiou et al. found that the phenomenon of LC3A SLSs is particularly relevant for NSCLC and high number of SLSs may be associated with poor prognosis, particularly in surgically resected stage I–II NSCLC [15]. whilst according to Schläfli et al. high levels LC3A SLSs was not associated with overall survival in stage IA-IIB NSCLC [16]. In HCC, Lee et al. showed that LC3 expression correlates with improved prognosis, regardless of tumor stage or liver function, suggesting LC3A to be an independent prognostic factor in HCC [17]. The study of human GBM revealed that both cytoplasmic overexpression LC3A and LC3A SLSs are frequently found in GBM patients, where it is also associated with poor survival outcomes [13].

Overall, autophagy is regulated in a cell and organ specific manner, it can be assessed by using conventional LC3A immunohistochemical staining, and it has been associated with worse clinical outcome in several solid tumors. To the best of our knowledge, none of these studies however examined the prognostic role of autophagy in patients with potentially curative NSCLC undergoing tumor resection followed by adjuvant chemotherapy. For this reason, the aim of our study was to assess the relationship between autophagy, by LC3A, and the survival outcomes in patients with resected, stage I-III NSCLC, who were additionally treated with adjuvant platinum-based combination chemotherapy.

2. Materials and methods

2.1. Patients and samples

The study included 116 non-small cell lung cancer patients who were diagnosed at the University Hospital, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic, between 1996–2000 and 2003-2011. Patient selection was based on the availability of well-documented treatment and follow up information. Corresponding archival tissue samples were obtained from the archives of the Department of Clinical and Molecular Pathology. From 116 patients, 42 patients were treated with surgical resection without adjuvant chemotherapy and 74 patients were treated with adjuvant platinum based combination chemotherapy after operation (56 patients with Carboplatin and Navelbine, 16 patients with Cisplatin and Navelbine; 2 patients with Cisplatin and Paclitaxel). Patient samples were re-examined and clinicopathological/demographic parameters including age, gender, clinical stage, depth of tumour invasion, lymph node metastasis, distant metastasis and differentiation were obtained. Patients were categorised according to the 7th WHO classification of tumors of the lung (2015) [19]. Detailed clinicopathological characteristics of patients and tumors are given in Table 1.

2.2. Tissue microarray construction

Tumour tissue microarrays (TMAs) were constructed using 116 formalin-fixed and paraffin-embedded primary NSCLC tissue samples. The tissue area for sampling was based on visual alignment with the corresponding H&E stained section on a slide. Two tissue cores (diameter: 1.00 mm; height: 3–4 mm) taken from a donor tumour block were placed in a recipient paraffin block with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). A core of normal tissue was punched from each case, and 4 μ sections of the resulting microarray block were used for immunohistochemical analysis.

Table 1
Clinicopathological characteristics of all non-small-cell lung cancer (NSCLC) patients. F, female, M, male, ADC, adenocarcinoma, SCC, squamous-cell carcinoma, LCC, large cell carcinoma, CHT, chemotherapy.

Total: 116		Frequency	Percentage
Gender	F	40	46.4
	M	76	53.6
Histology	ADC	44	37.9
	SCC	49	42.2
	LCC	23	19.8
Grade	G1	18	15.5
	G2	30	25.9
	G3	69	57.8
TNM Stage	I	32	27.6
	II	19	16.4
	III	65	56
Tumor size (T)	T1	28	24.1
	T2	69	58.6
	T3	15	12.9
	T4	4	3.4
Lymph node metastases (N)	N0	72	61.2
	N1	21	18.1
	N2	23	19.8
Treatment	Surgery	42	36.2
	Surgery + CHT	74	63.8

2.3. Immunohistochemical staining

Sections (4 μm) of formalin-fixed and paraffin-embedded (FFPE) tissue samples were deparaffinised in xylene and rehydrated using serial dilutions of alcohol. Heat mediated antigen retrieval was performed using automated Histos 5 rapid microwave histoprocessor (Milestone) in citrate buffer, pH6. Antibody against autophagy cleaved LC3A (APG8a, Abgent inc.) was used at dilution of 1:100. Tissue sections without primary antibody addition served as a negative control. In addition, normal kidney tissue was used as a postive control for LC3A staining, and normal lung tissue was used as an additional negative control. Stained slides were scanned by Olympus dotSlide Digital Virtual Microscopy System (Japan). The number of SLSs were evaluated on digitalised virtual slides, by two independent pathologists (M.G, J.Š.). In addition, membranous and cytoplasmic staining was assessed by the histoscore (Hscore) method (percentage of positive cells x intensity of staining (1, weak; 2, moderate; and 3, strong). The final histoscore ranged from 0 (minimum) to 300 (maximum)). Non-representative, damaged or non-evaluable cases were regarded as missing values.

2.4. NSCLC online data-base and survival analysis

The Kaplan-Meier plotter (KMplot, http://www.kmplot.com/analysis) is freely available and capable of assessing the effect of 54,675 genes on survival using 10,293 cancer samples. These include 2437 lung cancer samples with mean follow-up period of 40 months. The primary purpose of this tool is to conduct meta-analysis-based biomarker assessments [20]. We have checked the relatonship between LC3 A mRNA (gene symbol: MAP1LC3A) and NSCLC patient survival. In total, three different Affymetrix probe sets were available with relevant survival information. We used “auto select best cutoff” option, for the dichotomisation of MAP1LC3A mRNA expression level.

2.5. Statistical analysis of data

Groups were compared using the Mann Whitney *U* test and correlations were assessed using Spearman‘s rank testing. All analyses were performed using IBM SPSS statistical software version 22. $P < 0.05$ was considered statistically significant. Survival analysis was performed using Kaplan-Meier method and significance was evaluated using the log-rank test. For survival analysis, the number of SLSs and Hscore was dichotomised by using the respective medians. Primary study endpoint was OS, secondary study endpoint DFS. OS was defined as the period from the date of surgery until death or to the last date of follow-up; DFS was defined as the length of time between primary treatment and any sign of disease reccurence. Cases lost during follow-up and those ending in death from any cause other than lung cancer were censored. In addition, online software Cutoff Finder (Charite, Berlin, http://molpath.charite.de/cutoff/) was used for analysis of prognostically most important cut-off values [21].

3. Results

From 116 cases, 88 cases were evaluable for final analysis. LC3A staining was characterised by different expression patterns, including diffuse, cytoplasmic, membranous and perinuclear positivity as well as formation of SLSs. The staining patterns for LC3A and SLSs are shown in Fig. 1. Whilst membranous and perinuclear positivity of LC3A was rare (4 and 11 cases respectively), various levels of cytoplasmic expression (Hscore between 10–300) has been seen in the majority of cases (81/92%) with the median Hscore of 165. Out of 81 cytoplasmic LC3A positive cases, 64 tumors (79%) had a LC3A cytoplasmic expression above the median (Hscore > 165). The number of LC3A SLSs varied from 0 to 300, with the median number of 10. Frequency of SLSs above median (> 10) has been revealed in 42/48% cases from 88. There was no statistically significant association between either LC3A cytoplasmic positivity or the presence of LC3A SLSs and clinicopathological parameters including tumor grade, stage and histological subtype, despite the fact that LC3A cytoplasmic positivity was numerically higher in stage III tumors (Fig. 2). Hscore 130 was identified as the optimal threshold for LC3A and OS and DFS in the study population using the Cut-off finder software. However, using LC3A Hscore 130 as a threshold, high LC3A cytoplasmic expression did not reach the statistically significant association with a lower OS (HR = 2.22, P = 0.11) and lower DFS (HR = 2.44, p = 0.07) (Fig. 3A,B). On the other hand, using Cutoff Finder we have identified a statistically significant difference in OS and DFS according to the number of LC3A SLSs. In particular LC3 A SLSs > 20 were associated with shorter OS (HR = 2.27, p = 0.011) and DFS (HR = 2.27, p = 0.003) in whole group of NSCLC patients (Fig. 3C,D). The distribution of LC3A SLSs in different clinicopathological groups of NSCLC patients is given in Table 2.

KM plotter analysis for OS and DFS showed a statistically significant difference according to LC3A (MAP1LC3A) mRNA expression. High expression of MAP1LC3A mRNA was significantly associated with poor OS in three different Affymetrix probe sets (HR = 2.23, p = 1.4e-11; HR = 1.8, p = 1.1e-07; HR = 1.8, p = 4.8e-08) (Fig. 4A–C), as well as with poor DFS in three different Affymetrix probe sets (HR = 2.13, p = 0.00047; HR = 2.18, p = 0.00058; HR = 2.21, p = 0.00026) (Fig. 4D–F). We did not find any significant survival difference in different treatment groups of NSCLC patients, neither in our cohort, nor in KM plotter datasets.

4. Discussion

Clinical significance of authophagy in human NSCLC is incompletely understood. In the present study, we aimed to assess the role of autophagy in patients with potentially curative NSCLC undergoing surgery and adjuvant chemotherapy if indicated, using LC3A immunohistochmistry. We have found statistically significant association between LC3A SLSs and surivival outcomes in patients with resected, stage IB-III NSCLC. Particularly, high number of LC3A SLSs was significantly associated with poor OS and DFS (HR = 2.27, p = 0.011 and HR = 2.27, p = 0.003 respectively). In line with our study results, Karpathiou et al., also found a significant association between higher number LC3A SLSs and shorter OS and DFS in resected stage I-II NSCLC, but not in stage III NSCLC [15]. However, according to Schläfli et al. high levels of LC3A SLSs are not associated with OS in stage IA-IIB NSCLC [16]. In addition, an increased number of LC3A SLSs is associated with poor prognosis in breast [12], endometrial [11], colorectal [7] carcinomas and cutaneous squamous cell carcinomas [10]. Similar to the study from Karpathiou et al. [15], we did not find a statistically significant relationship between cytoplasmic LC3A expression and survival outcomes in our cohort of NSCLC patients. To define the prognostically important cutoff for SLSs we used online tool “cutoff finder“, designed by Budczies et al. from Charite medical university, Berlin, Germany [20]. Several other studies have employed the same method for prognostic studies in various tumors. Including one recent study in glioblatoma patients, where cutoff finder was used to define the prognostically important cutoff values for immunohistochemically detected proteins, such as bcl-2, cyclin D1, p16, p21, p27, p53, Sox11 and WT1 [4].

In addition to immunohistochemical analysis, we have also checked the relationship between LC3A mRNA expression and survival in publicly available NSCLC data set from KM plotter, which also provides the tool for survival analysis [22]. Analysis of all available Affimetrix probe sets showed that high expression of LC3A mRNA is significantly associated with OS (HR = 2.23, p = 1.4e-11; HR = 1.8, p = 1.1e-07; HR = 1.8, p = 4.8e-08) and DFS (HR = 2.13, p = 0.00047; HR = 2.18, p = 0.00058; HR = 2.21, p = 0.00026) (Fig. 4). LC3A mRNA expression is not an accepted method for assessing autophagy activity. However, our study results indicate that it might be used as an

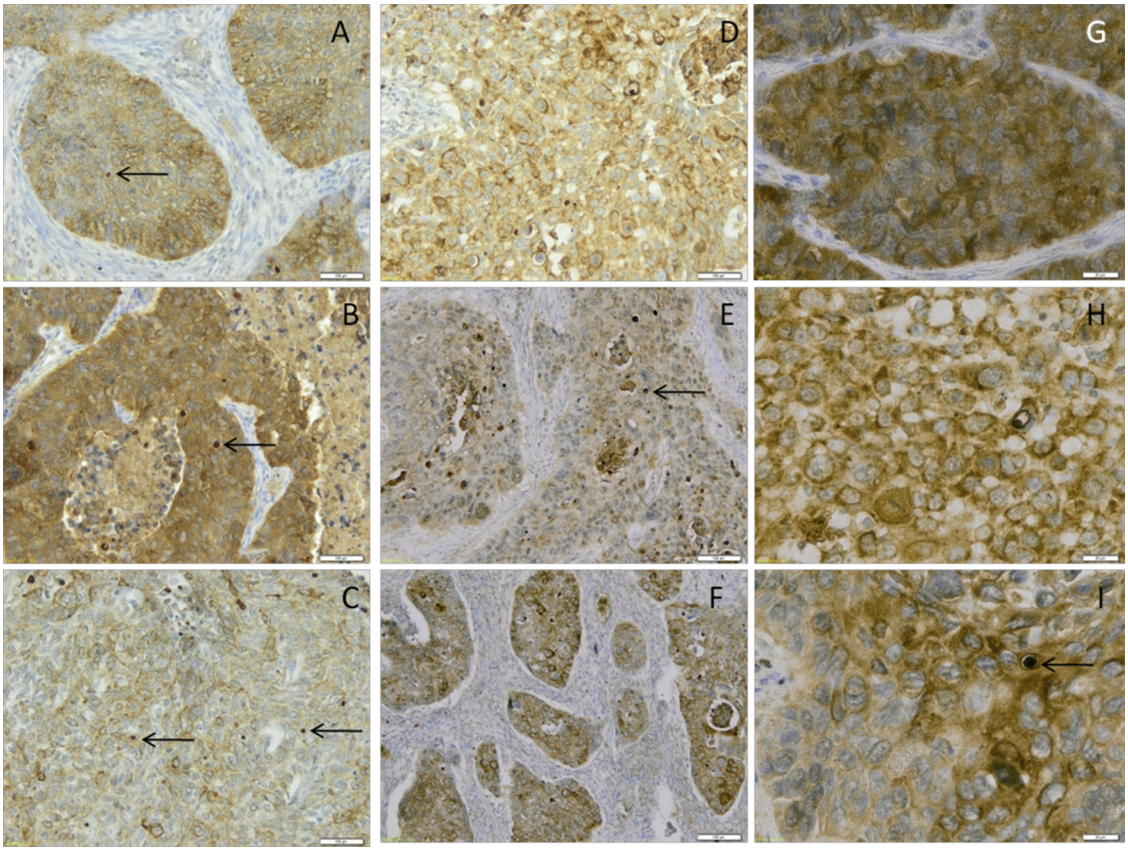

Fig. 1. The various immunohistochemical patterns of LC3A protein in lung cancer. Stone-like structures (SLSs) are marked with arrows. (A) Diffuse cytoplasmic positivity and SLSs in squamous-cell carcinoma. (B) Diffuse cytoplasmic positivity and SLSs in adenocarcinoma. (C) Membranous positivity and SLSs in adenocarcinomas. (D) Cytoplasmic and perinuclear positivity in adenocarcinomas. (E) SLSs and cytoplasmic positivity in solid adenocarcinomas. (F) SLSs and cytoplasmic positivity in squamous-cell carcinoma. (G) diffuse cytoplasmic positivity in adenocarcinomas. (H) Cytoplasmic and juxta-nuclear positivity in adenocarcinomas. (I) Diffuse cytoplasmic positivity and SLSs in adenocarcinomas.

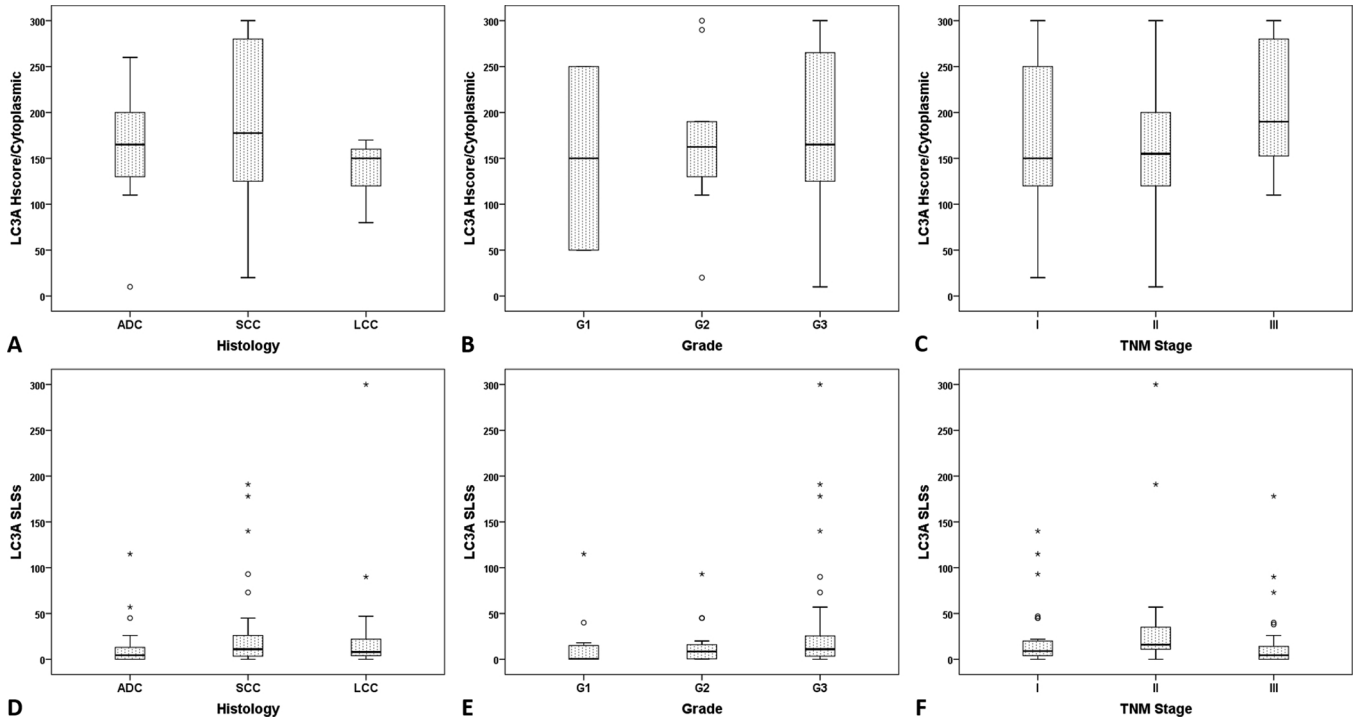

Fig. 2. Distribution of cytoplasmic LC3A (A,B,C) and LC3A stone-like structures (SLSs) (D,E,F) in different clinicopathological groups of non-small-cell lung cancer (NSCLC), including histological subtype, grade, and stage of the disease.

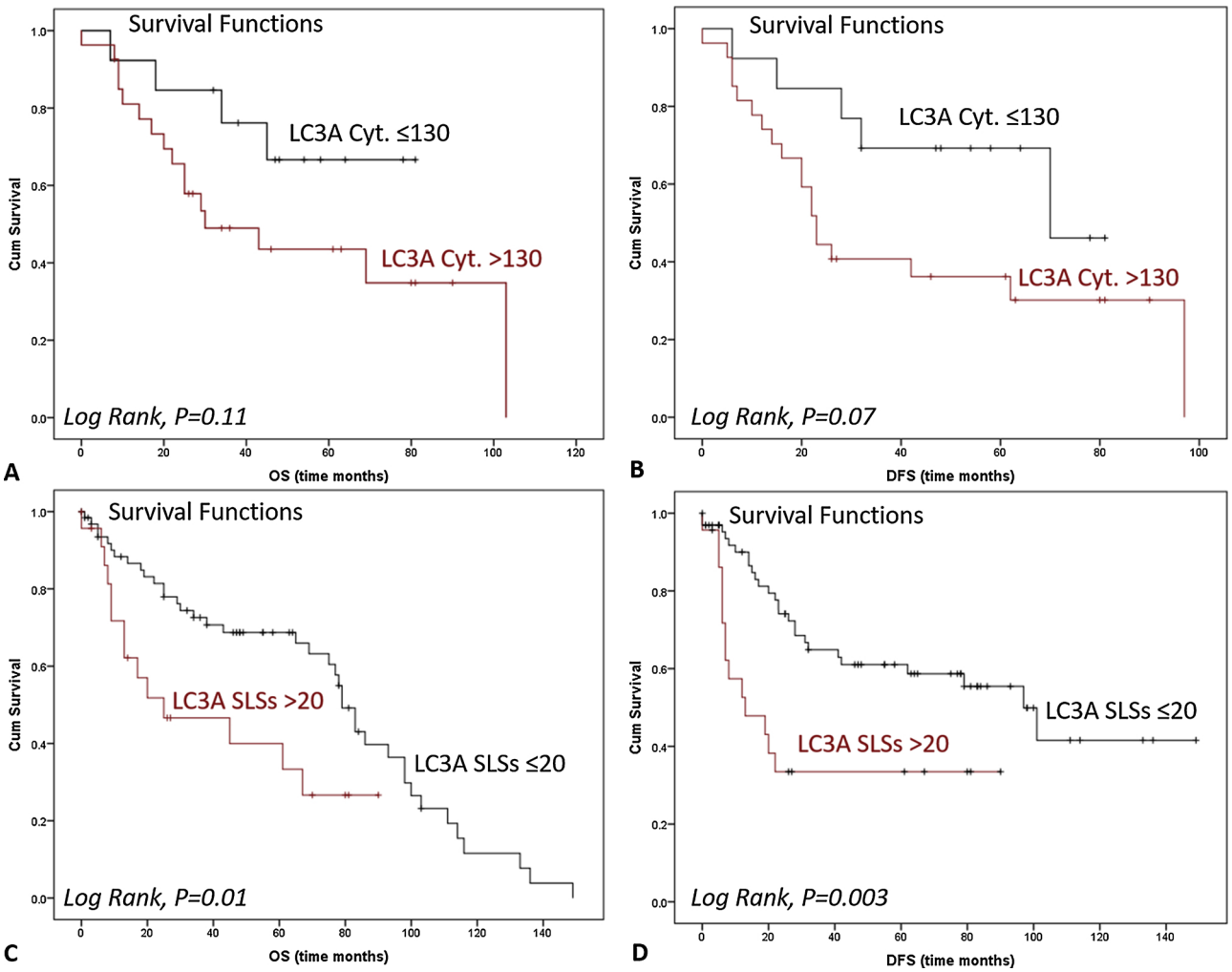

Fig. 3. Kaplan–Meier survival analysis. Panels A and B show the association between LC3A cytoplasmic expression and overall survival (OS) and disease-free survival (DFS) respectively. Panels C and D show the association between LC3A SLSs and OS and DFS respectively.

Table 2
Clinicopathological characteristics and distribution of LC3A stone-like structures (SLSs) in 88 non-small-cell lung cancer (NSCLC) patients available for staining evaluation. F, female, M, male, ADC, adenocarcinoma, SCC, squamous-cell carcinoma, LCC, large cell carcinoma, CHT, chemotherapy.

Total: 88		N/%	LC3A SLSs	
			≤20 N/%	> 20 N/%
Gender	F	31/35	25/80	6/20
	M	57/65	39/68	18/32
Histology	ADC	27/31	21/78	6/22
	SCC	40/45	28/70	12/30
	LCC	21/24	15/71	6/29
Grade	G1	12/14	10 /83	2/17
	G2	21/24	17/81	4/19
	G3	55/62	37/67	18/33
TNM stage	I	28/32	21/75	8/25
	II	18/20	10/56	8/44
	III	41/36	33/80	8/20
Tumor size (T)	T1	19/21	15/79	4/21
	T2	55/63	40/73	15/27
	T3	9/10	6/67	3/33
	T4	5/6	3/60	2/40
Lymph node metastases (N)	N0	55/63	40/73	15/27
	N1	18/20	13/72	5/28
	N2	15/17	11/73	4/27
Treatment	Surgery	26/29	23/88	3/12
	Surgery + CHT	62/71	49/79	13/21

additional prognostic parameter in patients with NSCLC. High level of total LC3A mRNA in tumor tissue, might also reflect an ongoing level of autophagy activity. However, this finding needs further confirmation on other independent NSCLC datasets. Comparison between LC3A mRNA level and LC3A immunohistochemical expression patterns on matching tumor samples would be also desirable. The predictive value of autophagy activity with regards to standart treatments has not been reported so far. In our study we also did not find any significant survival difference in a subgroup of adjuvantely treated patients, including NSCLC cohorts from our institution and KM plotter.

We have found the differential pattern of LC3A expression in NSCLC specimens, including diffuse cytoplasmic, membranous and perinuclear positivity, as well as the formation of SLSs (Fig. 1). In our NSCLC speciments, membranous and perinuclear positivity was rare, whilst 92% of patients revealed cytoplasmic LC3A positivity at least to some extent (Hscore ≥ 10), and above median count (n ≥ 10) of LC3A SLSs was seen in 48% of cases. These results further indicate the tumor specific regulation of autophagy activity. For example perinuclear pattern of LC3A staining which was quite rare in our NSCLC cohort, is frequent finding in neoplastic breast lesions [12] and in colorectal carcinoma [7]. On the other hand, LC3A SLSs are not detected in malignant lymphomas and reactive follicular hyperplasias [18]. We did not find a statistically significant association between either LC3A cytoplasmic expression or LC3A SLSs and clinicopathological variables. Including histological subtype, grade and stage of the disease. Although not significant, we have found a slightly higher number of LC3A SLSs in SCC and LCC,

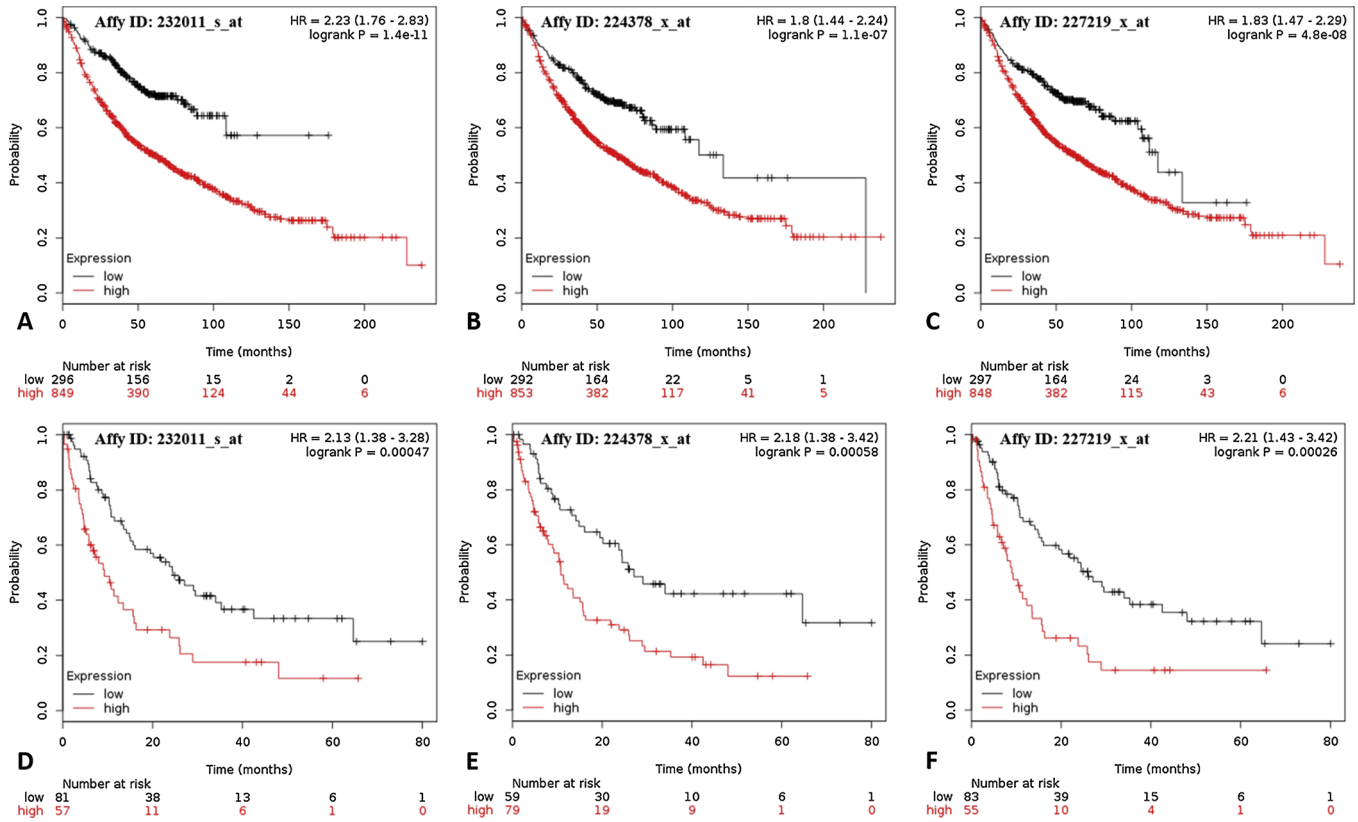

Fig. 4. Survival analysis of LC3A gene (MAP1LC3 A) expression on KM plotter. Panels A, B and C show the association between LC3A and overall survival (OS) in different Affymetrix probe sets; Panels D, E and F show the association between LC3A and disease-free survival (DFS) in different Affymetrix probe sets.

compared to ADC. Similarly, Karpathiou et al. who also found a high occurrence SLSs in SCC and LCC, but not in ADCs. However, with the difference from our study large number of SLSs was significantly correlated with nodal spread and lymphovascular invasion (LVI) [15].

The causes as well as the mechanisms of increased autophagy activity in malignant tumors are not well understood. It has been shown in experimental models that increased autophagy represents an adaptive response of lung cells to injurious stimuli, such as hypoxia, inflammation, ischemia, pharmaceuticals, or inhaled xenobiotics (i.e., air pollution, cigarette smoke) [23]. It has also been shown that, there is a relationship between endoplasmic reticulum (ER) stress and activation of autophagy [24,25]. ER stress response can be activated in normal or transformed lung cells or in fibroblasts as a general response to cigarette smoke or agents associated with acute lung injury [24,25]. Unfortunatelly, in our NSCLC dataset there was no information available about smoking status of patients, so we could not check if the increase in autophagy activity is related to smoking. Other potential limitations of our study are the smaller sample size and use of TMAs constructed from two 1 mm cores from each patient. However, the later might be less important issue. Even though, recent recommendations indicate higher robustness of using three 1 mm cores from each patient, we have a good experience in testing prognostic markers on TMAs, constructed from large number of NSCLC patients, using two 0.6 mm cores from each. We were even able to confirm high prognostic value of tumor infiltrating lymphocytes based on the investigation of mentioned TMAs (unpublished data). We also have not done more detailed characterisation of autophagy process in NSCLC, using other relevant autophagy markers. However, from clinical point of view, single and efficient marker of patient prognosis is always more wellcome, rather than combination of several biomarkers. Overall, our study emphasizes an important role of autophagy in NSCLC patient prognosis, and should be used as an inspiration for further, more in depth studies, for better understanding of the mechanisms of deregulated autophagy. Such studies might also identify an additional treatment targets in patients with NSCLC.

Author contribution

All authors contributed equally to preparing the manuscript.

Conflict of interest statement

The authors state that there are no conflicts of interest regarding the publication of this article.

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