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ORIGINAL ARTICLE

Association between autophagy and *KRAS* mutation with clinicopathological variables in colorectal cancer patients

Noel Jacques AWI[1], Hooi-Yeen YAP[1], Subasri ARMON[2], John Seng-Hooi LOW[3], Kaik-Boo PEH[4], Suat-Cheng PEH[1,3], C Soon LEE[5], Sin-Yeang TEOW[1]*

[1]Department of Medical Sciences, School of Medical and Life Sciences, Sunway University, Jalan Universiti, Bandar Sunway, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia, [2]Pathology Department, Hospital Kuala Lumpur, Jalan Pahang, 50586 Kuala Lumpur, Malaysia, [3]Sunway Medical Centre, Jalan Lagoon Selatan, Bandar Sunway, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia, [4]Mahkota Medical Centre, Mahkota Melaka, Jalan Merdeka, 75000 Melaka, Malaysia, [5]Discipline of Pathology, School of Medicine, Western Sydney University, Sydney, Australia.

Abstract

Autophagy is a host defensive mechanism responsible for eliminating harmful cellular components through lysosomal degradation. Autophagy has been known to either promote or suppress various cancers including colorectal cancer (CRC). *KRAS* mutation serves as an important predictive marker for epidermal growth factor receptor (EGFR)-targeted therapies in CRC. However, the relationship between autophagy and *KRAS* mutation in CRC is not well-studied. In this single-centre study, 92 formalin-fixed paraffin-embedded (FFPE) tissues of CRC patients (42 Malaysian Chinese and 50 Indonesian) were collected and *KRAS* mutational status was determined by quantitative PCR (qPCR) (n=92) while the expression of autophagy effector (p62, LC3A and LC3B) was examined by immunohistochemistry (IHC) (n=48). The outcomes of each were then associated with the clinicopathological variables (n=48). Our findings demonstrated that the female CRC patients have a higher tendency in developing *KRAS* mutation in the Malaysian Chinese population ($p<0.05$). Expression of autophagy effector LC3A was highly associated with the tumour grade in CRC ($p<0.001$) but not with other clinicopathological parameters. Lastly, the survival analysis did not yield a statistically significant outcome. Overall, this small cohort study concluded that *KRAS* mutation and autophagy effectors are not good prognostic markers for CRC patients.

Keywords: Autophagy proteins; *KRAS* mutation; prognosis; Malaysian; Indonesian; LC3A; LC3B; p62; colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) resulted in 9.2% of cancer-related death worldwide which is ranked second after lung cancer (18.4%) according to Global Cancer Observatory 2018, World Health Organization (WHO).[1] There were approximately 1.85 million new cases in 2018 which was ranked third after lung and breast cancers. In Malaysia, CRC is the second most common cancers and ranked third in terms of mortality rate.[1] A recent key review suggested that lifestyle modification, nutritional factors, screening and chemoprevention are crucial in controlling the CRC incidence and mortality rates.[2] Other emerging challenges are standardisation of molecular biomarker testing and guidance on personalised therapies in CRC which require global concerted efforts.[3,4]

The aetiology of CRC is complex and both genetic and environmental factor have huge impacts on it. One of the important pathways is *EGFR* (epidermal growth factor receptor) signalling pathway which involves *KRAS* (Kirsten rat sarcoma viral oncogene), *NRAS* (neuroblastoma ras viral oncogene homolog) and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) genes.[4,5] In the past decade, this pathway has shown to play pivotal role in CRC development and progression, and subsequently serve as a therapeutic target for

**Address for correspondence*: Sin-Yeang Teow, PhD, Senior Lecturer, Sunway University, Bandar Sunway, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia. Phone: +603 7491 8622 Ext. 7449. Fax: +603 5635 8633. Email: *ronaldt@sunway.edu.my*

cancer therapies especially in advanced stage or metastatic CRC (mCRC).[6] *KRAS* mutation occur in approximately 30-40% CRC and it results in tumour aggressiveness and confers poorer survival in CRC patients.[7] It also acts as an excellent predictive marker for the resistance of EGFR-targeted therapies.[7]

Since the cause of CRC is multifactorial, many other oncogenic proteins from other biological pathways have also been studied on their linkage with CRC and their potential use as biomarkers. For example, colon cancer secreted protein-2 (CCSP-2) was shown to be a promising marker for detection of metastatic or recurrent CRC[8] while activated EGFR and HER3 proteins could act as predictive markers for better overall survival in patients treated by anti-EGFR therapy.[9] Recently, autophagy which is a self-defensive mechanism that degrades and eradicates damaged cellular proteins or organelles, has shown to play a double-faced role in either promoting or suppressing the tumour growth in CRC.[10] This starvation-induced mechanism has also been extensively studied for its potential as prognostic and/or predictive biomarkers in CRC.[11]

Interestingly, an *in vitro* study showed that the expression of mutated *KRAS* upregulated the autophagy followed by the activation of MEK/ERK pathway in CRC, and resulted in cancer cell survival during starvation.[12] Furthermore, immunohistochemical analysis of CRC tissues by Schmitz *et al*. revealed that autophagy effectors including LC3, p62 and Beclin-1 could serve as promising prognostic markers.[13] However, these findings have not been further investigated and there is certainly a big gap of understanding between *KRAS* mutation and autophagy in CRC. This study aimed to evaluate the *KRAS* mutation status and autophagy expression of CRC tissues collected from a single centre. The findings were then correlated with the patients' clinicopathological parameters and their potential use as prognostic marker were investigated.

MATERIALS AND METHODS

Reagents

Antibodies were purchased from Abcam (Cambridge, UK) and Cell Signalling Technology (Danvers, MA, USA) for this study (Table 1). Beta-actin antibody was used as the assay control for IHC staining. All chemicals and solvents were purchased from Merck (Darmstadt, Germany).

Tissue sample and clinicopathological data collection

Archived formalin-fixed paraffin-embedded (FFPE) tissue blocks from 92 patients (42 Malaysian Chinese and 50 Indonesian) diagnosed and underwent colectomy at the Mahkota Medical Centre from the year 2013 to 2015 were retrieved for *KRAS* mutation study. Out of the 92 cases, tumour and adjacent normal mucosa blocks from 48 patients with complete clinical pathological data were chosen for autophagy staining. This study has been approved by Sunway University Research Ethics Committee (SUNREC 2017/051), Sunway Medical Centre Independent Research Ethics Committee (013/2017/ER), and National Medical Research Ethic Committee (NMRR-18-1137-42073).

Haematoxylin and Eosin (H&E) staining

The tissue blocks were sectioned at a thickness of 4 μm and placed on a silane-coated slides (MUTO #5116, Tokyo, Japan) before heating at 60˚C for 1 h. The paraffin was then removed using the decloaking chamber (Biocare Medical NxGen, Pacheco, CA, USA) and DepART solution (Biocare Medical #BRI4044G1, Pacheco, CA, USA) at 60˚C for 20 min. After the slides were washed, the sections were stained with haematoxylin (Leica #3801575, Wetzlar, Germany) for 8 min followed by washing for 5 min under running tap water. The tissues were then differentiated in 1 % acid alcohol (concentrated HCL in 70 % ethanol) for 30 sec and washed in running tap water for 1 min. Then, the slides were immersed in 0.1 M

TABLE 1: Details of antibodies used for autophagy examination in tissues

Antibody	Type	Host species	Dilution used	Clone	Brand
p62/ SQSTM1	Monoclonal	Rabbit	1:500	EPR18351	Abcam
LC3A	Monoclonal	Rabbit	1:1,000	D50G8	Cell Signalling Technology
LC3B	Monoclonal	Rabbit	1:1,000	D11	Cell Signalling Technology
Beta-actin	Monoclonal	Mouse	1:3,000	8H10D10	Cell Signalling Technology

sodium bicarbonate for 1 min to allow bluing followed by washing in running tap water for 5 min. The water was removed by dipping the slides in 95 % ethanol for 10 X. The tissues were counterstained by immersing the slides in eosin (Leica #3801600, Wetzlar, Germany) for 2 min followed by dehydration in 95 % ethanol for 5 min. Further dehydration of the tissues was performed through immersion of the slides in 2 changes of absolute ethanol for 5 min each. The tissues were then cleared in 2 changes of xylene for 5 min each before mounted by xylene-based mounting medium (Leica #3801732, Wetzlar, Germany). The slides were then verified and the tumour portion of each slide was marked by senior pathologists.

Immunohistochemistry (IHC)

Sections were prepared as described above. The paraffin removal and antigen retrieval were performed using the FLEX Target Retrieval Solution Low pH (Agilent Dako #K800521, Santa Clara, CA, USA) for LC3A and LC3B and High pH (Agilent Dako #K800521, Santa Clara, CA, USA) for p62/SQSTM1 staining. The slides were heated in the decloaking chamber at 110˚C for 30 min and washed in deionised water to remove the retrieval solution residue. The tissues were stained using the REAL Envision Detection System (Agilent Dako #K500711, Santa Clara, CA, USA) according to the manufacturer's instructions. The tissues were blocked by using the Endogenous Enzyme Block (Agilent Dako #S202386, Santa Clara, CA, USA) for 10 min. The tissues were then incubated overnight in the antibody solution according to the respective dilutions (Table 1) in the humidified slides chamber at 4 ˚C. The tissues were then incubated with the HRP-Polymer (Agilent Dako #5007, Santa Clara, CA, USA) for 30 min and the DAB+ chromogen (Agilent Dako #5007, Santa Clara, CA, USA) was applied for 5 min. The tissues were then counterstained with haematoxylin and dehydrated with ethanol and cleared with xylene before mounting. The IHC slides were then assessed by the senior pathologists for scoring. Each slide was scored for percentage of positivity and intensity of stained cells as follows: 0% (0), <10 % (1), 10-25 % (2), 26-50 % (3), 51-75 % (4), >75 % (5); negative (0), weak (1), mild (2), moderate (3), strong (4). The sum of positivity and intensity was used as a total staining score for each protein. Score of 0 was represented as 'negative' staining, while 1 to 5 and 6 to 9 were represented as 'low' and 'high' staining, respectively. Similar IHC protocol was used for all the antibodies following the optimised dilutions (Table 1).

KRAS mutation analysis

A total of 5 sections from each FFPE tissue at a thickness of 10 μm was collected for DNA extraction. Paraffin was dissolved by xylene and the DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of DNA was examined by checking the A_{260}/A_{280} ratio using SpectraMax QuickDrop micro volume spectrophotometer (Molecular Devices, San Jose, CA, USA). Generally, the DNA was diluted at a ratio of 1:10 for the *KRAS* mutation testing using Therascreen KRAS RGQ PCR Kit (Qiagen. Hilden, Germany) according to the manufacturer's protocol. This detection was performed on Rotor-Gene Q MDx 5plex HRM (Qiagen, Hilden, Germany) and analysed using Rotor-Gene Q Software, version 2.3.1.49 (Qiagen, Hilden, Germany).

Association and statistical analysis

The primary outcome was overall survival, which was defined as the time from the initiation of surgery to death due to the disease or to the date of the last follow-up. Significant differences in the clinical-pathological variables between each group were tested using the Fisher's exact or Chi-square tests. The distribution of overall survival was estimated using the Kaplan-Meier analysis and log-rank test. The analyses were performed using the Statistical Package for Social Sciences, version 26.0 (SPSS Inc, Chicago, IL, USA), and $p < 0.05$ (2-tailed test) was considered statistically significant.

RESULTS

Patient cohort analysis

This study cohort consists of 42 Malaysian Chinese and 50 Indonesian with unknown ethnicity, respectively. In combination, CRC was more prevalent in females (Table 2), CRC patients of Indonesian population had lower mean age (54.8-58.9) than the Malaysian Chinese population (62.7-65.7) In terms of the tumour characteristics, higher incidence was seen in advanced stage (stage III and IV), moderate tumour grade, tumour size less than 5cm, and in left colon than in rectum. Proximal colon (caecum, ascending, hepatic flexure and transverse colon) was termed as right colon while distal colon (splenic flexure, descending,

sigmoid and rectosigmoid colon) was termed as left colon. Higher frequencies were also found in T3 or T4 stage, histopathology with positive lymph nodes, and localised tumours without infiltrating lymphocytes (Table 2).

KRAS mutation and its prognostic value

DNA was extracted from the tissues and *KRAS* gene mutation was determined by PCR method. From the 92 patients, 44% had *KRAS* mutation, 12ASP (20%) being the most frequent mutation in codon 12 followed by 13ASP (14%) in codon 13 (FIG 1A). When assessed separately, Indonesian (50%) has a higher *KRAS* mutation rate than Malaysian Chinese (36.6%) (FIG 1B). Furthermore, the mutation was more frequent in the right side of colon (54.5%) compared to left colon (38.5%) and rectum (43.3%)

TABLE 2: *KRAS* mutation and the correlation with clinicopathological variables

Clinico-pathological variables	Malaysian Chinese				Indonesian			
	***KRAS* wildtype (n=27)**	***KRAS* mutated (n=15)**	**Total (n=42)**	***p* value**	***KRAS* wildtype (n=25)**	***KRAS* mutated (n=25)**	**Total (n=50)**	***p* value**
Gender								
Female	12	13	25	0.01*	14	11	25	0.572
Male	15	2	17		11	14	25	
Age (mean ± SD, age range)								
	62.7 ± 12.7 (36-86)	65.7 ± 10.9 (43-79)	42	0.79	58.9 ± 11.7 (30-82)	54.8 ± 11 (29-69)	50	0.202
Tumour grading								
Poor	2	0	2	0.551	1	3	4	0.304
Moderate	23	14	37		23	19	42	
Unknown	2	1	3		1	3	4	
Tumour size								
<5cm	17	9	26	0.141	16	14	30	0.837
>5cm	10	4	14		8	10	18	
Unknown	0	2	2		1	1	2	
Tumour site								
Colon	13	8	21	0.966	12	13	25	0.999
Rectum	14	6	20		13	12	25	
Unknown	0	1	1		0	0	0	
Staging								
I and II	9	3	12	0.485	5	6	11	0.999
III and IV	18	12	30		20	19	39	
Tumour stage								
T1 and T2	1	1	2	0.999	2	0	2	0.490
T3 and T4	26	14	40		23	25	48	
Lymph node metastases								
N-	10	3	13	0.314	5	6	11	0.999
N+	17	12	29		20	19	39	
Distant metastasis								
M-	25	15	40	0.530	23	19	42	0.247
M+	2	0	2		2	6	8	
Tumour infiltrating lymphocytes								
Present	8	4	12	0.999	11	9	20	0.773
Absent	19	11	30		14	16	30	

SD – standard deviation; N-/ N+ - Histopathology with negative or positive nodes; M-/ M+ - Not metastatic or metastatic; * - Statistically significant

(FIG 1C) in both populations combined. To assess the correlation of *KRAS* mutation with clinicopathological variables, Fisher's exact or Chi-square tests were performed. In the Malaysian Chinese population, *KRAS* mutation significantly correlated with the gender ($p<0.05$), but did not show any correlation with other clinicopathological variables (Table 2). Kaplan-Meier curves showed that the CRC patients with *KRAS* mutation exhibited poorer survival compared to the wild-type (FIG 2). However, the difference was not significant from the statistical analysis. This suggests that *KRAS* mutation does not have any prognostic value for the CRC patients.

Autophagy expression in colorectal cancer tissues and its prognostic value

FIG 3 demonstrates cytoplasmic and perinuclear expression of LC3A, LC3B, and p62 in malignant glands of colorectal adenocarcinoma from 48 patients. Different levels of expression (negative, low or high) was determined based on the IHC scores by the pathologists. Negative staining of p62 was not shown as all tissues showed positive staining. The scoring was then summarised and tabulated in Table 3. Overall, all tissues

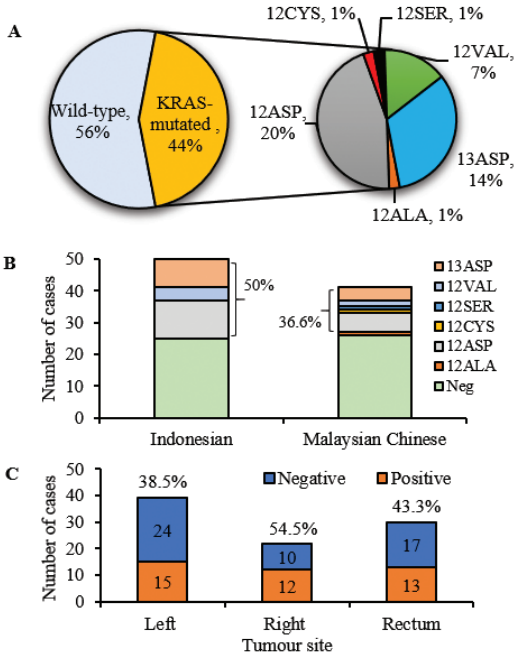
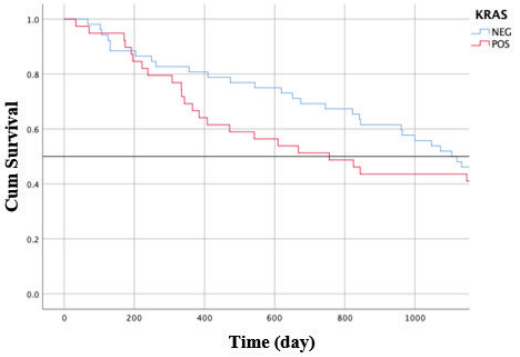


FIG. 1: *KRAS* mutation study of 92 colorectal cancer patients. (A) Frequency of *KRAS* mutation in: **(A)** codon 12 and 13; **(B)** Malaysian Chinese and Indonesian; and **(C)** tumour site in left or right colon or rectum



Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.104	1	.747
Breslow (Generalized Wilcoxon)	.865	1	.352
Tarone-Ware	.451	1	.502

Test of equality of survival distributions for the different levels of KRAS.

FIG. 2: Survival analysis of colorectal cancer patients with *KRAS* mutation.

expressed p62 and 93.8 % had high expression while only 14.6 % and 25 % of LC3A and LC3B expression were seen, respectively. Tissues which simultaneously express the autophagy markers were shown in FIG 4. 64.6% of p62-positive tissues expressed p62 only while 4.2% expressed LC3A, LC3B and p62. 10.4% co-expressed LC3A and p62 while 20.8% co-expressed LC3B and p62. Correlation study showed that LC3A expression was significantly correlated with the tumour grading ($p < 0.05$), but not with other clinical pathological variables (Table 3).

As all CRC tissues expressed p62, we examined whether this expression was cancer-specific using the adjacent non-cancer tissues from the 48 patients. Only 44 sets of non-tumour tissues were included in this study as the rest of blocks failed to be retrieved. Our findings showed that 94.5 % of the non-cancer tissues had p62 staining (86.4 % showing high and 9.1 % showing low expression) (Table 4). The p62 expression in tumour tissues was associated with the adjacent non-cancer tissues ($p < 0.05$). On the other hand, LC3A and LC3B (only one case showed low expression) were not or mildly expressed in the adjacent non-tumour tissues (Table 4). Further, Kaplan-Meier survival analysis was performed and the outcome showed that the three autophagy proteins were not suitable to be used as prognostic markers (FIG 5).

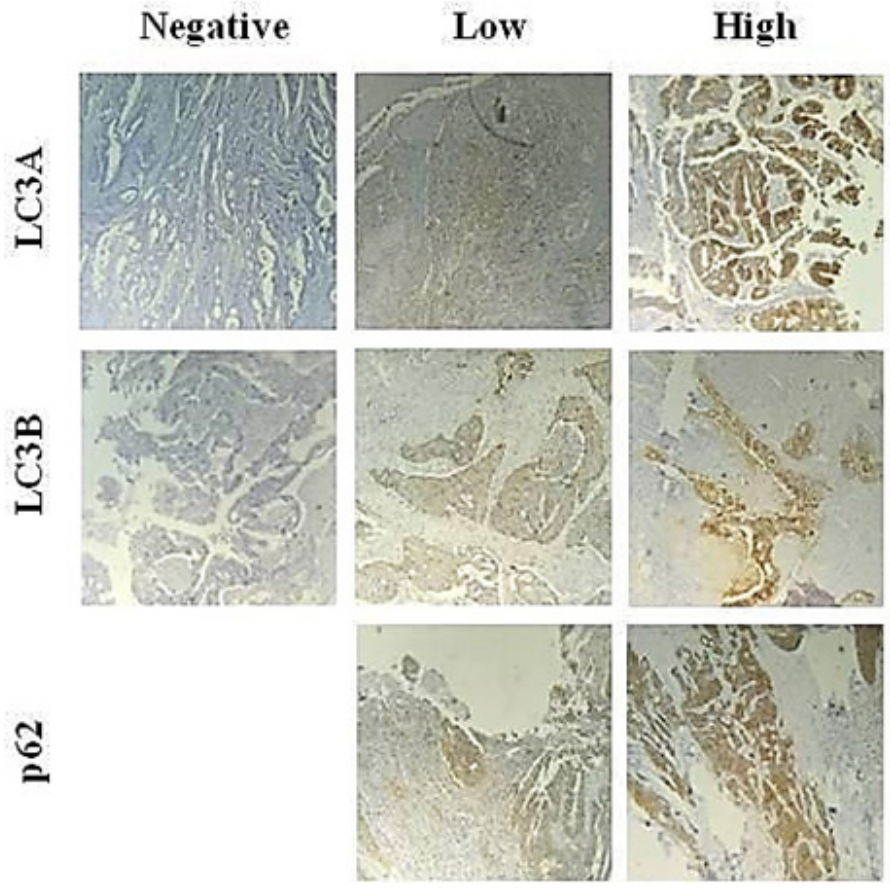


FIG. 3: Different expression of LC3A, LC3B and p62 in colorectal cancer tissues. Negative staining of p62 is not shown as all tissues express p62. Images were captured at x400 magnification.

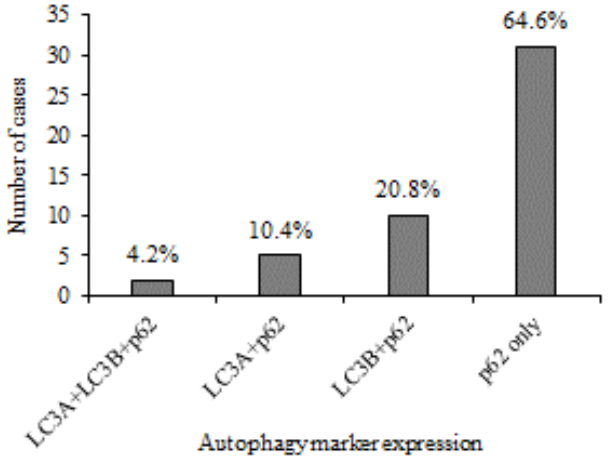


FIG. 4: Simultaneous expression of p62, LC3A and/or LC3B in colorectal cancer tissues.

TABLE 3: Immunostaining for p62, LC3A, and LC3B expression and the correlation with clinicopathological variables

Clinico-pathological variables	p62			LC3A				LC3B			
	Low (n=3)	High (n=45)	*p* value	Neg. (n=41)	Low (n=3)	High (n=4)	*p* value	Neg. (n=36)	Low (n=10)	High (n=2)	*p* value
Gender											
Female	1	28	0.554	25	1	3	0.527	23	4	2	0.198
Male	2	17		16	2	1		13	6	0	
Age											
<59	1	20	0.999	18	2	1	0.546	16	5	0	0.423
>59	2	25		27	1	3		20	5	2	
Tumour grading											
Poor	1	4	0.375	1	3	1	**0.0001***	3	1	1	0.305
Moderate	2	37		36	0	3		29	9	1	
Unknown	0	4		4	0	0		4	0	0	
Tumour size											
<5cm	1	28	0.437	27	2	0	0.061	20	8	1	0.628
>5cm	2	14		11	1	4		13	2	1	
Unknown	0	3		3	0	0		2	0	0	
Tumour site											
Colon	2	29	0.966	26	2	3	0.987	23	6	2	0.817
Rectum	1	15		14	1	1		12	4	0	
Unknown	0	1		1	0	0		1	0	0	
Staging											
I and II	1	8	0.472	8	0	1	0.667	8	1	0	0.536
III and IV	2	37		33	3	3		28	9	2	
Tumour stage											
T1 and T2	0	2	0.999	2	0	1	0.837	1	1	0	0.573
T3 and T4	3	43		39	3	3		35	9	2	
Lymph node metastases											
N-	1	9	0.512	8	0	2	0.235	9	1	0	0.446
N+	2	36		33	3	2		27	9	2	
Distant metastasis											
M-	3	40	0.999	36	3	4	0.621	31	10	2	0.394
M+	0	5		5	0	0		5	0	0	
Tumour infiltrating lymphocytes											
Present	1	32	0.227	27	3	3	0.45	24	7	2	0.61
Absent	2	13		14	0	1		12	3	0	
***KRAS* status**											
KRAS wildtype	1	23	0.999	20	2	2	0.836	17	5	2	0.384
KRAS mutated	2	22		21	1	2		19	5	0	

Neg. – Negative; N-/ N+ - Histopathology with negative or positive nodes; M-/ M+ - Not metastatic or metastatic;
* - Statistically significant

DISCUSSION

KRAS mutation is one of the frequent *RAS* gene mutations in solid tumours such as pancreas, lung and CRC. Mutated *KRAS* mainly drives malignant transformation in cancers while acting as an important biomarker of resistance to anti-EGFR therapy which is a targeted therapy for mCRC.[4,5] Our studied CRC cohort showed 44% of *KRAS* mutation which is consistent to the consensus global rate of 30 to 50%.[14,15] Interestingly, the Indonesian patient cohort alone has higher *KRAS* mutation rate of 50%. This

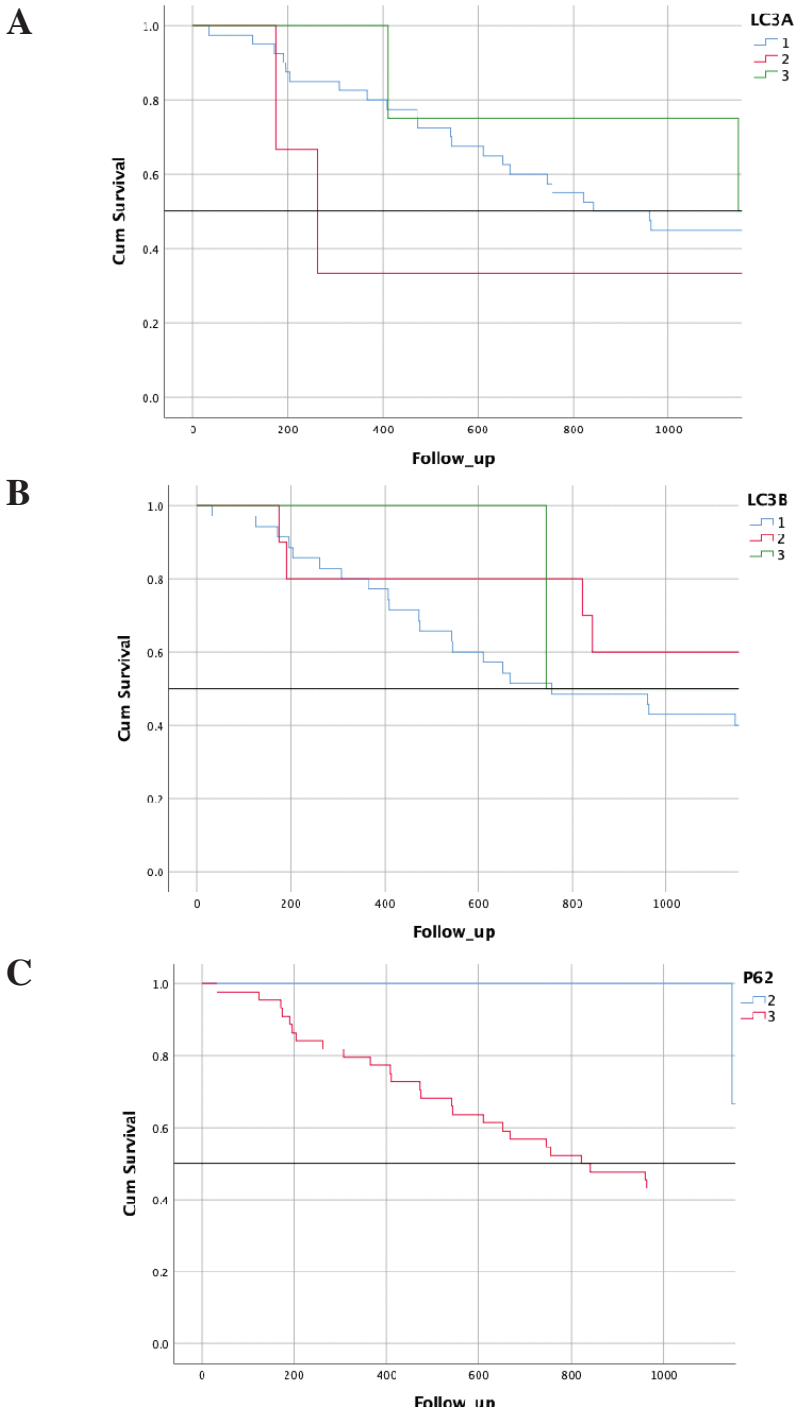


FIG. 5: Survival analysis of colorectal cancer patients with **(A)** LC3A; **(B)** LC3B; and **(C)** p62 expression.

TABLE 4: Immunostaining of LC3A, LC3B and p62 in tumour and adjacent non-tumour tissues

Autophagy marker	Expression level	Tissue type				*p* value
		Tumour (n = 48)		Non-tumour (n = 44)		
		Number (n)	Percent (%)	Number (n)	Percent (%)	
LC3A	Negative	41	85.4	44	100	NA
	Low	3	6.3	0	0	
	High	4	8.3	0	0	
LC3B	Negative	36	75	43	97.7	0.137
	Low	10	20.8	1	2.3	
	High	2	4.2	0	0	
p62	Negative	0	0	2	4.5	**0.002***
	Low	3	6.3	4	9.1	
	High	45	93.7	38	86.4	

NA – not applicable; * – Statistically significant

could be due to the small sample size. Among the mutations, 12ASP was the most common mutation which is consistent to the findings from RASCAL (Kirsten RAS in-Colorectal-Cancer Collaborative Group) and RASCAL II studies.[14,15] From the association studies, our findings showed that *KRAS* mutation was significantly associated with female gender in Malaysian Chinese only, but not in Indonesian cohort and other clinicopathological characteristics. This finding is consistent with another study with larger sample size involving 226 CRC Chinese patients in China.[16] However, there was a study involving 1,735 patients in France showed higher frequency of *KRAS* mutation in males.[17] Consistent to a previous study,[18] *KRAS* mutation was not associated with the prognosis in CRC patients. Several studies demonstrated that specific mutations in codon 12 might have prognostic values.[19,20] However, this question can not be addressed in this study due to the limitation of small sample size. It would also be valuable to determine other mutations such as *NRAS*, *BRAF* and PIK3CA and important features such as microsatellite instability (MSI) status in association with *KRAS* when assessing its prognostic implication as described elsewhere.[21,22]

To our best knowledge, this is the only study with Malaysian and Indonesian cohort focusing on the clinicopathological and prognostic implications of autophagy proteins in CRC. Out of the three proteins, LC3A was found to be associated with the tumour grade of CRC. This is in line with a previous study showing the association of another LC3 isoform, LC3B with the tumour grade.[23] LC3 protein is expressed as three splice variants- LC3A, LC3B and LC3C with unique tissue distribution.[24] LC3A was not expressed in adjacent normal tissues, so as LC3B in which only 1 of 44 non-cancer tissues showed weak-positive expression (Table 4). This finding is contradictory to a study which demonstrated that LC3B was expressed both in cancer cells and normal epithelial cells.[23] Specifically, the same study showed that the LC3B-II expression was significantly higher in cancer tissue than in normal tissue. The LC3 is proteolytically cleaved by a protease to form LC3-I which is then conjugated with ATGs to a phosphatidylethanolamine (PE) moiety to generate LC3-II.[10] LC3-II is inserted into the membrane of autophagosomes and undergoes degradation upon autolysosome fusion.[10] Through Western blot assay, the LC3-I/LC3-II ratios are usually determined to corelate to the number of autophagosomes.[25] It would be interesting to study the subtype of LC3 protein and its association with the clinical pathological features in future investigations.

Another autophagy protein p62, on the other hand is a substrate for LC3 to facilitate degradation during autophagy.[10] In this study, expression of p62 was correlated in both tumour and non-tumour tissues. In contrast, it has been described elsewhere that the p62 level was higher in the tumour tissues than those in normal tissues.[26,27] On top of that, we

found that there were intense p62 staining in the nerve plexus cells both in the tumour and non-tumour tissues (data not shown). This has been previously reported in the colon tissues but in another autophagy marker, LC3.[23,28] In the survival analysis, autophagy proteins did not show any prognostic value in CRC patients which is contrasting numerous large-scale studies.[26,29] This might be due to the variation of sample size or the ethnicities of studied cohort which deserve future studies. In this study, we conclude that autophagy effectors LC3A, LC3B and p62 are not associated with the patient clinicopathologic features (except for LC3A and tumour grading) and cannot be used as prognostic markers. Further investigations are warranted to validate these findings with a larger scale of CRC cohorts.

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Authors' contribution: Sin-Yeang Teow and C Soon Lee conceptualized the work. Noel Jacques Awi and Hooi-Yeen Yap performed the experiments, data analysis and wrote the manuscript draft. Subasri Armon and Suat-Cheng Peh performed the data analysis. John Seng-Hooi Low, Kaik-Boo Peh and Sin-Yeang Teow reviewed and edited the manuscript.

Conflict of interest: The authors declare no conflicts of interest.

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