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Autophagy-related Proteins as a Prognostic Factor of Patients With Colorectal Cancer

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Objectives: Autophagy plays a dual role in tumorigenesis. In the initial stages, it promotes cell survival and suppresses carcinogenesis, whereas in cancer development, it induces cancer cell survival. In this study, we investigate the role of autophagy as a protective or tumor suppressor mechanism in colorectal cancer (CRC) cell lines and evaluate its role as a potential biomarker in human tumor samples.

Materials and Methods: The data of 68 patients with CRC treated at our Department from January 1 to December 31, 2016 were analyzed. Immunohistochemistry evaluation of p62, LC3B, Beclin-1, and Rab-7 in formalin-fixed paraffin-embedded tissue samples was performed and their expression was correlated with clinicopathologic characteristics, mutation status, and therapeutic approach. The χ^2 was used to test an association among categorical variables. Survival curves were estimated using the Kaplan-Meier method and differences were assessed using the log-rank test. Colo-205, HT29, SW-480, and Caco-2 cell lines were also used so as to test the autophagy markers with oxaliplatin, irinotecan, hydroxychloroquine, and 3-methyladenine.

Results: Overexpression of Beclin-1 is associated with poor survival ($P = 0.001$) in patients with CRC treated with chemotherapy, irrespective of the stage and mutational status. Rab-7 is also correlated with progression-free survival (PFS) ($P = 0.088$). Oxaliplatin (10 and 20 μM) and irinotecan (10 and 20 μM) inhibit autophagy in microsatellite stable (MSS) CRC cell lines. The inhibition of autophagy in MSS CRC cell lines after treatment with oxaliplatin and irinotecan is further identified through monodancylcadaverine staining. Moreover, inhibition of autophagy with molecules such as hydroxychloroquine (20 μM) and 3-methyladenine (5 mM) was identified by the accumulation of p62 and LC3B.

Conclusions: Beclin-1 is an independent prognostic factor of overall survival and PFS. Also, Rab-7 is identified as an independent prognostic factor of PFS. Besides, several chemotherapeutic drugs such as oxaliplatin and irinotecan inhibit autophagy in MSS CRC cell lines in a similar way like hydroxychloroquine and 3-methyladenine. Thus, in MSS patients who develop chemoresistance, a combination of other therapies that include an autophagy inhibitor could be more beneficial. Further clinical trials are needed to investigate these therapeutic strategies.

Key Words: autophagy, Beclin-1, chemotherapy, colorectal cancer

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Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide. It is estimated that, by 2030, CRC diagnosis will increase by $>50\%$.[1] As metastases are expected in 20% of patients with CRC, the development of new markers for more effective therapeutic options is pivotal.[2,3] CRC represents one of many malignancies in which autophagy, a necessary catabolic process, has been identified to play an essential role in tumorigenesis.

Autophagy-related genes (ATGs) play a crucial role in facilitating the regulation of autophagy.[4] Several proteins such as Beclin-1 (Atg6), MAP1LC3B (Atg8), p62/SQSTM1, and the Ras-related protein Rab-7 have been identified as vital elements of autophagy in cancer.[5] Beclin-1 is rarely mutated in the majority of tumors and it is associated with the initiation of autophagy through interaction with PI3k. LC3B-I protein through lipidation is converted into LC3-BII. LC3-BII is associated with the formation of autophagic vesicles and is used as an indicator of autophagy. Another essential protein for autophagy is p62/SQSTM1, which targets packaging and delivery proteins for autophagic digestion. This protein has been identified as a crossroad of apoptosis, autophagy, and cancer. Rab-7 is involved in endocytosis, a process in which some of its steps are similar to those of the maturation of autophagosome.[6]

A plethora of studies support the idea of the dual role of autophagy in CRC. Autophagy plays a crucial role in energy homeostasis of cells, which is required for several cellular functions, such as angiogenesis,[7] migration,[8] proliferation, and epithelial-mesenchymal transition phenotype.[9] Autophagy is enhanced in the hypoxic region of already established tumors where the energy demands are elevated.[10] Furthermore, cancer cells of high-graded tumors seem to be linked to autophagy to maintain their energy balance.[11] The impact of autophagy in cancer patients' response to chemotherapy is already known. Elevated levels of autophagy are associated with inadequate response to chemotherapeutic drugs and dismal survival rates.[12,13]

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In several cancer types, including CRC, a single-nucleotide polymorphism, in ATGs, such as ATG16L1, is connected with the reduction of autophagy and a significant negative predictive value for patients' survival with metastatic disease.[14] In contrast, several other studies identified the positive impact of monoallelic deletion or total loss of other ATGs.[15] UVRAG proteins are linked to BECN1 and function as autophagy regulators.[16] The mutation of UVRAG reduces autophagy, resulting in enlarged cancer cell proliferation in CRC cells.[17] Moreover, BIF-1 proteins that are associated with BECN1 have been observed to turn into abnormal or absent in a range of cancer types, such as CRC.[18]

Furthermore, *KRAS*, an essential oncogene in CRC development, is strongly associated with autophagy.[13] Under stressful conditions such as hypoxic tumor regions, cancer cells of KRAS-dependent tumors use autophagy to support growth and maintain an energy balance.[19] Inhibition of upregulated autophagy in KRAS-dependent tumors decreases cell proliferation and promotes tumor suppression.[20] The increasing amount of dysfunctional proteins and cellular organelles along with the inhibition of autophagy increase the risk of malignancy. Lastly, several studies with a knockout of different ATGs, Beclin-1 or AMBRA, have justified that low levels of autophagy are essential for cell survival.[21,22]

All these studies support the controversial role of autophagy in these mechanisms as either a tumor promoter or tumor suppressor. The controversial role of autophagy in cancer as a cytoprotective or tumor suppressor mechanism needs to be further investigated.[23,24] The aim of this study was to assess the impact of autophagy-related proteins on the survival rate of patients with CRC and the potential autophagy mechanism in CRC cell lines.

MATERIAL AND METHODS

Patients Characteristics

The data of 41 (aged 34 to 81) patients with CRC treated at our Department from January 1 to December 31, 2016 were studied. For these patients, there were available data regarding tumor histology grade, TNM classification, and mutation status of the genes *KRAS* (48.8%), *NRAS* (9.8%), and *BRAF* (7.4%), and on whether they were microsatellite instability (MSI) positive (7.4%). Molecular analyses were performed on patient samples before they received any treatment. In addition, there was available information regarding their treatment protocol (chemotherapy and/or radiotherapy). By the time of the data evaluation (December 2017), 4 patients (9.8%) had died because of their disease (Table 1).

TABLE 1. The Data of 68 Colorectal Cancer Enrolled Patients' Samples, n (%)

Sex	
Male	25 (60.9)
Female	16 (39.1)
Tumor size	
pT2	6 (14.6)
pT3	24 (58.5)
pT4	11 (26.9)
Lymph nodes	
pN0	16 (39.0)
pN1	15 (36.6)
pN2	10 (24.4)
Distant metastases	
pM0	26 (63.4)
pM1	15 (36.6)
KRAS	
Negative	21 (51.2)
Positive	20 (48.8)
NRAS	
Negative	37 (90.2)
Positive	4 (9.8)
BRAF	
Negative	38 (92.6)
Positive	3 (7.4)
MSI	
Stable	38 (92.6)
High	3 (7.4)

MSI indicates microsatellite instability.

DNA Extraction From Formalin-fixed Paraffin-embedded Tissues and Molecular Analysis

Sections of 10-μm thickness were cut from paraffin-embedded tissue blocks. DNA was extracted from the selected tissue areas following a standard DNA extraction kit protocol (NucleoSpin Tissue, Macherey-Nagel, Duren, Germany). The extracted DNA was quantitated on a Picodrop microliter spectrophotometer. Samples were screened in duplicates for mutations of *KRAS, NRAS*, and *BRAF*, using a real-time polymerase chain reaction approach followed by high-resolution melting analysis on a Light Cycler 480 (Roche Diagnostics, GmbH, Germany).[25] Polymerase chain reaction products positive by high-resolution melting analysis were purified and subjected to Sanger sequencing and/or pyrosequencing. MSI status was evaluated by molecular analysis of sensitive mononucleotide MSI markers (BAT25, BAT26, NR24, and NR21) and confirmed by analysis of MMR protein expression.[26]

Immunochemistry

Immunohistochemistry of p62, LC3B, Beclin-1, and Rab-7 was performed on 5-μm-thick formalin-fixed, paraffin-embedded tissue samples. The sections were microwave heated with 10 mM citrate buffer (pH 6.0) for antigen retrieval (p62, LC3, and Rab-7). For Beclin-1, antigen retrieval was carried out with a hot water bath at pH 9.0. Three percent H_2O_2 was applied to quench the endogenous peroxidase. Tissue sections were incubated at 4°C overnight with one of the following primary antibodies: SQSTM1/p62 (Cell Signaling #88588; 1:200 dilution), LC3B (Cell Signaling #3868; 1:200 dilution), Beclin-1 (Invitrogen #MA5-15825; 1:100 dilution), and Rab-7 (Invitrogen #PA5-72549; 1:100 dilution). Sections were subsequently incubated with SignalStain Boost Detection Reagent in a humidified chamber for 30 minutes at room temperature. The sections were developed with diaminobenzidine and counterstained with hematoxylin.

Immunohistochemistry Evaluation

H-score evaluated the immunoreactivity of p62, LC3B, Beclin-1, and Rab-7 according to the intensity and percentage of positively stained cells. Tissues without any staining were rated as 0, with faint staining as 1, with moderate staining as 2, and with intense staining as 3. The H-scores were determined by multiplying the intensity score by the percentage of positively stained cells. Tumors with an immunoreactive score of 0 to 100 were evaluated as negative, and those with 101 to 300 were classified as positive.[27–29]

Statistical Analysis

Statistical analysis was performed with SPSS22 software (SPSS Inc., Chicago, IL). Pearson's χ^2 test was used to evaluate the correlation of p62, LC3B, Beclin-1, and Rab-7 expressions with clinicopathologic parameters of patients with CRC. Univariate survival analysis was performed according to the

Kaplan-Meier method, and survival was compared using the log-rank test. Differences were considered very significant if a *P*-value was < 0.05 (2-tailed) and a statistical trend if a *P*-value was <0.1 (2-tailed).

Cell Lines

Colo-205 (CCL-222), HT29 (HTB-38), SW-480 (CCL-228) human colon adenocarcinoma, and Caco-2 (ATCCHTB-37) colon intermediate adenoma cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines used in this study were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, L-glutamine, vitamins, penicillin, and streptomycin antibiotics and amino acids (all from Invitrogen). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. All experiments were under the approval of the Ethics Committee of our University.

Western Blot

After the incubation time, radioimmunoprecipitation assay buffer is used for the preparation of whole-cell lysates. The protein concentration was determined using the Bradford method (Bio-Rad, 5000006). A total of 25 μg of protein was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Whatman; Scheicher & Schuell, Dassel, Germany). Membranes were incubated with the primary antibodies overnight at 4°C. After the incubation time, membranes were washed with Tris Buffered Saline with Tween 20 and then incubated with the appropriate secondary antibody, for 1 hour at 24°C.[30] Antibodies targeting SQSTM1/p62 (Cell Signaling #8025), LC3B (Cell Signaling #3868), Beclin-1 (Cell Signaling #3777), Rab-7 (Cell Signaling #9367; Cell Signaling, Danvers, MA), and Actin (sc-8035; Biotechnology Inc., Santa Cruz, CA) were used. The signal of the antibodies was identified with the enhanced chemiluminescence and specific detection system (Amersham Biosciences, Uppsala, Sweden) after exposure to Fuji Medical X-Ray Film. The number of protein levels was measured using specific software (ImageQuant software, Amersham Biosciences). The normalization of protein levels was against actin. We performed 3 independent experiments and the SD is presented. The amount of loading protein for western blot is 25 μg of the sample in a total volume of 20 μL. ImageJ is used for the quantification of protein bands.

2-dimensional Culture

For the 2-dimensional culture, cells (5000 cells/well) were grown on coverslips in 24-well plates in medium, at 37°C. The cells were treated with 10 and 20 μM of oxaliplatin or irinotecan for 24 hours. For the confocal analysis, the cells were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline, and immediately analyzed in confocal to detect the autophagic vacuoles. Monodancylcadaverine (MDC) is an autofluorescent marker that preferentially accumulates in autophagic vacuoles. MDC accumulation in autophagic vacuoles is because of a combination of ion trapping and specific interactions with vacuole membrane lipids. Cell cytoskeleton was stained with phalloidin (Alexa Fluor 546, A22283, Life Technologies).

RESULTS

Expression of Autophagy Markers in CRC Tissues

The expression of 4 autophagy markers p62, LC3, Beclin-1, and Rab-7 was successfully performed in CRC human tissues. Then, a pathologist with no knowledge of the clinical data scored all immunohistochemical staining, according to the staining intensity and the percentage of positively stained tumor cells (Fig. 1). Furthermore, the normal mucosa was stained with the same autophagic markers. It appears that normal mucosa has null or very low expression of the 4 autophagy markers lymphocytes appear to have positive staining (Supplementary 1, Supplemental Digital Content, http://links.lww.com/AJCO/A286).

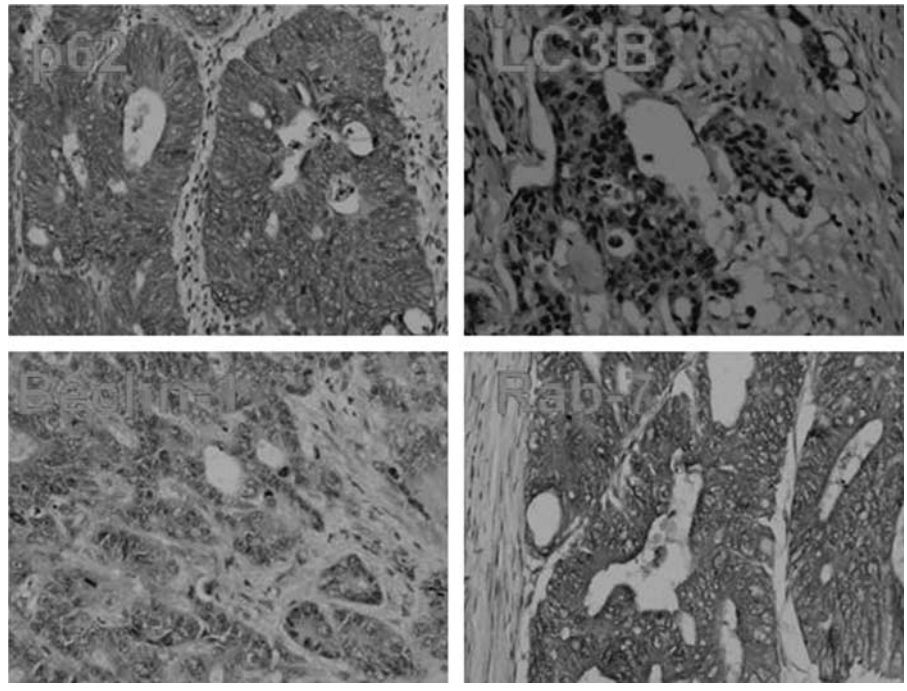

FIGURE 1. Immunohistochemical staining of p62, LC3B, Beclin-1, and Rab-7 in human colorectal carcinomas (×40).

TABLE 2. Comparison of Clinical, Molecular, and Treatment Factors With Autophagy Marker

	p62		
Factors	**Low H-score**	**High H-score**	***P***
Sex			
Male	21	4	0.352
Female	15	1	
Age (y)			
< 65	15	3	0.439
65 >	21	2	
Tumor			
T2	4	2	0.133
T3	21	3	
T4	11	0	
Node			
N0	15	1	0.573
N1	13	2	
N2	8	2	
Metastasis			
M0	23	3	0.866
M1	13	2	
Stage			
2	8	1	0.985
3	15	2	
4	13	2	
KRAS			
No	19	2	0.592
Yes	17	3	
NRAS			
No	33	4	0.410
Yes	3	1	
BRAF			
No	33	5	0.503
Yes	3	0	
MSI			
No	33	5	0.503
Yes	3	0	
Chemotherapy			
No	4	1	0.569
Yes	32	4	
Radiotherapy			
No	30	5	0.323
Yes	6	0	
	LC3		
Sex			
Male	20	5	0.706
Female	12	4	
Age (y)			
< 65	12	6	0.119
65 >	20	3	
Tumor			
T2	3	3	0.147
T3	19	5	
T4	10	1	
Node			
N0	12	4	0.931
N1	12	3	
N2	8	2	
Metastasis			
M0	20	6	0.819
M1	12	3	
Stage			
2	6	3	0.638
3	14	3	
4	12	3	
KRAS			
No	17	4	0.645
Yes	15	5	
NRAS			
No	28	9	0.264
Yes	4	0	
BRAF			
No	29	9	0.340
Yes	3	0	
MSI			
No	29	9	340
Yes	3	0	
Chemotherapy			
No	3	2	0.298
Yes	29	7	
Radiotherapy			
No	27	8	0.735
Yes	5	1	
	Beclin-1		
Sex			
Male	20	5	0.534
Female	14	2	
Age (y)			
< 65	15	3	0.629
65 >	19	4	
Tumor			
T2	4	2	0.476
T3	21	3	
T4	9	2	
Node			
N0	12	4	0.239
N1	10	3	
N2	12	0	
Metastasis			
M0	21	5	0.629
M1	13	2	
Stage			
2	6	3	0.338
3	15	2	
4	13	2	
KRAS			
No	17	4	0.731
Yes	17	3	
NRAS			
No	31	6	0.657
Yes	3	1	
BRAF			
No	31	7	0.414
Yes	3	0	
MSI			
No	31	7	0.414
Yes	3	0	
Chemotherapy			
No	2	3	0.006
Yes	32	4	
Radiotherapy			
No	28	7	0.229
Yes	6	0	
	Rab-7		
Sex			
Male	19	6	0.942
Female	12	4	
Age (y)			
< 65	15	3	0.308
65 >	16	7	

TABLE 2. (*continued*)

	p62		
Factors	**Low H-score**	**High H-score**	***P***
Tumor			
T2	5	1	0.697
T3	17	7	
T4	9	2	
Node			
N0	11	5	0.715
N1	12	3	
N2	8	2	
Metastasis			
M0	19	7	0.619
M1	12	3	
Stage			
2	6	3	0.758
3	13	4	
4	12	3	
KRAS			
No	14	7	0.172
Yes	17	3	
NRAS			
No	27	10	0.232
Yes	4	0	
BRAF			
No	30	8	0.077
Yes	1	2	
MSI			
No	28	10	0.307
Yes	3	0	
Chemotherapy			
No	4	1	0.807
Yes	27	9	
Radiotherapy			
No	26	9	0.633
Yes	5	1	

MSI indicates microsatellite instability.

Association of Autophagy Markers Expression With Clinicopathologic Characteristics, Molecular Status, and Therapeutic Approach

None of the autophagy markers were found to correlate with their expression with pathologic factors after analysis through the χ^2 test. Also, their expression levels were not correlated with mutations in *KRAS*, *NRAS*, and *BRAF* genes, and with MSI-positive tumors (Table 2). However, patients with CRC treated with chemotherapy and Beclin-1 expression showed a statistically significant correlation ($P = 0.006$).

Effects of Autophagy Markers Expression on Overall Survival (OS) and Progression-free Survival (PFS)

Patients with low levels of Beclin-1 expression showed a statistically greater therapeutic benefit in terms of OS (log-rank test, $P = 0.001$) and PFS (log-rank test, $P = 0.069$) than patients with high levels of Beclin-1 expression. Patients with low Rab-7 expression seemed to have better PFS compared with those with high expression (log-rank test, $P = 0.088$) (Figs. 2A, B).

Oxaliplatin and Irinotecan Inhibit Autophagy in Microsatellite stable (MSS) CRC Cell Lines

Four different MSS colon adenocarcinoma and intermediate adenoma cell lines (Caco-2, Colo-205, HT29, and SW-480) were examined, regarding autophagic properties, after treatment with 10 and 20 μM of oxaliplatin and irinotecan for 24 hours. The protein levels of Beclin-1, LC3B, p62, and Rab-7 were measured with western blot analysis. In both CRC cell lines, Caco-2 and Colo-205 cell lines, autophagy was inhibited after treatment with oxaliplatin and irinotecan, as confirmed with the increased protein levels of p62 despite the enhancement of Beclin-1 (Fig. 3A). In another CRC cell line, HT29, a dose-dependent pattern of the reduction of Beclin-1 occurred after treatment with irinotecan and 20 μM of oxaliplatin. Furthermore, autophagy inhibition was confirmed by the increasing levels of p62, despite the increasing levels of LC3 at the same treatment points (Fig. 3A). In SW-480 CRC cell lines, treatment with irinotecan and oxaliplatin decreased Beclin-1 and p62 protein levels in all treatment points. Moreover, in the same cell line, both drugs increased the total amount of LC3 (Fig. 3A). Also the marker of endocytosis Rab-7 was further tested.

In Caco-2 and Colo-205, the presence of both drugs (irinotecan and oxaliplatin) led to a decrease in the protein levels of Rab-7. In HT29, is revealed a dose-dependent pattern of the reduction of Rab (Fig. 3A and Table 3).

As an additional confirmation of autophagy inhibition, MDC staining revealed the presence of autophagic vacuoles in a high percentage of phalloidin-stained cells. In all CRC cell lines, treatment with the chemotherapeutic drugs (irinotecan and oxaliplatin) significantly decreased the presence of autophagic vacuoles, identified through the detection of MDC staining. The quantification of MDC in each cell line is also presented (Fig. 3B).

The Levels of Autophagic Markers After Effective Inhibition of Autophagy

Two inhibitors of autophagy (20 μM hydroxychloroquine [HCQ] and 5 mM of 3-methyladenine [3-MA]) were used for 24 hours in CRC cell lines to identify the protein levels of these autophagic markers after inhibition of autophagy in different stages—3-MA inhibits autophagy by blocking autophagosome formation through inhibition of type III phosphatidylinositol 3-kinases and HCQ prevents lysosomal acidification. Thus, 3-MA and HCQ inhibit the initiation and the autophagy flux in different stages, respectively. In the Caco-2 cell line, HCQ and 3-MA increased Beclin-1.

Moreover, the inhibition of autophagy after treatment with these 2 inhibitors was identified by the increased expression of p62 and the increased total amount of LC3 (Fig. 4). In Colo-205, HCQ and 3-MA inhibit autophagy as it was identified through the reduction of protein levels of Beclin-1 (and enhancing protein levels of p62). In addition, in the same cell line, HCQ increased and 3-MA decreased the total protein of LC3 (Fig. 4). In HT29, HCQ increased and 3-MA reduced the protein levels of Beclin-1. Besides, LC3 and p62 protein levels were increased after treatment with both inhibitors. In SW-480 CRC cell line, Beclin-1 was decreased after treatment with both autophagy inhibitors. The protein levels of p62 and the total amount of LC3 were increased.

The increasing ratio of LC3II/I and p62 in all cell lines after treatment with both inhibitors confirmed the inhibition of autophagy (Fig. 4).

The autophagy-dependent endocytotic process was tested through the protein levels of Rab-7. In Caco-2 and Colo-205 cell lines, Rab-7 is increased. In HT29, treatment with HCQ and 3-MA decreased the protein levels of Rab-7. In SW-480 CRC cell line, HCQ and 3-MA increase and decrease the protein levels of Rab-7, respectively (Fig. 4).

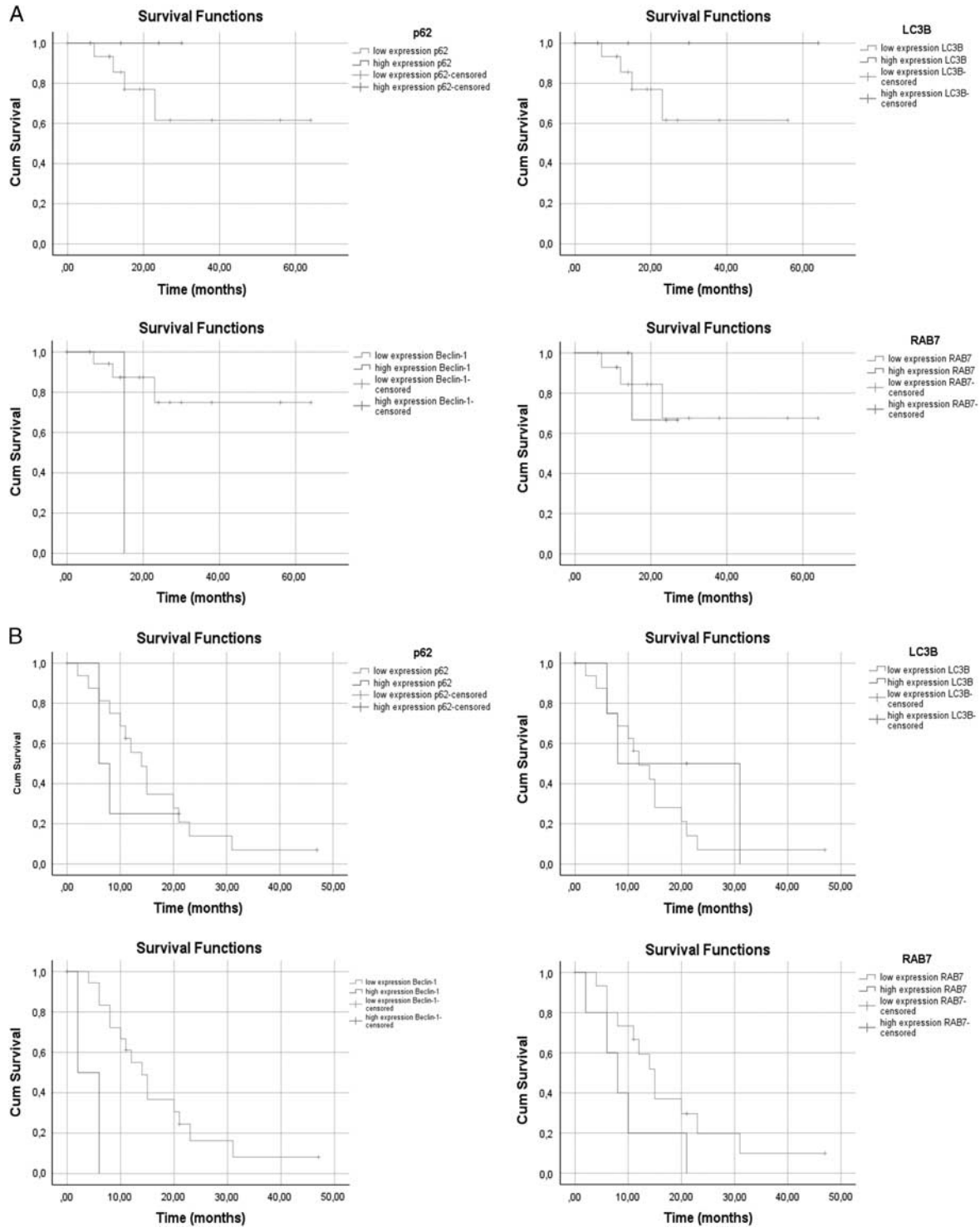

FIGURE 2. A, Kaplan-Meier estimates of overall survival of patients with CRC with low and high expression of autophagy markers p62, LC3B, Beclin-1, and Rab-7. High expression of Beclin-1 reduces life span. B, Kaplan-Meier estimates of PFS of patients with CRC with low and high expression of autophagy markers p62, LC3B, Beclin-1, and Rab-7. In low expression of Beclin-1 and Rab-7, we observed better PFS. CRC indicates colorectal cancer; PFS, progression-free survival.

DISCUSSION

Autophagy is a mechanism involved in both the survival and growth of cancer cells.[11,31] In our experiments, we have shown that in the majority of MSS CRC cell lines, oxaliplatin and irinotecan inhibit autophagy in the later phases of autophagosome formation. Our results are consistent with other studies that report worse OS and

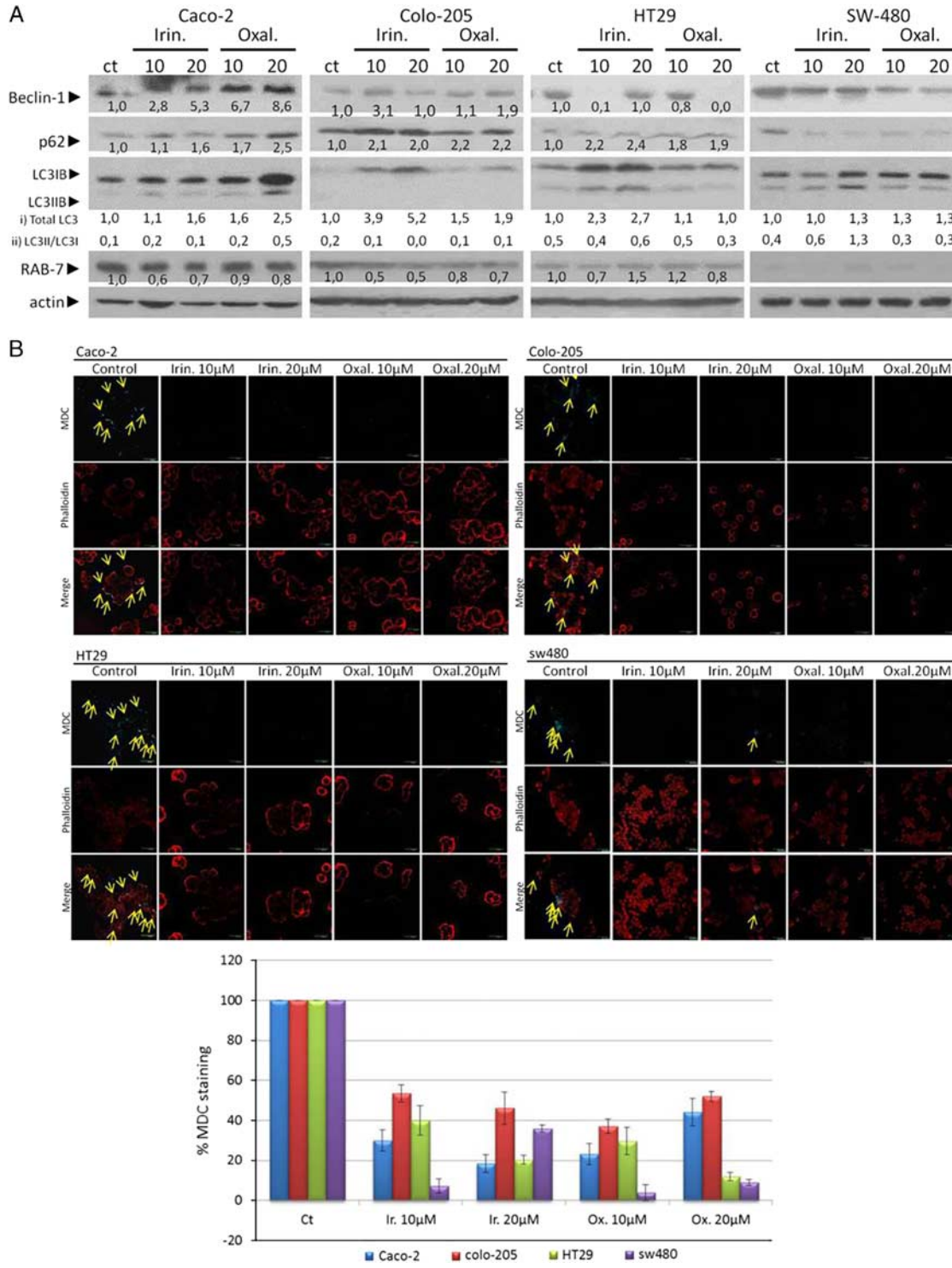

FIGURE 3. Oxaliplatin and irinotecan inhibit autophagy in microsatellite stable colorectal cancer cell lines. Western blot analysis after 24-hour exposure of cells in 10 and 20 μM of oxaliplatin or irinotecan. The protein levels of Beclin-1, p62, LC3, and Rab-7 were identified by specific antibody. The quantification of LC3 reflects the whole protein levels as compared with the untreated sample in each cell line (i) and the ratio of LC3II/LC3I in each sample separately (ii). Protein levels were normalized against actin (A). The formation of autophagic vacuoles in Caco-2, Colo-205, HT29, and SW-480 cells because of treatments was determined with 0.1 mM of MDC (light blue) through confocal microscopy, whereas phalloidin staining (red) was used for cytoskeleton detection. The yellow arrows show the autophagic vacuoles. The graph represents the quantification of MDC in each cell line (B). MDC indicates monodansylcadaverine.

TABLE 3. The Effect of Irinotecan and Oxaliplatin on Beclin-1 and Autophagy Activation as it was Identified Through the Protein Levels of p62 and LC3B after 24 Hours

	Irinotecan		Oxaliplatin	
	10 μM	20 μM	10 μM	20 μM
Caco-2				
Beclin-1	INCR	INCR	INCR	INCR
Autophagy	IN	IN	IN	IN
Colo-205				
Beclin-1	INCR	NC	INCR	INCR
Autophagy	IN	IN	IN	IN
HT29				
Beclin-1	DE	NC	DE	DE
Autophagy	IN	IN	IN	IN
SW-480				
Beclin-1	DE	DE	DE	DE
Autophagy	Act.	Act.	Act.	Act.

Act indicates activation; DE, decreased; INCR, increased; IN, inhibition.

PFS after chemotherapy in patients with CRC with a high expression of Beclin-1 compared with patients with low Beclin-1 expression.[32,33]

Autophagy is characterized by the formation of the autophagosome, a double-membrane structure that is strongly associated with the LC3B protein. In several solid tumors, including CRC, LC3B staining is usually associated with high levels of autophagy.[34] It seems from our experiments that chemotherapeutic drugs, such as oxaliplatin and irinotecan, inhibit autophagy at later stages of autophagosome formation. It has been observed that the process of autophagy is inhibited at later stages, as shown by the accumulation of both p62 and LC3B, although autophagy is triggered in the initial stages as observed by the increased protein levels of Beclin-1. The accumulation of LC3B and p62 in CRC cell lines after treatment with one of these drugs confirms the inhibition of autophagy in our model. p62 plays a crucial role in autophagosome formation and delivery of ubiquitinated cargoes to the autophagosome for autophagic degradation.[35,36] The protein itself is degraded and is used as an autophagy marker, as during inhibition of autophagy, p62 accumulates, and in contrast, decreased levels of p62 are observed when autophagy is induced.[37,38] The inhibition of autophagy in MSS CRC cell lines after treatment with oxaliplatin and irinotecan is further identified through MDC staining a molecule that preferentially accumulates in autophagic vacuoles because of a combination of ion trapping and specific interactions with membrane lipids.[39]

Another critical protein in the maturation of the autophagophore is Rab-7.[40] Rab-7 is responsible for the delivery of cargoes and participates in the fusion step of the autophagophore with endocytic vesicle and lysosomes. Thus, Rab-7 is a multifunctional regulator of both autophagy and endocytosis.[41,42] According to this study, oxaliplatin and irinotecan seem to affect the protein levels of Rab-7 after the inhibition of autophagy in later stages in MSS CRC cell lines. The value of Rab-7 as a marker is rising as patients with CRC who show high expression of Rab-7 have better PFS compared with those with low expression.

To study the levels of primary autophagy markers during inhibition of autophagy, we incubated MSS cell lines with 2 inhibitors of autophagy. 3-MA inhibits the initiation of autophagy and HCQ blocks autophagy at a subsequent stage.[43,44] Several clinical trials on patients with metastatic colorectal cancer have shown that chloroquine and HCQ are useful only when they are combined with chemotherapeutic agents, inhibitors of histone deacetylases and antiangiogenic agents.[45,46] Inhibition of autophagy with molecules such as HCQ and 3-MA is identified by the accumulation of p62 and LC3B in CRC cell lines.[47,48] Furthermore, 3-MA inhibits the first steps of autophagy and seems to reduce the protein levels of Beclin-1 in 3 out of 4 used MSS CRC cell lines. Oxaliplatin and irinotecan can inhibit autophagy in MSS CRC cell lines in a similar manner as HCQ and 3-MA. Chemotherapy (oxaliplatin and irinotecan) inhibits the cytoprotective mechanism of autophagy in a later stage, as we demonstrated by measuring the protein expression levels of autophagy markers such as

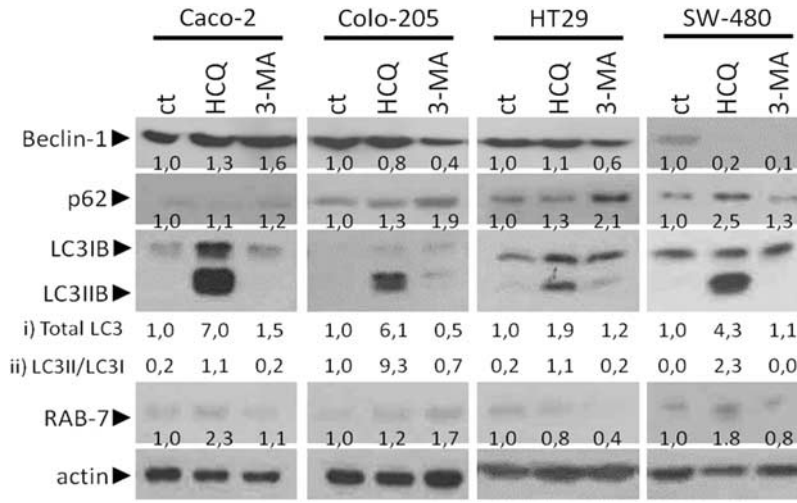

FIGURE 4. The protein levels of autophagy markers after treatment with 5 mM of 3-MA and 20 μM of HCQ in colorectal cancer cell lines. Western blot analysis after 24-hour exposure of cells in 5 mM of 3-MA and 20 μM of HCQ. The protein levels of Beclin-1, p62, LC3, and Rab-7 are presented. The quantification of LC3 reflects the whole protein levels as compared with the untreated sample in each cell line (i) and the ratio of LC3II/LC3I in each sample separately (ii). Protein levels were normalized against actin. HCQ indicates hydroxychloroquine; 3-MA, 3-methyladenine.

Beclin-1, p62, LC3B, and Rab-7 in samples of patients with CRC.

CONCLUSIONS

The present study supports the hypothesis that in patients with CRC who are treated with chemotherapy, induction of Beclin-1 expression and worse OS and PFS are correlated. Also, patients with CRC who show high expression of Rab-7 have better PFS compared with those with low expression. Also, several chemotherapeutic drugs such as oxaliplatin and irinotecan inhibit autophagy in MSS CRC cell lines in a similar way like HCQ and 3-MA. Thus, we can conclude that patients who have CRC, irrespective of their stage and tumor mutational status, should be tested for both microsatellite stability and autophagy markers Beclin-1 and Rab-7 as independent prognostic factors. For MSS patients who have undergone chemotherapy should combine treatment with inhibitors of autophagy, or treatments such as immunotherapy. Further clinical trials are needed to investigate these therapeutic strategies.

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