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The influence of irradiation on autophagy process in normal and malignant colorectal epithelia

Constantin Daniel Uscatu[1,2], Francisc Mixich[1], Theodor Viorel Dumitrescu[3], Elena-Raluca Nicoli[1], Ionela Cristina Deliu[4], George Mihail Man[5], Iancu Emil Pleşea[2,6]

[1]Department of Cellular and Molecular Biology, University of Medicine and Pharmacy of Craiova, Romania

[2]Department of Pathology, Emergency County Hospital, Craiova, Romania

[3]Department of Surgery, Emergency County Hospital, Craiova, Romania

[4]Department of Gastroenterology, University of Medicine and Pharmacy of Craiova, Romania

[5]Department of Pathology, Emergency County Hospital, Pitesti, Romania

[6]Department of Pathology, University of Medicine and Pharmacy of Craiova, Romania

Abstract

Aim: The authors assessed the influence of preoperative radiotherapy on autophagy process using a quantitative assessment of LC3 expression on both normal and tumoral colorectal tissues. *Materials and Methods*: Normal and malignant tissue samples were taken from 50 patients that underwent surgery for colorectal adenocarcinoma of which 11 received preoperative radiotherapy. Tissue samples were included in paraffin and sections were immunomarked for LC3 expression. LC3 percentage was assessed with dedicated software on 10 randomly selected fields with 40× objective from both normal and malignant tissue samples of each patient. The resulting data were assessed and compared with a statistical apparatus. *Results*: LC3 was overexpressed in tumoral tissue as compared with normal one. The LC3 percentage is different from person to person and the higher it is in normal epithelium, the higher will be in tumoral epithelium of the same person, regardless the irradiation. The LC3 expression levels are decreasing from tumoral non-irradiated epithelia to normal irradiated epithelia. LC3 expression in tumoral cells is granular, with particular perinuclear disposal and often "annular" pattern. *Conclusions*: The autophagy process has a basal level in the normal tissue, with interindividual variability. The autophagy process proved to be upregulated in the tumoral cells, with a particular morphological expression, namely the presence of cytoplasmic coarse granules disposed in an "annular" pattern. Preoperative radiotherapy is downregulating the autophagy process both in normal and tumoral tissue but to a lesser extent in the latter.

Keywords: colorectal cancer, autophagy, LC3, preoperative irradiation.

Introduction

Autophagy is an important cellular function, which provides recycling of aged and degraded cytoplasmic structural components [1–4]. Three forms of autophagy are described: macroautophagy, microautophagy and chaperone-mediated autophagy. The most studied one is macroautophagy. In this form of autophagy, the cytoplasmic constituents that need to be digested are wrapped in double-membrane vesicles named autophagosomes [5, 6]. Autophagosomes fuse with lysosomes in order to ensure the degradation of engulfed cytoplasmic particles *via* lysosomal enzymes [7].

A key component of autophagy machinery, microtubule-associated protein 1 light chain 3 (MAP1LC3) is needed for the cascade of autophagy events to work properly and autophagosomes to fuse with lysosomes. This protein is synthesized as a precursor form of LC3 that undergoes a cleavage at its C-terminal end. From this process results a cytoplasmic form, LC3-I, that is expressed in a diffuse pattern. This is further conjugated with phosphatidylethanolamine, resulting LC3-II isoform, which binds to the inner and outer membrane of autophagosomes, constituting a reliable marker for autophagy detection [5, 8, 9].

An important number of articles, few of them, however, on human tissues and fewer on normal and malignant colorectal tissues (the above-cited papers) described different protocols for the evaluation of autophagy process on paraffin-embedded tissue samples, but, for all we know, none of them offered a quantitative assessment of LC3 but a qualitative one. Therefore, the aim of our study was to present a method for an appropriate quantitative assessment of LC3 expression on both normal and tumoral paraffin-embedded tissues, and to assess the influence of preoperative radiotherapy on autophagy process.

Materials and Methods

Tissue samples of colorectal adenocarcinoma and normal mucosa were taken from 50 patients that underwent surgery. All patients were informed about their participation in this study and a written consent was provided by every patient. The initial group was divided in two subgroups depending on the presence or not of the preoperative radiotherapy as follows: Subgroup A – 39 patients without radiotherapy and Subgroup B – 11 patients with radiotherapy before the surgery.

Two tissue samples were taken from every patient:

one from the tumor tissue and another from normal colorectal mucosa away from the tumor region.

The samples were processed using the classical histological technique (fixation in 10% buffered formalin and embedment in paraffin). Two serial sections, 3 μm thick were cut from each paraffin block.

First section was stained with Hematoxylin–Eosin (HE) for diagnosis orientation (Figure 1).
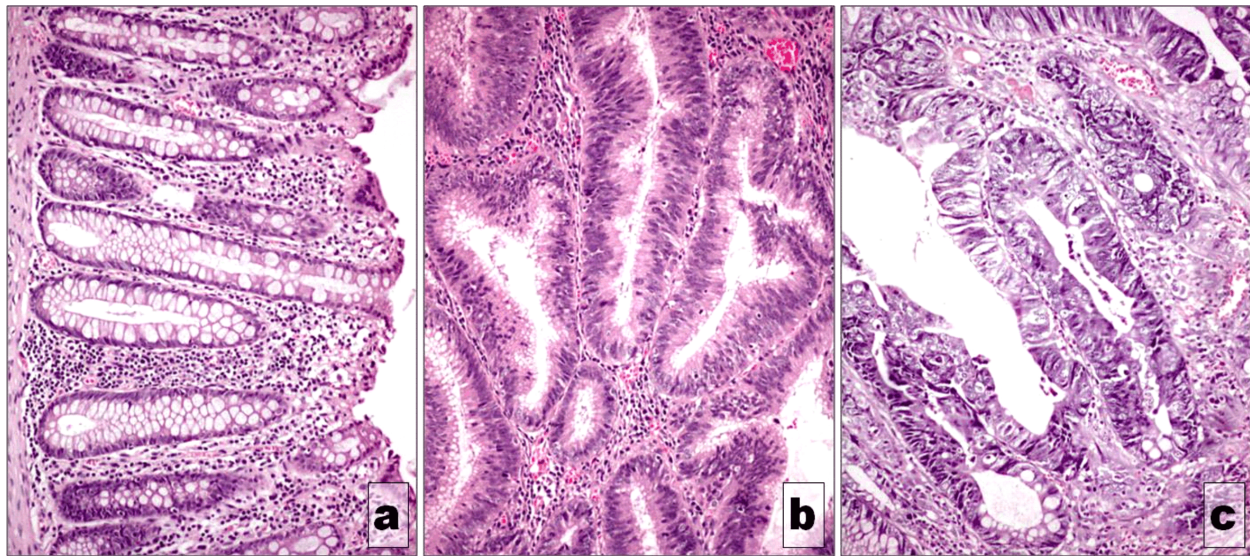

Figure 1 – *(a) Normal colonic mucosa; (b) Non-irradiated adenocarcinoma; (c) Irradiated adenocarcinoma. HE staining, ×100.*

Second section was stained for LC3 expression using Abcam, rabbit anti-human polyclonal anti-LC3A/B antibody (ab58610, 1:50 dilution) capable of detecting both LC3-I and LC3-II isoforms. The immunohistochemistry technique used included the following steps: dewaxing with xylene; rehydration in graded ethanol solutions; blocking of endogenous peroxidase with 6% H_2O_2; microwave antigen retrieval at 650 W in citrate buffer with pH 6.0 for 20 minutes; washing in phosphate-buffered saline (PBS) with pH 7.0; blocking of non-specific binding sites with 3% Nonfat-Dried Milk Bovine (Sigma-Aldrich) for 30 minutes, at 250^0C; incubation with primary antibody (LC3) for 16 hours at 250^0C; washing in PBS; incubation with EnVision (Dako) at 250^0C for 30 minutes; antibody detection using 3,3'-diaminobenzidine (DAB) for nine minutes, at 250^0C; counterstaining with Hematoxylin.

Tissue slides were analyzed using an Olympus CX 31 microscope equipped with a ColorView II camera and AnalySis Pro 5.0 software calibrated for this microscope. Ten randomly selected fields with 40× objective and noted F1, F2 .... F10 were taken of each immunomarked slide of both normal and tumoral tissue in order to ensure a correct assessment of the LC3 percentage on the slide section. The intratumoral selected fields belonged to both central and peripheral areas of the tumor.

LC3 protein expression level was quantitatively assessed in two steps using the image analysis software. In the first step, regions of interest (ROIs) including only normal or tumoral epithelial tissue were defined, excluding all the other types of tissues and the glandular lumens (Figure 2 – upper central frame). Further, ROIs areas were determined and noted with EA1, EA2 ... EA10.

In the second step, the LC3 area, noted with LC3-A1, LC3-A2 ... LC3-A10 was determined only from the epithelial component, either normal or tumoral using the next threshold (HIS): Hue: 0–180 / Intensity: 0–255 / Saturation: 0–255, which excluded areas stained with Hematoxylin (Figure 2 – lower central frame).
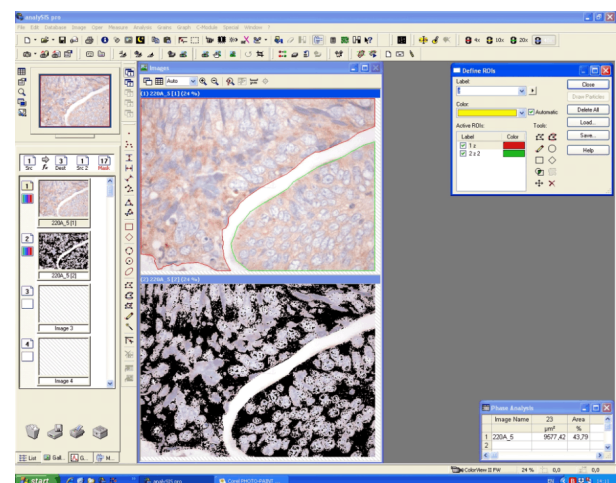

Figure 2 – *Quantitative assessment of LC3 levels – AnalySis Pro 5.0 Measurement tool window. Upper image: determination of ROIs. Lower image: selection of LC3 covered area with HIS preset values.*

The mean LC3 expression level for each case was computed for both normal and tumoral areas following the next formula:

$$\frac{EA1 + EA2 + ... + EA10}{LC3\text{-}A1 + LC3\text{-}A2 + ... + LC3\text{-}A10} \times 100$$

The lowest value (MIN), the highest value (MAX), average value (AV), and standard deviation (STDEV) were determined. For graphical representation, MAX, AV + STDEV, AV, AV - STDEV and MIN were used.

All measurements were exported and processed in Excel module of Microsoft Office Professional 2003. For data statistical analysis, Student's *t*-test, Pearson's correlation test and ANOVA test from XLSTAT version 3.02 were used (with significance level α=0.05 for both

tests). The graphs showing the evolution patterns of LC3 expression in normal and tumoral tissue were done with the "Graph" tool included in the "Excel" module of the Microsoft Office Professional 2003 software package and XLSTAT software version 3.02.

⊟ Results

The group of patients was composed of 31 males and 19 females, age ranging from 45 to 85 years old.

In the first step, we compared LC3 levels in normal and tumoral tissue in each of the two subgroups. A first general remark was that the mean values of LC3 percentage from both tumoral and epithelial cells area were different from one patient to another.

In the group without preoperative irradiation, the LC3 protein was clearly overexpressed in tumoral epithelial cells as compared with normal epithelial cells.

Thus, the AV value of LC3 expression amount was higher in tumoral tissue than in normal tissue. The interval comprising the majority of values was almost similar in tumoral and normal tissue due to almost equal STDEV values. This difference was statistically validated by Student's t-test, whose p-value was <0.01, meaning a very highly significant correlation (Figure 3A – left and Figure 4).
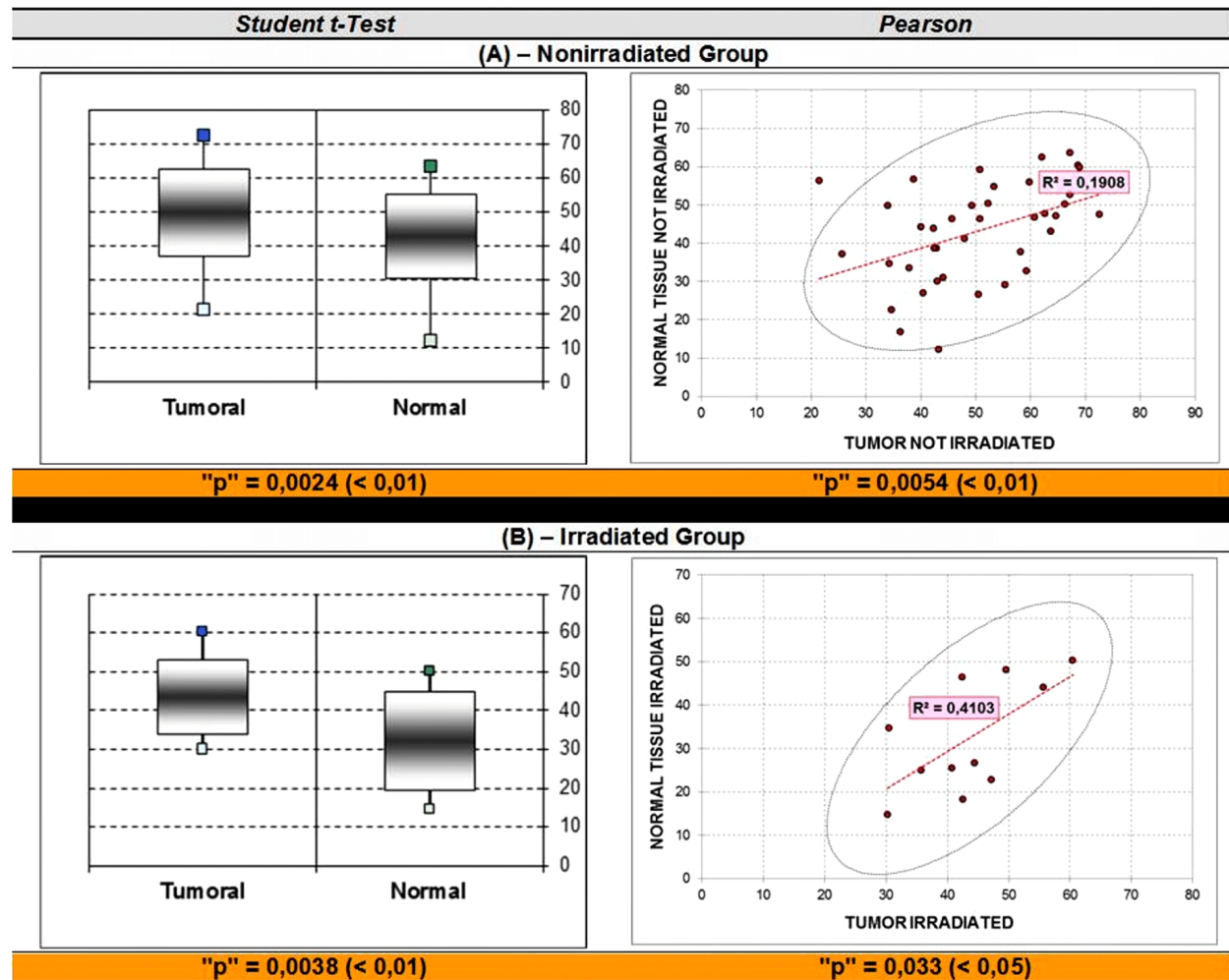

Figure 3 – *Statistical parameters of the studied groups.*

Moreover, Pearson's correlation test showed that the levels of LC3 amount in normal and tumoral tissue are in a direct correlation, meaning that the higher is the level in the normal epithelium the higher is in tumoral epithelium too, correlation validated by the p-value <0.01, meaning a very highly significant correlation (Figure 3A – right).

The situation was almost the same in the group with preoperative irradiation, meaning that the AV value of LC3 expression amount was also higher in tumoral tissue than in normal tissue. The interval comprising the majority of values was narrower in tumoral tissue due to a smaller STDEV value.

The difference was also statistically validated by Student's t-test, whose p-value was <0.01, meaning a significant correlation (Figure 3B – left and Figure 5).

Pearson's correlation test showed that the levels of LC3 amount in normal and tumoral tissue are also in a direct correlation, like in the other subgroup, correlation validated by the p-value <0.05, meaning a significant correlation (Figure 3B – right).

When we compared together the mean values of LC3 expression in both subgroups and in both normal and tumoral epithelial tissue, we observed two interesting situations. The first remark was that LC3 mean values of the tumoral epithelia (both non-irradiated and irradiated) were higher than LC3 mean values in the normal epithelia (either non-irradiated or irradiated). The second remark was the general descending trend of the LC3 mean value from the tumoral non-irradiated epithelia to the normal irradiated epithelia (Figure 6).
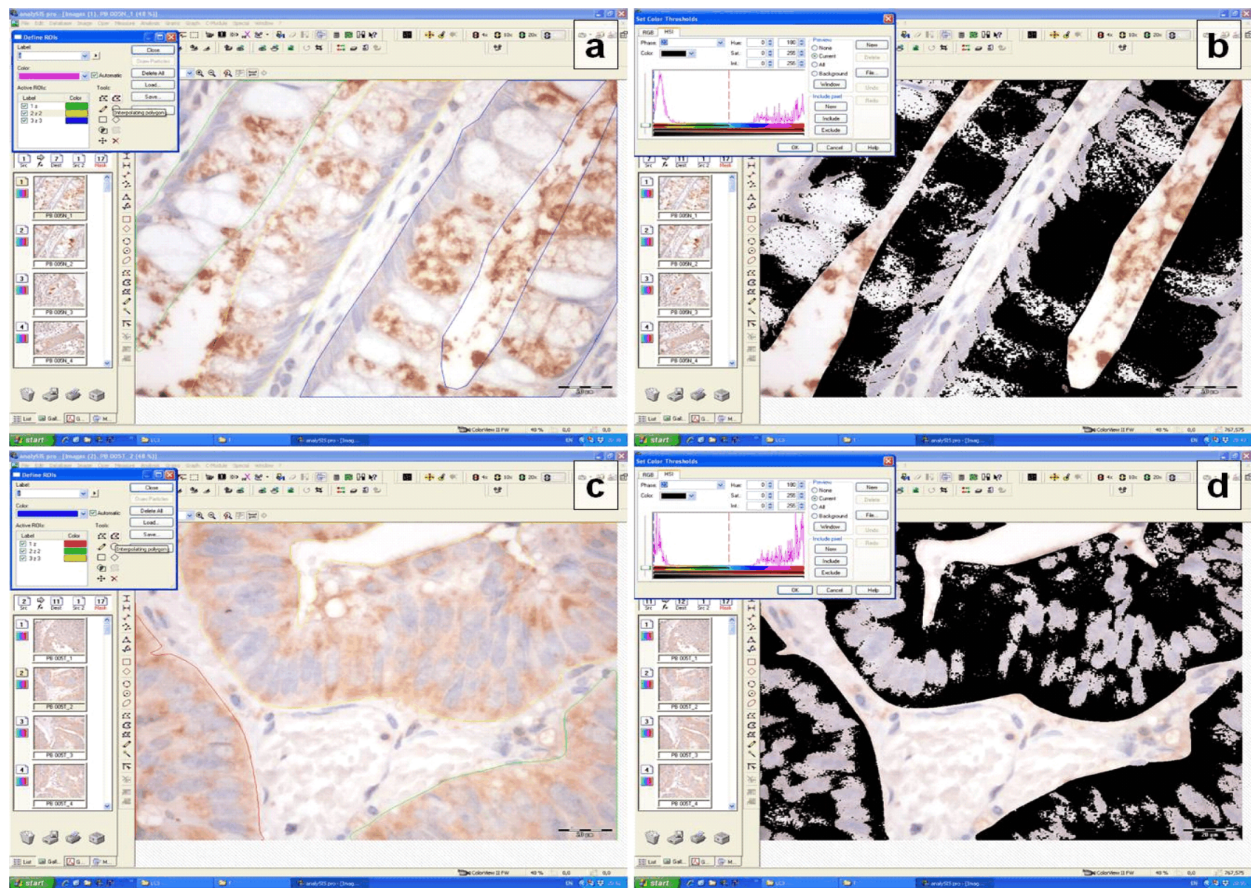
Figure 4 – *Non-irradiated subgroup: (a and c) Determination of ROIs; (b and d) Selection of LC3 covered area with HIS preset values.*
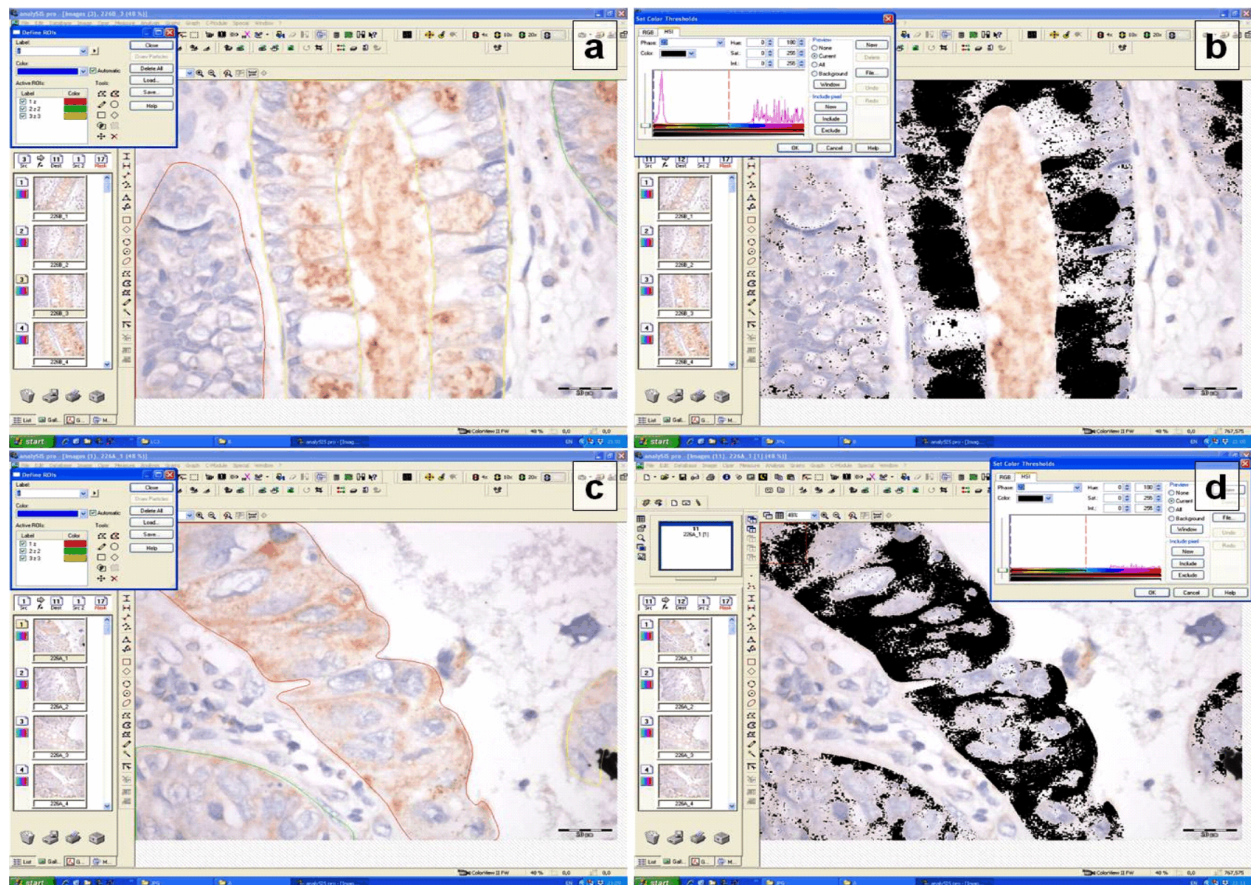

Figure 5 – *Irradiated subgroup: (a and c) Determination of ROIs; (b and d) Selection of LC3 covered area with HIS preset values.*
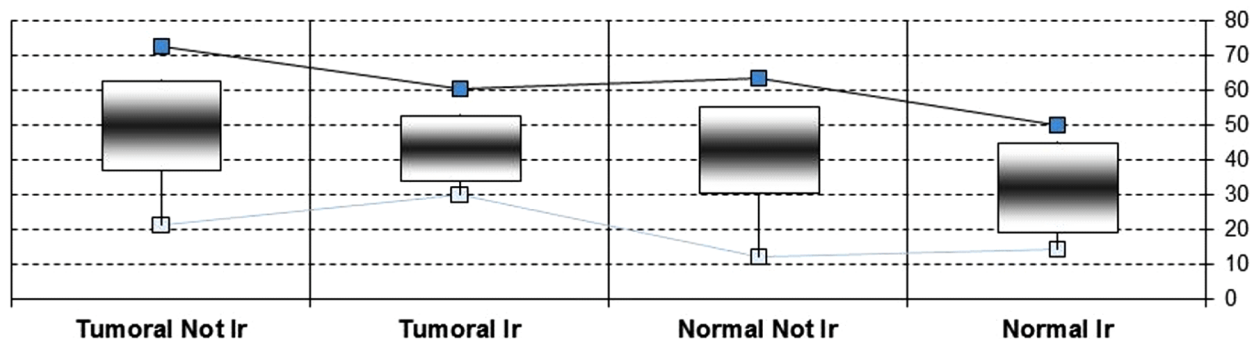
Figure 6 – *Synopsis of statistical parameters of all subgroups.*

The ANOVA test validated this decreasing general trend and the existence of differences between the LC3 mean values of the two subgroups and the two epithelial populations. However, the Student's *t*-test did not validate statistically the difference between the groups of tumoral epithelia (non-irradiated and irradiated) and the small difference between LC3 mean values of irradiated tumoral cells and non-irradiated normal cells but validated the difference between the groups of normal epithelia (non-irradiated and irradiated) (Table 1).

Table 1 – *Staining procedures used in the study*

Statistics	*P*-value			
Group	*Tumoral Non-Irradiated (T-NI)*	*Tumoral Irradiated (T-I)*	*Normal Non-Irradiated (N-NI)*	*Normal Irradiated (N-I)*
ANOVA single factor	0.0005 (<0.001) [Fcrit.(2.69)<F(6.33)]			
Student's *t*-test	*Group*	*T-NI*	*T-I*	*N-NI*
	T-I	0.0731 (>0.05)		
	N-NI	0.002 (<0.01)	0.90 (>0.05)	
	N-I	0.0008 (<0.001)	0.003 (<0.01)	0.02 (<0.05)

Some authors considered that the intracellular condensations of immunomarked material they called "autophagy puncta" would correspond to the LC3-II isoform bound to the autophagosomes [3, 5, 9]. Therefore, in the next step of our study we tempted a qualitative assessment of the LC3 distribution in the cytoplasm of both normal and malignant epithelial cells.

In the normal epithelium LC3 had no or diffuse expression in the basal cellular pole that could correspond to LC3 I isoform, and a coarse granular feature at the apical pole, the so-called "autophagy puncta" (Figure 7, a and b).

In the tumoral epithelium, in turn, the cytoplasm of almost all cells presents both a diffuse expression of LC3 and "autophagy puncta", placed especially in the vicinity of nucleus, most often irregularly but, sometimes, disposed in a "circular" pattern (Figure 7, c and d).
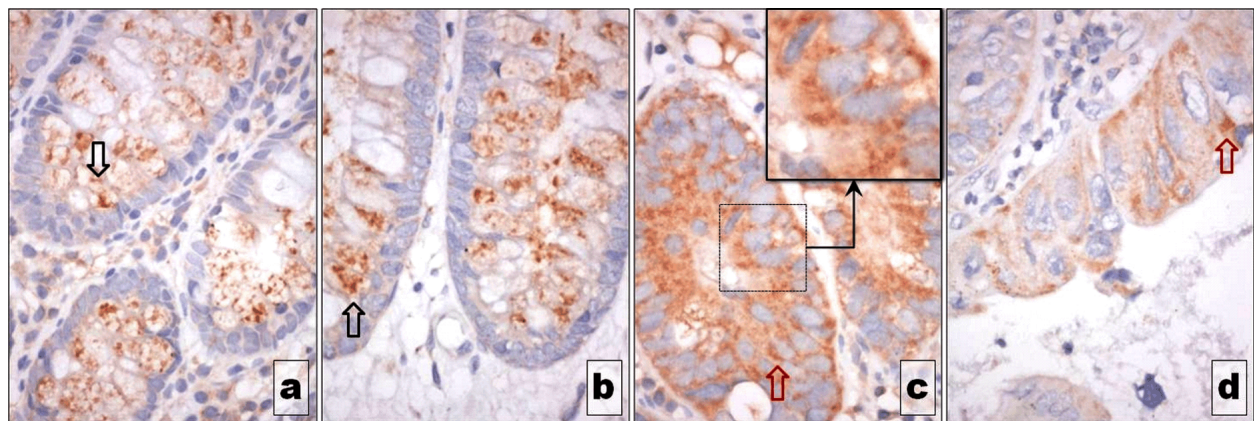

Figure 7 – *LC3 agglomerations in normal epithelium (a – non-irradiated, b – irradiated) – black arrows. "Autophagy puncta" in tumoral epithelium (c – non-irradiated, d – irradiated) – red arrows. LC3 particular arrangement in a circular pattern demarcating a vesicular structure – black rectangle in the right upper corner of (c) image. LC3 immunomarking, ×400.*

⊟ Discussion

A first observation resulted from our analysis was that the basal expression level of LC3 varied from one person to another which could lead to the assumption that LC3 expression is genetically regulated but this imposes further studies in order to be proved.

As we mentioned above, there are few studies in the literature concerning the assessment of autophagy process in human colorectal carcinoma on paraffin-embedded tissue samples. In all of them, the evaluation is qualitative and records an overexpression of LC3 in the tumoral tissue as compared with the normal one [3, 5, 7, 9].

Our quantitative determinations of LC3 protein expression level confirmed the differences of the autophagy process intensity between the tumoral proliferation and normal epithelium previously observed. Moreover, we found this difference, even more pregnant, between tumoral proliferations and colonic epithelia irradiated before the surgical intervention.

The direct correlation between LC3 levels in tumoral proliferation and normal colonic epithelium of the same case revealed by the Pearson's correlation test proves that the intensity level of the autophagy process in the tumoral proliferation is depending on the intensity of the same process in the normal epithelium, fact that could support the assumption that autophagy process is genetically regulated.

Comparative analysis of statistical data as determined by Student's *t*-test concerning the autophagy process revealed decreasing trend from its highest intensity in the non-irradiated tumoral proliferation to its lowest intensity in irradiated normal epithelium. However, the intensity of autophagy process is higher in tumoral proliferations than in normal epithelia regardless the preoperative irradiation.

Preoperative irradiation induced a downregulation of autophagy process. This decrease is less prominent in tumoral proliferations than in normal epithelia, as Student's *t*-test results proved. However, more studies on larger series with and without preoperative radiotherapy are needed in order to have a better confirmation of the preoperative radiotherapy influence on LC3 expression level in the tumoral tissues.

The qualitative analysis of LC3 distribution in the cytoplasm defined two different patterns: in normal cells, the LC3 expression was predominantly granular, with agglomerations near the apical pole rather than in the perinuclear space; in tumoral cells, the expression is higher as compared with normal cells, and is both diffuse in the cytoplasm and granular, with coarse granules, with peri-nuclear disposition and, sometimes, annular arrangement. Further studies are needed to elucidate if the different above-described morphological features represent the presence of different LC3 isoforms in the cytoplasm.

⊟ Conclusions

The preliminary study of LC3 expression in colonic normal and tumoral epithelial cells pointed out the existence of a basal level of the autophagy process in the normal tissue, with interindividual variability. The autophagy process proved to be upregulated in the tumoral cells, with a particular morphologic expression, namely the presence of cytoplasmic coarse granules disposed in an "annular" pattern. Preoperative radiotherapy is downregulating the autophagy process both in normal and tumoral tissue but to a lesser extent in the latter.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Corresponding author
Iancu Emil Pleşea, Professor, MD, PhD, Department of Pathology, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Department of Pathology, Emergency County Hospital of Craiova, 1 Tabaci Street, 200642 Craiova, Romania; Phone/Fax +40251–306 109, e-mail: pie1956@yahoo.com