UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA

DOCTORAL SCHOOL

PHD THESIS

SUMMARY

Autophagy in colorectal cancer

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CONTENT

KEYWORDS

- Colorectal Cancer
- Autophagy
- LC3
- BECN1
- BCL 2
- CD 34
- CD 31
- CD 105

KNOWLEDGE

Chapter I: Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer type worldwide among men and second among women, accounting for about 10% of the overall incidence of cancers (1-3). In terms of mortality, colorectal cancer is the fourth globally (2).

In Romania colorectal cancer mortality doubled in the past two decades, CRC is currently the second cause of death after lung cancer (4). Currently, the Ministry of Health estimated the incidence of CRC at 10.1 new cases per 100,000 men and 7.3 cases per 100,000 women (5).

Chapter II: Autophagy

Autophagy (from the Greek "auto" = own and "phagein" = to eat (6)) is a basal catabolic mechanism characterized by cellular auto digestion, removal of cellular components (such as organelles and proteins) weathered or degraded and toxic waste resulted from cellular metabolism, achieved by the action of lysosomes (7.8). Autophagy (or auto phagocytosis) is a physiological process that occurs in normal cells at a basic level, ensuring homeostasis and cell survival under special conditions, by degradation and recycling of cellular compounds (8). Hypoxia, oxidative stress and nutrient deprivation are among the special conditions that trigger autophagy, which ensures cell survival (9).

Chapter III: The role of autophagy in cancer

The current accepted hypothesis presents autophagy as having a dual, contradictory role in carcinogenesis. First, autophagy is a supervisory mechanism in normal cells, protecting them from malignant transformation by removing damaged proteins and organelles, and by reducing DNA damage, reactive oxygen species and mitochondrial abnormalities. However, autophagy can support the formation of malignant cells by providing nutrients, necessary for the growth of malignant cells, in hypoxic conditions and by inhibiting cell death and increasing resistance to therapy (10,11).

Malignant cells` response to autophagy during metastasis is different depending of tumor stage. Autophagy can help reduce metastasis in the early stages of malignant cells` dissemination by promoting inflammatory immune response against the tumor. In addition, autophagy limits tumor necrosis and the expansion of dormant malignant cells (which lead to the formation of micrometastases) and at the same time affecting the senescence caused by oncogenes (12). Autophagy seems to support the formation of metastases in advanced stages of cancer, by increasing survival of malignant cells detached from the primary tumor (which is possible due to lack of extracellular matrix) and supporting the dissemination of malignant cells to distant sites from the primary tumor, due to transition of tumoral cells (which lack connection with the extracellular matrix factors) into a dormant stage until appropriate conditions for cell growth and division (12:13).

Chapter IV: The main autophagy genes involved in colorectal cancer

- LC3/LC3-II:
  - overexpression in advanced stages (14);
  - overexpression is associated with aggressiveness (15);
  - perinuclear overexpression is associated with a positive prognosis (16);
  - elevated levels in the DLD-1 and SW480 (CRC cell lines) treated with inhibitors of autophagy (17);
  - elevated levels in CRC cell lines treated with 5-fluorouracil (18);
  - elevated levels in CRC cell lines treated with 5-fluorouracil and radiotratate (19);
  - low levels are associated with a good prognosis and response to treatment (20,21);
  - lack of expression is associated with a poor prognosis and a low survival (22).
- BECN1/Beclin1:
  - overexpression is inversely proportional to metastasis (23);
  - overexpression is associated with a good prognosis (24);
  - overexpression is associated with increased survival in patients treated with 5-fluorouracil (25);

- low levels are associated with an increased survival in advanced stages at patients treated with cetuximab (20,21);
- low levels are associated with a poor outcome and low survival (22);
- low levels are associated with lymph-vascular invasion (26).

➢ BCL2/Bcl2:

- increased levels are associated with metastasis and invasion (27);
- increased levels ar associated with resistance to paclitaxel (28).

Chapter V: Autophagic medication in colorectal cancer

Autophagy inhibitors may have antineoplastic effects, for example: suppressing the protective effect of autophagy by treatment with 3-methyl adenine it has been reported to increase the therapeutic efficacy of cisplatin and 5-FU in digestive cancers, including colon cancer (29).

Autophagy modulators may become a target for increasing the effectiveness of anticancer therapies, either alone or in combination with other chemotherapeutic agents (30).

PERSONAL CONTRIBUTIONS

Chapter VI: The objectives

The main goal is the finding of a potential new therapeutic targets by studying the key gene involved in autophagy (LC3 BECN1 and BCL2), aiming at the same time, the possible association with other targets already used in colorectal cancer therapy. Objectives:

- assessing the role of autophagy: promoter or tumor suppressor by studying the expression level of LC3 at primary tumor;

- evaluating BCL-2 and BECN1 gene expression to observe their influence on regulation of autophagy in primary tumor compared to normal tissue and their role in tumor development;

- assessing blood supply in the tumor compared to normal colorectal mucosa.

Chapter VII: Material and Methods

The material was collected from patients diagnosed and operated for colorectal cancer, two samples being collected: one from the malignant tumor and other from normal tissue.

Three different methods (Immunohistochemistry, Western Blot and Real Time PCR) were used to evaluate the gene and protein expression of the targets of interest, in order to achieve objectives.

Chapter VIII: LC3 expression in colorectal cancer

LC3 protein expression on paraffin sections obtained from normal tissue and tumor revealed an highly significant LC3 level overexpression of tumor tissue ($p < 0.001$ for Student t test) as opposed to the normal (which was considered as a reference level for LC3 expression), with an average LC3 value higher in the tumor compared to the average of healthy tissue. Also, the Pearson correlation test showed that the levels of LC3 in normal tissue and in tumor are in a direct correlation ($p < 0.001$), as the level of LC3 is higher in normal epithelium, the more it will be higher in the malignant epithelium. This leads to the conclusion that the level of expression of LC3 protein is genetically regulated in normal cells, ranging from individual to individual, and is up-regulated in malignant cells.

The quantitative evaluation of LC3 protein by Western blot revealed a high level of expression of isoform LC3-II (considered to be a marker for autophagy) in the tumor tissue compared to normal, Student's t test adjusted by the Wilcoxon factor was significantly ($p < 0.05$); LC3-II expression averages were 0.107 g/µl in normal tissue and 0.504 g/µl in the tumor. Autophagy is up-regulated in malignant tissue, participating actively in the carcinogenic, unlike healthy tissue, were autophagy is regulated at a basal level, ensuring an optimal functioning.

Evaluation of MAP1LC3 gene expression found no statistically significant difference between the mean values of LC3 gene expression in normal tissue (0,484) and the mean values for tumor tissue (0.770), with a p coefficient of Student t test of 0,283.

Chapter IX: Gene expression LC3, BCL2 and BECN1 in colorectal cancer

The comparation of LC3 and BECN1 gene expression levels resulted in an average of 1,164 (standard deviation 1.461) for LC3 and 0.9286 (standard deviation 0.6774) for BECN1, this difference being statistically significant (Student t test - p = 0.5304), which supports the assumption that autophagy is up-regulated in malignant colorectal tissue, BECN1 is overexpressed in tumor compared to normal tissue, unlike BCL2 which is overexpressed in normal tissue. When comparing BCL2 and BECN1 expression levels, we obtained an average of 1,047 (standard deviation 0.9857) for BECN1 and 0.8638 (standard deviation 1.753) for BCL2, Student t test was highly significant (p <0.0001), which shows an promoter effect of the autophagic process on malignant tissue compared to normal epithelium, an effect that appears to play an important role in carcinogenesis support.

Chapter X: The relationship between autophagy and neovascularization in colorectal cancer

Immunohistochemical staining of sections from the tumor showed a mean of CD34 and CD31 labeled vessels higher in the tumor base compared to the surface of the tumor, and a higher average of the vessels marked with CD105 in the tumor surface compared to the base area of the tumor. The difference between the averages of the results was highly significant (ANOVA: p <0.0001).

Neoproliferation vessels marked with CD105 were compared with quantitative assessment of LC3 in the same areas, observingtheir correlation. Spearman correlation test (p = 0.9588) did not reveal any relation between the process of neovascularization and autophagic process, the number of neo-vessels being increased in cases with a high, and cases with a low percentage of LC3, no trend (upward or downward) of the correlation existing.

The above observation brings an important argument on the possibility of combining anti-autophagic therapy with antiangiogenic therapy, the two types of therapies targeting different mechanisms involved in carcinogenesis, which do not seem to influence each other.

Chapter X: FINAL CONCLUSIONS

1. The results obtained in this study show an augmentation of the autophagic process in malignant colorectal tissue compared to normal one.
2. BECN1, promoter gene of autophagic process, is overexpressed in tumor tissue compared with BCL2, inhibiting gene of autophagy process, the results indicate that autophagy promotes progression of carcinogenesis.
3. Tumor neovascularization, measured using the CD105 marker, does not correlate with autophagic process, the two systems not influencing each other.
4. Our results support the fact that the autophagic process may be a new therapeutic target for colorectal cancer therapy, anti-autophagy therapy alone or in combination with anti-angiogenic therapy represents a future treatment option for colorectal cancer.

Bibliography

1. BERNARD W. STEWART CPW: **World Cancer Report 2014**, vol. 1, 2014 edn. Lyon: International Agency for Research on Cancer; 2014.
2. **World Health Organization** [http://www.who.int/mediacentre/factsheets/fs297/en/]
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics**. *CA: A Cancer Journal for Clinicians* 2011, **61**(2):69-90.
4. Ait Ouakrim D, Pizot C, Boniol M, Malvezzi M, Boniol M, Negri E, Bota M, Jenkins MA, Bleiberg H, Autier P: **Trends in colorectal cancer mortality in Europe: retrospective analysis of the WHO mortality database**. *BMJ* 2015, **351**:h4970.
5. **Ministerul Sănătații** [www.ms.ro/documente/1216%20Anexa%202_8720_6579.doc]
6. **Autophagy** [https://en.wikipedia.org/wiki/Autophagy]
7. Levine B, Kroemer G: **Autophagy in the Pathogenesis of Disease**. *Cell* 2008, **132**(1):27-42.
8. Lin N-Y, Beyer C, Gießl A, Kireva T, Scholtysek C, Uderhardt S, Munoz LE, Dees C, Distler A, Wirtz S *et al*: **Autophagy regulates TNFα-mediated joint destruction in experimental arthritis**. *Annals of the Rheumatic Diseases* 2012, **72**(5):761-768.
9. Mizushima N, Levine B, Cuervo AM, Klionsky DJ: **Autophagy fights disease through cellular self-digestion**. *Nature* 2008, **451**(7182):1069-1075.
10. Panda PK, Mukhopadhyay S, Das DN, Sinha N, Naik PP, Bhutia SK: **Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics**. *Seminars in Cell & Developmental Biology* 2015, **39**:43-55.
11. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, Codogno P, Debnath J, Gewirtz DA, Karantza V *et al*: **Autophagy in malignant transformation and cancer progression**. *The EMBO Journal* 2015, **34**(7):856-880.
12. Kenific CM, Thorburn A, Debnath J: **Autophagy and metastasis: another double-edged sword**. *Current Opinion in Cell Biology* 2010, **22**(2):241-245.
13. Su Z, Yang Z, Xu Y, Chen Y, Yu Q: **Apoptosis, autophagy, necroptosis, and cancer metastasis**. *Molecular Cancer* 2015, **14**(1):48.

14. Chen Z, Li Y, Zhang C, Yi H, Wu C, Wang J, Liu Y, Tan J, Wen J: **Downregulation of Beclin1 and Impairment of Autophagy in a Small Population of Colorectal Cancer**. *Dig Dis Sci* 2013, **58**(10):2887-2894.
15. Zheng HY, Zhang XY, Wang XF, Sun BC: **Autophagy enhances the aggressiveness of human colorectal cancer cells and their ability to adapt to apoptotic stimulus**. *Cancer biology & medicine* 2012, **9**(2):105-110.
16. Giatromanolaki A, Koukourakis MI, Harris AL, Polychronidis A, Gatter KC, Sivridis E: **Prognostic relevance of light chain 3 (LC3A) autophagy patterns in colorectal adenocarcinomas**. *Journal of Clinical Pathology* 2010, **63**(10):867-872.
17. Sato K, Tsuchihara K, Fujii S, Sugiyama M, Goya T, Atomi Y, Ueno T, Ochiai A, Esumi H: **Autophagy Is Activated in Colorectal Cancer Cells and Contributes to the Tolerance to Nutrient Deprivation**. *Cancer Research* 2007, **67**(20):9677-9684.
18. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H: **Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model**. *European Journal of Cancer* 2010, **46**(10):1900-1909.
19. Schonewolf CA, Mehta M, Schiff D, Wu H, Haffty BG, Karantza V, Jabbour SK: **Autophagy inhibition by chloroquine sensitizes HT-29 colorectal cancer cells to concurrent chemoradiation**. *World journal of gastrointestinal oncology* 2014, **6**(3):74-82.
20. Guo G-F: **Autophagy-related proteins Beclin-1 and LC3 predict cetuximab efficacy in advanced colorectal cancer**. *World Journal of Gastroenterology* 2011, **17**(43):4779.
21. Yang M, Zhao H, Guo L, Zhang Q, Zhao L, Bai S, Zhang M, Xu S, Wang F, Wang X *et al*: **Autophagy-based survival prognosis in human colorectal carcinoma**. *Oncotarget* 2015, **6**(9):7084-7103.
22. Choi JH, Cho Y-S, Ko YH, Hong SU, Park JH, Lee MA: **Absence of Autophagy-Related Proteins Expression Is Associated with Poor Prognosis in Patients with Colorectal Adenocarcinoma**. *Gastroenterology Research and Practice* 2014, **2014**:1-10.
23. Zhang M-Y, Gou W-F, Zhao S, Mao X-Y, Zheng Z-H, Takano Y, Zheng H-C: **Beclin 1 Expression is Closely Linked to Colorectal Carcinogenesis and Distant Metastasis of Colorectal Carcinoma**. *IJMS* 2014, **15**(8):14372-14385.
24. Yang Z, Ghoorun RA, Fan X, Wu P, Bai Y, Li J, Chen H, Wang L, Wang J: **High expression of Beclin-1 predicts favorable prognosis for patients with colorectal cancer**. *Clinics and Research in Hepatology and Gastroenterology* 2015, **39**(1):98-106.
25. Li B-X, Li C-Y, Peng R-Q, Wu X-J, Wang H-Y, Wan D-S, Zhu X-F, Zhang X-S: **The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers**. *Autophagy* 2009, **5**(3):303-306.
26. Zaanan A, Park JM, Tougeron D, Huang S, Wu T-T, Foster NR, Sinicrope FA: **Association of beclin 1 expression with response to neoadjuvant chemoradiation therapy in patients with locally advanced rectal carcinoma**. *International journal of cancer* 2015, **137**(6):1498-1502.
27. Koehler BC, Scherr A-L, Lorenz S, Urbanik T, Kautz N, Elssner C, Welte S, Bermejo JL, JÃ¤ger D, Schulze-Bergkamen H: **Beyond Cell Death â€“ Antiapoptotic Bcl-2 Proteins Regulate Migration and Invasion of Colorectal Cancer Cells In Vitro**. *PLoS ONE* 2013, **8**(10):e76446.
28. Wu S, Wang X, Chen J, Chen Y: **Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells**. *Biochemical and Biophysical Research Communications* 2013, **434**(4):898-903.
29. Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T, Kuwano H: **Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells**. *Ann Surg Oncol* 2009, **16**(3):761-771.
30. Radogna F, Dicato M, Diederich M: **Cancer-type-specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target**. *Biochemical Pharmacology* 2015, **94**(1):1-11.