The Influence of Tumor Microenvironment on ATG4D Gene Expression in Colorectal Cancer Patients

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Abstract
Despite great progress in research on the subject, the involvement of autophagy in colorectal cancer (CRC) pathogenesis (initiation, progression, metastasis) remains obscure and controversial. Autophagy is a catabolic process, fundamental to cell viability and connected with degradation/recycling of proteins and organelles. In this study, we aimed at investigating the relative expression level of mRNA via Real-Time PCR of 16 chosen genes belonging to Atg8 mammalian orthologs and their conjugation system, comprising GABARAP, GABARAPL1, GABARAPL2, MAP1LC3A, MAP1LC3B, MAP1LC3C, ATG3, ATG7, ATG10, ATG4A, ATG4B, ATG4C, ATG4D, and three genes encoding proteins building the multimeric ATG16L1 complex, namely ATG5, ATG12, and ATG16L1, in 73 colorectal tumors and paired adjacent normal colon mucosa. Our study demonstrated the relative downregulation of all examined genes in CRC tissues in comparison to adjacent noncancerous mucosa, with the highest rate of expression in both tumor and non-tumor tissues observed for GABARAPL2 and the lowest for MAP1LC3C. Moreover, in patients with advanced-stage tumors and high values of regional lymph nodes, statistically significant downregulation of ATG4D expression in adjacent normal cells was observed. Our study confirms the role of autophagy genes as cancer suppressors in colorectal carcinogenesis. Furthermore, in regard to the ATG4D gene, we observed the influence of tumor microenvironments on gene expression in adjacent colon mucosa.

Keywords Colorectal cancer · Autophagy · ATG · LC3 · GABARAP · Relative expression

Introduction

Colorectal cancer (CRC) is among the leading causes of cancer-related death in the world (in fourth place among malignancies) [1]. In developed countries with high/very high human development indices (HD1), its incidence is higher. Although great progress has been made over the past decades in cancer prevention and identification as well as in anticancer therapy, including personalized therapy, the survival rate of CRC patients is still unsatisfying. However, in countries with high/very high HDIs (e.g., the USA, New Zealand), mortality rates have been stabilizing or declining, whereas in medium- to high-HDI counties (Eastern Europe, Asia) both incident and mortality rates have rapidly
increased [1]. An understanding of cancer pathogenesis, along with a search for new prognostic biomarkers, may influence improvement in prognoses and may also enable the discovery of new potential targets for anticancer therapies. Much recent research has indicated autophagy as a potential new biomarker in CRC development [2].

Maintenance of cellular homeostasis plays an extraordinarily significant role in the normal growth and development of living organisms. In eukaryotic cells, autophagy maintains intracellular balance through the bulk degradation and recycling of redundant or damaged cytoplasmic proteins, aggregates, and/or organelles [3, 4]. Moreover, autophagy is involved in other physiological processes such as cell development, tumor suppression, immune defense, and response to stress; thus its deregulation has been linked to many diseases, including cancer [5]. This highly conserved process is strictly coordinated by core autophagy proteins encoded by more than 30 Autophagy-related (ATG) genes. The first step in this mechanism is the formation of an autophagosome, beginning with the creation of a cup-shaped membrane structure also known as an isolation membrane or phagophore. During elongation, the concave part of a phagophore becomes the luminal side of an autophagosome inner vesicle [6]. The next step in autophagy leads to the elongation and maturation of autophagosomes which fuse to lysosomes; thus, their contents are degraded by acidic hydrolases [7]. In autophagosome elongation, the pivotal role is played by mammalian homologs of the yeast Atg8 (autophagy-related 8) protein family. These homologs comprise two subfamilies: the microtubule-associated protein 1 light chain 3 MAP1LC3 including LC3A, LC3B, and LC3C, and the gamma-aminobutyric acid receptor-associated protein GABARAP, composed of three members: (i) GABARAP, (ii) GABARAPL1/GEC1/ATG8L (GABARAP-like protein 1/guinea pig endometrial glandular epithelial cells-1/Atg8-like protein), and (iii) GABARAPL2/GATE-16 (GABARAP-like protein 2/Golgi-associated ATPase enhancer of 16 kDa) [8–10]. All of these proteins contain highly conserved sequences (from yeasts to mammals), suggesting their important role in living cells [11]. Moreover, proteins belonging to these two subfamilies contain a conserved C-terminal glycine which is vital in autophagy [12].

The MAP1LC3 family is involved in phagophore elongation, whereas the GABARAP family is required in the later stages of autophagosome formation [12]. Apart from autophagy, Atg8 orthologs mediate in various intracellular trafficking processes [11]. The expression of Atg8 homolog proteins varies, depending on tissue type: e.g., LC3C is expressed mainly in the lungs, GABARAPL1 in the nervous system, GABARAPL2 in the brain, and GABARAP in the endocrine glands [11, 12].

Two ubiquitin-like conjugation systems are involved in the processing of Atg8 family members. One involves ATG7 (an E1-like enzyme) and ATG10, the other ATG3 (E2-conjugating enzyme) and ATG7; both mediate in the conjugation of the ATG16L1 complex (consisting of ATG5, ATG12, and ATG16). These enzymes, along with the Atg16L1 complex, are involved in the conjugation of phosphatidylethanolamine to yeast Atg8 human orthologs simultaneously with proteolytic maturation, which is dependent on ATG4 [13]. A certain role in the lipidation and delipidation of Atg8 homologs is played by the family of ATG4 cysteine proteases. Therefore, ATG4 activity is expected to be thoroughly regulated. At least, four human homologs are known to be involved in this process: (i) ATG4A (autophagin-2), (ii) ATG4B (autophagin-1), (iii) ATG4C (autophagin-3), and (iv) ATG4D (autophagin-4). Autophagins are responsible for the cleavage of various substrates of human Atg8 homologs [10, 14]. In most normal tissues, the expression of ATG4s is detected at low levels, with the highest level of expression of ATG4 proteins being observed in skeletal muscles as well as in the brain, heart, liver, pancreas, and testes [14]. Increased expression of ATG4 proteins has been shown to be connected with both the progression and suppression of tumor development as well as with cancer therapy resistance. Thus, these proteins may play a role as a potential/novel target in cancer therapy [14].

Therefore, in this study we aimed to investigate the mRNA expression of genes encoding pivotal proteins engaged in autophagosome elongation, such as GABARAP, GABARAPL1, GABARAPL2, MAP1LC3A, MAP1LC3B, MAP1LC3C, ATG3, ATG4A, ATG4B, ATG4C, ATG4D, ATG5, ATG7, ATG10, ATG12, and ATG16L1, in colorectal cancer tissue and paired adjacent normal colon mucosa.

Materials and methods

The studied group was characterized in our previously published paper [15]. Briefly, samples of cancer tissue and paired normal adjacent mucosa obtained from 73 patients during surgery were collected and stored in accordance with recommended procedures. The clinical characteristics of the study group as well as the methodology of molecular and statistical analyses were precisely described previously [15]. Informed consent was obtained from each individual participant included in the study. The study design was accepted by the Wrocław Medical University Ethical Committee (approval number KB-822/2012).

In brief, mRNA was isolated from tumors as well as from noncancerous adjacent tissues and, following quality control, synthesis of cDNA was carried out. We employed the RealTime Ready Assay with Universal ProbeLibrary (Roche) with GABARAP (ID 117150), GABARAPL1 (ID 118062), GABARAPL2 (ID 117687), MAP1LC3A (ID 144005), MAP1LC3B (ID 144582), MAP1LC3C (ID 117687), and MAP1LC3D (ID 144005).
144902, ATG3 (ID 118148), ATG4A (ID 109561), ATG4B (ID 109269), ATG4C (ID 109546), ATG4D (ID 109054), ATG5 (ID 125999), ATG7 (ID 120541), ATG10 (ID 138547), ATG12 (ID 118103), and ATG16L1 (ID 113714) genes. Moreover, for normalization of Real-Time PCR results, three reference genes were applied [15].

The $2^{-\Delta\Delta C_{T}}$ method, Student’s t test, the Wilcoxon signed-rank test, Spearman’s correlation coefficient, and the Benjamini-Hochberg procedure were used for statistical analysis.

Results

In both normal and tumor tissue, the highest levels of gene expression were observed for GABARAPL2, followed by MAP1LC3B and MAP1LC3A (see Table 1), the lowest for MAP1LC3C (see Table 1).

For the ranking of genes according to the mean relative mRNA expression in all downregulated genes, see Table 2; for the ranking according to statistical significance, see Table 3.

### Table 1: Ranking of genes according to their absolute expression

| Gene                | $R_N$ | $\Delta N$  | 95% CI         | $R_T$ | $\Delta T$  | 95% CI         |
|---------------------|-------|-------------|----------------|-------|-------------|----------------|
| GABARAPL2           | 1     | 2.3859      | (2.2342, 2.5376)| 1     | 3.2986      | (3.1267, 3.4705)|
| MAP1LC3B            | 2     | 3.0610      | (2.8849, 3.2371)| 2     | 3.7988      | (3.6073, 3.9903)|
| MAP1LC3A            | 3     | 4.1686      | (3.9522, 4.3850)| 3     | 4.5209      | (4.3016, 4.7402)|
| ATG12               | 4     | 4.2312      | (3.9492, 4.5132)| 4     | 4.8904      | (4.5544, 5.1228)|
| ATG3                | 5     | 4.6081      | (4.5220, 4.6942)| 5     | 4.8905      | (4.7625, 5.0183)|
| ATG5                | 6     | 4.8188      | (4.7017, 4.9359)| 6     | 5.2499      | (5.1097, 5.3901)|
| ATG4D               | 7     | 5.8584      | (5.6341, 6.0827)| 7     | 6.0616      | (5.8688, 6.2544)|
| ATG4A               | 8     | 5.9106      | (5.7112, 6.1100)| 8     | 6.2979      | (6.0823, 6.5135)|
| ATG4C               | 9     | 5.9419      | (5.5782, 6.3056)| 10    | 6.5358      | (6.3620, 6.7096)|
| ATG7                | 10    | 6.3002      | (6.1761, 6.4333)| 9     | 6.3739      | (6.2110, 6.5368)|
| GABARAPL1           | 11    | 6.3489      | (6.0533, 6.6445)| 12    | 7.5241      | (7.2348, 7.8134)|
| GABARAP             | 12    | 6.9673      | (6.7999, 7.1347)| 13    | 7.7140      | (7.4961, 7.9319)|
| ATG4B               | 13    | 7.0813      | (6.8649, 7.2977)| 11    | 7.3425      | (7.1191, 7.5659)|
| ATG10               | 14    | 7.5374      | (7.3685, 7.7063)| 14    | 8.4026      | (8.1960, 8.6092)|
| ATG16L1             | 15    | 9.0196      | (8.6257, 9.4135)| 15    | 9.9371      | (9.6926, 10.1816)|
| MAP1LC3C            | 16    | 9.9120      | (9.4596, 10.3644)| 16    | 10.8150     | (10.0179, 11.6121)|

$R_N$ and $R_T$ show the rankings for healthy and tumor cells, respectively. Similarly, $\Delta N$ and $\Delta T$ are the delta scores for healthy and tumor cells, respectively.

### Table 2: Ranking of genes according to mean relative fall in expression

| Pos. | Gene            | $\Delta C_{T}$ | 95% CI       | $2^{-\Delta C_{T}}$ | 95% CI       |
|------|-----------------|----------------|--------------|----------------------|--------------|
| 1    | GABARAPL1       | −1.1751        | (−1.5688, −0.7815) | 2.2581               | (1.7189, 2.9666) |
| 2    | ATG16L1         | −0.9175        | (−1.3111, −0.5239) | 1.8888               | (1.4379, 2.4813) |
| 3    | GABARAPL2       | −0.9128        | (−1.1513, −0.6742) | 1.8827               | (1.5957, 2.2211) |
| 4    | MAP1LC3C        | −0.9029        | (−1.4695, −0.320)  | 1.8698               | (1.0916, 3.2032) |
| 5    | ATG10           | −0.8651        | (−1.1210, −0.6092) | 1.8215               | (1.5254, 2.1750) |
| 6    | GABARAP         | −0.7467        | (−1.0763, −0.4281) | 1.6780               | (1.4420, 1.9524) |
| 7    | MAP1LC3B        | −0.7378        | (−0.9945, −0.4810) | 1.6676               | (1.3957, 1.9924) |
| 8    | ATG12           | −0.6074        | (−0.9147, −0.3001) | 1.5235               | (1.2312, 1.8852) |
| 9    | ATG4C           | −0.5939        | (−1.0017, −0.1861) | 1.5093               | (1.1377, 2.0024) |
| 10   | ATG5            | −0.4311        | (−0.5961, −0.2660) | 1.3483               | (1.2025, 1.5116) |
| 11   | ATG4A           | −0.3873        | (−0.6409, −0.1338) | 1.3079               | (1.0972, 1.5593) |
| 12   | MAP1LC3A        | −0.3523        | (−0.6398, −0.0648) | 1.2766               | (1.0459, 1.5581) |
| 13   | ATG3            | −0.2823        | (−0.4308, −0.1337) | 1.2161               | (1.0971, 1.3480) |
| 14   | ATG4B           | −0.2612        | (−0.5318, 0.0094)  | 1.1985               | (0.9935, 1.4457) |
| 15   | ATG4D           | −0.1932        | (−0.4720, 0.0856)  | 1.1433               | (0.9424, 1.3870) |
| 16   | ATG7            | −0.0737        | (−0.2671, 0.1197)  | 1.0524               | (0.9204, 1.2034) |
When the Benjamini-Hochberg procedure for multiple testing was applied, each of the 13 significant differences explained above remained significant (tests used: Student’s *t* test and Wilcoxon rank test).

## Associations with expression levels in tumor tissue

### Age

The expression levels of *ATG4B* and *MAP1LC3A* were negatively correlated with age (older people had higher scores on average) (Spearman’s correlation coefficient $R = -0.250$; $p = 0.033$).

### Sex

The expression levels of *ATG7* and *GABARAPL1* were higher among males (lower mean scores): $p = 0.006$ and $p = 0.044$, respectively (see Table 4). These differences were not significant when the Benjamini-Hochberg procedure was applied.

### Metastasis (M)

The expression level of *ATG3* was lower when M = 1 ($p = 0.008$) (see Table 5). These differences were not significant when the Benjamini-Hochberg procedure was applied.

| Pos. | Gene      | $p$ value         | Expression in Tumor |
|------|-----------|-------------------|---------------------|
| 1    | GABARAPL2 | $9.2408 \times 10^{-10}$ | Lower               |
| 2    | ATG10     | $1.2169 \times 10^{-9}$ | Lower               |
| 3    | GABARAPL1 | $5.5105 \times 10^{-8}$ | Lower               |
| 4    | GABARAP   | $7.0458 \times 10^{-8}$ | Lower               |
| 5    | ATG5      | $1.9192 \times 10^{-7}$ | Lower               |
| 6    | MAP1LC3B  | $6.2283 \times 10^{-7}$ | Lower               |
| 7    | ATG16L1   | $3.2873 \times 10^{-6}$ | Lower               |
| 8    | ATG12     | 0.0001977         | Lower               |
| 9    | ATG3      | 0.0003003         | Lower               |
| 10   | ATG4A     | 0.002327          | Lower               |
| 11   | ATG4C     | 0.003696          | Lower               |
| 12   | MAP1LC3A  | 0.01457           | Lower               |
| 13   | MAP1LC3C  | 0.03195           | Lower               |
| 14   | ATG4B     | 0.05435           | No significant change |
| 15   | ATG4D     | 0.1673            | No significant change |
| 16   | ATG7      | 0.4473            | No significant change |

| Sex   | ATG7   | GABARAPL1 |
|-------|--------|-----------|
| Female | Mean 6.60244085 | 7.82499514 |
| N      | 36     | 36        |
| Standard deviation | 0.663860195 | 1.268416464 |

| Male  | Mean 6.15161525 | 7.23126054 |
| N     | 37     | 37        |
| Standard deviation | 0.690844545 | 1.199491078 |

| Total  | Mean 6.37394021 | 7.52406116 |
| N      | 73     | 73        |
| Standard deviation | 0.710193119 | 1.261277719 |

## Primary tumor (T)

T was positively correlated with expression levels at *GABARAPL1* (high T was correlated with low scores) (Spearman’s correlation coefficient $R = -0.250$; $p = 0.033$).

## TNM advancement

The level of advancement was negatively associated with expression at *MAP1LC3A* (Spearman’s correlation coefficient $R = 0.350$; $p = 0.002$).

## Regional lymph nodes (N)

High values of N (2 and 3) were associated with low levels of expression of *MAP1LC3A* in tumor cells ($p = 0.012$, analysis of variance, see Table 6).

| M    | ATG3               |
|------|--------------------|
| 0    | 4.84813801         |
| N    | 0.558581575        |
| 1    | 5.34750717         |
| N    | 0.326935149        |
| Total| 4.89668779         |
| N    | 0.558998761        |
Associations with expression levels in “normal” cells (adjacent normal tissue)

Age

Expression of GABARAP (Spearman’s correlation coefficient $R = -0.256; p = 0.029$) and of ATG7 (Spearman’s correlation coefficient $R = -0.241; p = 0.040$) was positively correlated with age (older people had, on average, lower scores). None of these correlations were significant when the Benjamini-Hochberg procedure was applied.

Sex, location, M

There was no significant association between sex, location of the tumor, the value of M, or the difference in expression levels between healthy and tumor cells of any gene.

Primary tumor (T)

T was negatively correlated with expression in healthy cells in the two following genes: ATG4B (Spearman’s correlation coefficient $R = 0.279; p = 0.018$) and ATG5 (Spearman’s correlation coefficient $R = 0.255; p = 0.029$).

TNM advancement

Low levels of expression of ATG4D were associated with stage III (analysis of variance, $p = 0.006$) (see Table 7).

Regional lymph nodes (N)

High values of N (2 and 3) are associated with low levels of expression of ATG4D in healthy cells ($p = 0.027$, analysis of variance). Nx was excluded from the analysis (see Table 8).

Associations with differences in expression levels between tumor and healthy cells

Age was positively correlated with the fall in expression levels between healthy and tumor cells of the three following genes: ATG4B (Spearman’s correlation coefficient $R = 0.352; p = 0.002$), and MAP1LC3A (Spearman’s correlation coefficient $R = 0.279; p = 0.017$). The association between age and the fall in expression of ATG4B remained significant when the Benjamini-Hochberg procedure for multiple testing was applied.

Regional lymph nodes (N)

$N=1$ is associated with a lack of difference in levels of expression of ATG4D between healthy and tumor cells. Other values of N are associated with lower levels of expression in tumor cells than in healthy cells ($p = 0.038$, analysis of variance) (see Table 9). Nx was excluded from the analysis.

Discussion

Altered expression (either under- or over-) of autophagy genes may be an important factor in the development and progression (metastases formation) of various human
malignancies [16, 17]. Despite the immense efforts exerted in this area, the role of autophagy in carcinogenesis remains controversial. Although dysregulation of the key autophagy proteins Beclin 1 and LC3 have been studied in a wide variety of tumors, the role of these proteins in colorectal cancer is ambiguous. The first study on LC3 expression in gastrointestinal cancers (esophageal, gastric, and colorectal) employing immunohistochemistry was performed by Yoshikawa et al., who showed that, in all examined cancer types, LC3 expression was significantly elevated in comparison to normal adjacent tissue. Interestingly, no significant association was found between clinicopathological parameters and LC3 expression [18]. Subsequent studies have also reported upregulation of LC3 expression in CRC tissues as well as in CRC cell lines [19–21]. However, in most cases the specificity of the relevant LC3 antibodies has not been examined. Moreover, other studies, including ours, have produced contradictory results [15, 22].

In this study, we found relative downregulation of all 16 examined genes. Among genes with extremely low levels of expression were MAP1LC3A, MAP1LC3B, and MAP1LC3C. Intriguingly, Bai et al. showed that LC3AvI was commonly silenced at the transcriptional level in numerous cancer cell lines due to epigenetic changes. Notably, other research revealed that BNIP3, another gene engaged in the regulation of autophagy, is also silenced in CRC cell lines by the methylation of CpG islands as well as by histone deacetylation [23]. Hence, these observations implied epigenetic modifications as an important factor modifying autophagy expression in cancer cells.

Yang et al. demonstrated underexpression of Beclin 1 and LC3B in the central area of a tumor compared to the adjacent noncancerous mucosa. Additionally, expression of the analyzed proteins has been shown to be strongly associated with patients’ survival. Thus, it has been hypothesized that the expression patterns of the autophagic proteins in the adjacent noncancerous “normal” tissues may also serve, albeit to a lesser degree, as predictive markers of the clinical outcomes of diseases. This is possible due to the strong affect exerted by the tumor’s microenvironment on autophagic flux in the adjacent mucosa, which may influence the promotion of tumor proliferation [24]. However, we found no association between normal cells and expression of MAP1LC3A, MAP1LC3B, or MAP1LC3C.

Nevertheless, in regard to the specific tumor microenvironment, we found statistically significant downregulation of ATG4D expression in adjacent normal cells in patients with high tumor stages and high values for regional lymph nodes. ATG4D encodes a protein belonging to the ATG4 mammalian family (the class of four cysteine proteases, ATG4A–D) presenting endopeptidase activity, vital for later stages of autophagosome maturation and fusion with lysosomes [25]. ATG4D, one of the above-mentioned four proteases, contains a domain (DEVD) cleaved by caspase-3. The truncated form of ATG4D, compared to its full-length form, shows increased priming and delipidating activity against GABARAPL1 [26]. It has been shown that silencing of ATG4D expression sensitizes HeLa cells and lead to starvation-induced cell death, indicating that ATG4D-dependent autophagy administers to cellular survival [9, 26]. Furthermore, ATG4D has been linked to apoptosis pathways, as its overexpression may induce this process. In cells treated with hydrogen peroxide, cleaved ATG4D is recruited to dysfunctional mitochondria.

In conclusion, the expression of ATG4D and other ATG4 isoforms may regulate the post-translational activation of the LC3/GABARAP family proteins [9, 26, 27].

Our analysis indicates that, in patients with advanced stages of CRCs, the expression level of this vital enzyme is lower in normal tissues. Therefore, in this case, we probably observed a “field effect” and thus hypothesized that ATG4D plays a very important role in CRC development.

Interestingly, our study demonstrated that, in tumor cells, the low level of expression of ATG4B as well as of the previously mentioned MAP1LC3A was associated with age. This observation suggests that decreasing levels of enzyme ATG4B, which mediates the conversion of pro-LC3 isoforms to the LC3-I form (a substrate for subsequent lipidation to LC3-II), may be connected with both cancer development and the aging of patients.

ATG4B is involved in post-translational processing of LC3; however, our results indicate that depleted expression of ATG4B mRNA may trigger the reduced expression of genes encoding post-translational target for this gene. Because of the critical role of ATG4B in the autophagy process, an inhibitor of this protease has been proposed as a promising tool for monitoring the treatment not only of cancers but also of other diseases [28]. Nonetheless, to date, no data confirming the prognostic value of any ATG4 family member in cancers have been published [8].

Our research has shown that GABARAPL2 is the most prominently expressed gene in both normal and tumor tissue. The differing expressions of highly homologous proteins from the GABARAP family are observed in both developing and adult tissues. These proteins play an important role in various cellular processes, including receptor transport, cell proliferation, and autophagy [29]. GABARAPL1 is expressed in numerous tissues, including the brain, heart, skeletal muscles, kidney, ovary, and blood lymphocytes. It has been shown that estrogens may influence the expression of GABARAPL1 and hence may be involved in a variety of estrogen-sensitive diseases such as breast cancer [12]. The expression of the GABARAP family has been studied, *inter alia*, in breast cancer (BC). In cancer tissue, only the GABARAPL1 mRNA level was shown to be significantly reduced, whereas those of GABARAP and GABARAPL2...
were not. The downregulation of GABARAPL1 in BC has been linked to gene silencing via promoter hypermethylation and histone H3 deacetylation. Hence, it has been proposed that epigenetic inhibitors could be used along with classical chemotherapeutics in BC therapy [29]. Notably, in our study we found the GABARAPL1 gene to be the most silenced.

Hitherto, only one research project has examined the prognostic significance of the GABARAP protein in colorectal cancer, thus showing that high levels of expression of this protein were connected to poor differentiation and to shortened overall survival in CRC patients [30]. As of now, there is very little information on the mRNA levels in genes encoding ATG16L complex in colorectal cancer. We found variable mRNA expression levels in genes encoding proteins creating this complex, such as high ATG12, median ATG5, and low ATG16L1. ATG5 and ATG12 create an ubiquitin-like conjugate which interacts with ATG16L1 in a noncovalent manner and creates a multimeric complex crucial for elongation of the pre-autophagosomal membrane [31]. Experimental studies on mice models have shown that heterozygous deletion of ATG5 is involved in intestinal tumor growth and sensitizes tumor cells to chemotherapy [32]. Moreover, it has been demonstrated that ATG5 silencing by specific short interfering RNAs may strengthen the efficacy of chemotherapeutic agents during combination therapy. Interestingly, interferon-gamma has revealed a strong inhibition effect on adenomas of ApcMin/+ATG5+/−mice without obvious side effects. Therefore, a new anticancer treatment strategy based on interferon-gamma and ATG5-targeted inhibition has been proposed [32]. Strong downregulation of ATG5 expression in CRC samples as well as in colorectal cell lines was observed as used both Western blot analysis and IHC assay [33]. Moreover, a reduced level of the conjugated form ATG5–ATG12 in CRC samples in comparison to normal mucosa has been observed (data not shown) [33]. Our observations may indicate that ATG16L1 plays a regulatory role in the ATG16L complex because of its very limited expression in both normal and cancer tissue.

**Conclusions**

To conclude, in our research, we observed relative mRNA downregulation of all examined genes in colorectal cancer tissues. In normal cells of patients with advanced cancer stages, we found statistically significant downregulation of the ATG4D gene. A mean relative decrease in expression was most obvious for GABARAPL1. These findings may indicate the genes in question as potential biomarkers as well as putative targets in CRC diagnosis and therapy. However, our limited cohort and lack of biological/functional analyses indicate that further characterization is required.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest (including financial and non-financial).

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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