Nuclear Localisation of Autophagic p62 and Associated Cytoplasmic Beclin-1 and Bcl-2 Expressions in Adenomas and Adenocarcinomas of the Colorectal Regions

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Abstract

Background: Autophagy is an important biological process that is involved in cellular homeostasis and survival. Derailment of some cellular autophagic processes affects normal cellular function, resulting in cancers and other disorders. Autophagy related proteins are Beclin-1, a human tumour suppressor, Bcl-2 and p62 have been characterised in most cancers. Particularly, a number of studies have reported a loss of Beclin-1 and up regulation of Bcl-2 and p62 in breast cancers. However, studies regarding the expression of these proteins in colorectal adenoma-adenocarcinoma transformation sequence are yet to be described. In this study, we examined the expression patterns of Beclin-1, Bcl-2 and p62 in both colorectal adenomas and adenocarcinomas.

Methods: Immunohistochemistry was performed on formalin-fixed paraffin embedded tissue sections from 14 patients with colorectal tumours and the expression patterns were semi-quantitatively evaluated based on the intensity of staining and the percentage of tumour cells stained.

Results: Cytoplasmic Beclin-1 and p62 expression patterns ranged from moderate to high in both tubular adenomas and adenocarcinomas as compared to normal colonic mucosa. Cytoplasmic Bcl-2 expression was moderately expressed in tubular adenomas but negative to low expression was observed in the adenocarcinomas. This study also provided, for the first time, nuclear localization of p62 in only the colorectal adenocarcinomas.

Conclusion: Beclin-1, Bcl-2 and p62 may be up regulated in the transition of colorectal adenomas to adenocarcinomas.

List of Abbreviations: Bcl-2: B-Cell Lymphoma 2; Bcl-xL: B-cell lymphoma Extra Large; BH2/3: Bcl-2 Homology-2/3; HTA: Human Tissue Act; p62: Nuclearporin 62; ROS: Reactive Oxidative Stress (ROS); TBST: Tris Buffer Saline/ Tween-20; Vps34: Vacuolar Protein Sorting 34.

Keywords: Autophagy; Beclin 1; p62; Bcl-2; Immunohistochemistry; Colorectal; Adenoma; Adenocarcinoma

Background

Autophagy, an integral part of cellular homeostasis, is essentially crucial in ensuring the survival of cells or organisms through recycling of unwanted proteins and/or generation of energy for urgent need. To prevent the cellular milieu from clogging up, damaged or "out-of-work" organelles and misfolded proteins are sequestered in double-membrane vesicles called autophagosomes and delivered to cytosolic lysosomes for degradation of intracellular contents [1].

Under stress conditions, such as nutrient starvation and oxygen deprivation, autophagy is induced in eukaryotic cells to generate nutrients and energy. Even though the induction of autophagy might ensure cellular survival and homeostasis, excessive degradation of cellular contents could drain the cells of nutrients and energy, resulting in cell death [2].

Autophagy, a tightly controlled complex cellular process, recruits several protein in initiation, vesicle nucleation, fusion and degradation. An important protein, Beclin 1, which complexes with Vps34, is recruited at the site of autophagosome formation through phosphorylation of Ambral [3].

Beclin 1 is mono-allelically deleted in human breast, ovarian and prostate cancers but overexpressed in colorectal and gastric cancers [4]. In addition, mice with knockout Beclin 1 gene have been shown to have high incidence of tumour growth, indicating Beclin 1 may be the tumour suppressor [5]. Li et al., found an increased expression of Beclin 1 in colorectal cancer cells, and this was shown to correlate with
a longer survival in cancer patients, suggesting that Beclin 1 may play a role in cell death of cancer cells [6].

However, studies by Koukourakis et al., have shown that over-expression of Beclin 1 correlated with tumour aggressiveness and invasion. They proposed that Beclin 1 induction of autophagy does supresses cancer growth but when it is over-expressed it may play a role in tumour aggressiveness and invasion under adverse conditions. Beclin 1 has a Bcl-2 homology-2 (BH3) domain that is able to bind to BH1-BH2 domains of antiapoptotic proteins Bcl-2 and Bcl-xL. [7].

The binding leads to inhibition of autophagy, suggesting that Beclin 1 may mediate in a cross talk between autophagy and apoptosis [8]. Bcl-2-Beclin 1 complex leads to the inhibition of autophagy-associated cell death [9]. Studies have shown the loss of Bcl-2 in colon cancers [10].

Another equally important protein that is involved in autophagy, p62, serves as a scaffold for cargoes degradation [11]. The p62 protein is involved in cellular stress, proliferation, survival and inflammation [12]. It has recently been a focus of research due to its dynamic role in cellular homeostasis and carcinogenesis. Its interaction with membrane bound LC3 regulates the degradation of harmful proteins aggregates and organelles via autophagy [13].

Thus, the inhibition of autophagy results in the accumulation of p62, leading to increase in levels of intracellular reactive oxidative stress (ROS) that facilitates genomic instability, a condition that may favour malignant transformation [14].

Over expression of p62 has been associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease, as well as many solid tumours [14,15]. However, its role in the colorectal adenoma-adenocarcinoma transition sequence is yet to be clarified. Although autophagy has been linked with colorectal tumour development, there are limited studies so far done in this area. In addition, a study on the expression patterns of Beclin 1, Bcl-2 and p62 in the colorectal adenoma-adenocarcinoma transformation sequence to our knowledge is unavailable.

In this study therefore, the expression patterns of autophagy related proteins, Beclin 1, Bcl-2 and p62 were assessed immunohistochemically in both colorectal adenomas and adenocarcinomas.

Methods

Samples

Formalin fixed paraffin embedded (FFPE) of colorectal tissue samples, consisting of 7 tubular adenomas and 7 adenocarcinomas (Duke's Stage B) were retrieved from Queens Hospital, Romford, UK using SNOMED codes with an ethical approval from the Research Ethics sub-Committee of the University of Westminster, in compliance to the Human Tissue Act (HTA). Tissue samples were given designated HTA codes, which were used throughout this study.

Haematoxylin & Eosin (H&E) and Immunohistochemical staining

Haematoxylin and Eosin staining was performed according to standard protocol to confirm the histological representation of the colorectal tumour tissues. Another set of FFPE tissue sections used in H&E staining, were immunostained for Beclin 1, Bcl-2 and p62 as briefly described here. Sectioned tissues were dewaxed, hydrated and blocked with 30% hydrogen peroxide in methanol for 10 min to deactivate any endogenous peroxidase activity.

Beclin 1 and Bcl-2 antigens were retrieved using citrate buffer, pH 6.0, in a pressure cooker for 2 min. Antigen retrieval for p62 was achieved in citrate buffer, pH 6.0, in a microwave. Following antigen retrieval, sections were incubated in 2.5% normal horse serum for 20 min to block for any non-specific binding sites.

Sections were immunostained with diluted primary antibodies (Table 1) for 60 min followed by incubation with ImmPRESS reagent (Vector Laboratories Ltd) for 30 min. Colour development was achieved in Vector Impact BAD reagent for Beclin 1, Bcl-2, and p62. The immunostained sections were counterstained with haematoxylin, dehydrated, cleared and mounted. All the washings were performed with Tris buffer saline/0.05% Tween-20 (TBST).

Staining without the primary antibody was performed as a negative control. Staining of the small lymphocytes in the mantle zone of the lymphoid follicle was used as an internal positive control for Bcl-2 and staining at the germinal centre was used as internal positive control for both Beclin 1 and p62.

Table 1: Primary antibodies, clone, dilutions and source.

| Antibody | Dilution | Clone | Source |
|----------|----------|-------|--------|
| Beclin 1 | 1:400    | Rabbit monoclonal (EPR1733Y) | Abcam, UK |
| Bcl-2    | 1:200    | Mouse monoclonal (3.1) | Leica BIOSYSTEMS, UK |
| p62      | 1:600    | Mouse monoclonal (SQSTM1) | Abcam, UK |

Semi-quantitative assessment of immunohistochemical staining

Expression status was evaluated by scoring both intensity and proportion of tumour cells stained for Beclin 1, Bcl-2 and p62. The intensity of the staining was scored as 0 (no staining), 1+ (mild staining), 2+ (moderate staining) or 3+ (strong staining). The positive tumour cells were recorded after examining the entire tissue section at 100x magnification. The proportions of tumour cells stained were scored as follows; 0 for no staining, 1 for <25% of tumour stained, 2 for 25-50%, 3 for 51-75% and 4 for 76-100%.

The sum of the scores for intensity and proportion of tumour stained were taken as the measure of the positivity and described as the following: 0 for negative, 1-2 for low expression, 3-4 for moderate expression and 5-6 for high expression. Considering the distribution of nuclear staining in the tumour cells, positive nuclear expression for p62 was defined as the expression of each protein in >20% of tumour cells. To assess for reproducibility, all the slides were blindly rescored and tested for systematic errors using Wilcoxon test.

Results

This study examined the expression patterns of autophagy related proteins in adenoma and adenocarcinoma of colorectal tissues. H&E staining was performed on 14 colorectal tissues, which were histologically grouped into colorectal adenoma (7) and colorectal adenocarcinoma, Duke Stage B (7). Immunostaining for Beclin1, Bcl-2
and p62 was performed and the expression levels, using a Quick Score scale, were quantified.

The levels of expression of Beclin 1, Bcl-2 and p62 in both colorectal adenoma and adenocarcinoma cases are shown (Table 2).

**Cytoplasmic expression of Beclin 1 in colorectal adenomas and adenocarcinomas**

Beclin 1 expression pattern in colorectal adenoma and adenocarcinoma was investigated using immunohistochemistry. Of the 7 tubular adenoma cases, 1 case showed a moderate Beclin 1 expression whereas 6 showed a high expression level of Beclin 1 (Table 2). There were 3 cases each for moderate and high expression levels in adenocarcinoma of colorectal origin (Table 2). In both adenoma and adenocarcinoma, the Beclin 1 was localised in the cytoplasm (Figure 1). There was no Beclin 1 expression in the control case in which the primary antibody was omitted (Figure 1A).

The normal colonic cells showed moderate cytoplasmic staining at the epithelial layer (Figure 1B) but a strong cytoplasmic expression was observed in the more dysplastic glands of the tubular adenomas (Figure 1C). Additional internal positive control was achieved by the staining of the germinal centre of lymphoid follicle. There was a moderate even distribution of Beclin-1 in tubular adenoma (Figure 1D).

Higher expressions of Beclin 1 were observed in colorectal adenocarcinomas (Figures 1E and 1F). The average expression levels, as revealed by Quick score scale; indicated tubular adenoma had higher expression level than that of adenocarcinoma.

| Protein Expression | Tumour phenotype, number |
|--------------------|--------------------------|
|                    | Tubular Adenoma | Duke B Adenocarcinoma |
| Cytoplasmic Beclin 1 |              |                          |
| Negative           | 0             | 1                         |
| Low                | 0             | 0                         |
| Moderate           | 1             | 3                         |
| High               | 6             | 3                         |
| Total              | 7             | 7                         |
| Cytoplasmic Bcl-2  |              |                          |
| Negative           | 0             | 5                         |
| Low                | 0             | 2                         |
| Moderate           | 5             | 0                         |
| High               | 2             | 0                         |
| Total              | 12            | 7                         |
| Cytoplasmic p62    |              |                          |
| Negative           | 0             | 0                         |
| Low                | 0             | 0                         |
| Moderate           | 3             | 0                         |
| High               | 4             | 7                         |
| Total              | 7             | 7                         |
| Nuclear p62        |              |                          |
| Negative           | 7             | 2                         |
| Positive           | 0             | 5                         |
| Total              | 7             | 7                         |

Table 2: Expression patterns of autophagy related proteins in colorectal tumours.

**Cytoplasmic Bcl-2 expression in colorectal cancers**

In order to understand the role of Bcl-2 in the pathogenesis of colorectal tubular adenoma and adenocarcinoma, FFPE sections were immunostained for Bcl-2. Five and two cases, respectively, showed moderate and high Bcl-2 expression levels in adenoma. In contrast, there were 2 cases of adenocarcinoma that showed low expression levels of Bcl-2; and 5 adenocarcinoma cases showed no Bcl-2 expression (Table 2). Negative control was achieved by the omission of the primary antibody (Figure 2A). There was a moderate expression of Bcl-2 at the base of the crypts of normal colonic mucosa cells, adjacent to tumour cells (Figure 2B). Small lymphocytes around the mantle zone of the lymphoid follicles and infiltrating lymphocytes within the tumour stroma were stained intensely for Bcl-2 (Figure 2C). Moderate to high expressions of Bcl-2 were observed in all tubular adenoma cases but for the adenocarcinoma cases, negative to low expressions were observed (Figure 2E and 2F). The expression of Bcl-2 in both colorectal adenoma and adenocarcinoma was localised in the cytoplasm. As revealed by the Quick Score scale, there was a significant reduction of Bcl-2 expression in colorectal adenocarcinoma than tubular adenoma.
Nuclear and cytoplasmic expression of p\textsuperscript{62} in colorectal cancers

Several roles are associated with p\textsuperscript{62} in cancer biology. In this study we examined the localization and expression levels of p\textsuperscript{62} in colorectal adenoma and adenocarcinoma. All the 7 colorectal adenocarcinoma cases showed a high p\textsuperscript{62} cytoplasmic expression whilst 3 and 4 adenoma cases showed moderate and high p\textsuperscript{62} cytoplasmic expression respectively. A negative control was performed by omission of the primary antibody in the staining procedure, and it showed no staining for p\textsuperscript{62} (Figure 3A). Normal colonic mucosa showed moderate staining of the epithelial cells for p\textsuperscript{62} (Figure 3B). Moderate p\textsuperscript{62} expression was observed only in the cytoplasm of tubular adenoma (Figure 3C). Unlike tubular adenoma, which showed cytoplasmic p\textsuperscript{62} expression, there was p\textsuperscript{62} expression in both the cytoplasm and the nucleus of the adenocarcinomas cases. A high p\textsuperscript{62} nuclear expression with moderate p\textsuperscript{62} cytoplasmic expression was observed in colorectal adenocarcinoma (Figure 3D). There were high p\textsuperscript{62} cytoplasmic expression and low p\textsuperscript{62} expression in the nucleus of colorectal adenocarcinoma (Figure 3E). In contrast, Figure 3F shows moderate to high p\textsuperscript{62} nuclear expression. In colorectal tubular adenoma, p\textsuperscript{62} was mainly and exclusively cytoplasmic compared to colorectal adenocarcinoma, in which p\textsuperscript{62} was localised in both the cytoplasm and the nucleus. The average p\textsuperscript{62} cytoplasmic expression level in colorectal adenocarcinoma, as indicated in Figure 4, was higher than in tubular adenoma.

Discussion

Colorectal adenomas are lesions that can potentially be transformed to the malignant form, however, it has been estimated that about 95% of colorectal adenocarcinomas are known to arise from these pre-existing lesions [16]. Transformation of normal cells to benign...
adenoma and to malignant colorectal adenocarcinoma involves several cellular processes, including autophagy.

Autophagic processes are essentially involved not only in normal physiological aspect of cells but also in cancer pathogenesis. Studies of several autophagy related biomarkers including Beclin 1, Bcl-2 and p62 have shown that deregulation of autophagy may play a role in colorectal adenoma-adenocarcinoma transformation sequence.

In this study, the expression levels of Beclin 1 in normal colon tissue showed a moderate expression of Beclin 1 at the mucosa region. This is consistent with other studies [6,17], indicating that the presence of Beclin 1 as normal component of the normal colonic mucosa and might be involved in initiating basal level of autophagy necessary for cellular homeostasis. Furthermore, we observed that the cytoplasmic Beclin 1 expression ranged from moderate to high expression in tubular adenomas, which was in line with studies by Giatromanolaki et al., where high-grade dysplasia tubular adenomas with or without morphological features of malignant transformation were assessed for Beclin 1 expression. It was speculated that up-regulation of Beclin 1 may occur during the transformation of colorectal adenomas to invasive cancer.

In colorectal adenocarcinomas, Beclin 1 expression levels were from moderate to high. It is considered that Beclin 1 overexpression frequently occurs, but loss or inactivation of Beclin 1 may not occur in tumours of the gastrointestinal tract [4], which is in contrast with the findings [18], where the expression of Beclin 1 is lost in breast and human lung cancer, respectively. However, a low level expression of Beclin 1 was reported in some colorectal cancer subtypes [19]. The possible explanation to these different patterns of expression is that Beclin 1 expression is dependent on the tumour types and can be influenced by other environmental conditions.

Even though we report here of cytoplasmic Beclin 1 expression, others have reported of nuclear localisation of Beclin 1 in some colorectal cancer subtypes [6]. It will be interesting to establish the clinical significance of Beclin 1 localisation in colorectal cancers and its interconnectivity with chemotherapy and prognosis.

The expression pattern of another prominent autophagy protein Bcl-2 was examined in colorectal adenoma and adenocarcinoma. The expression of Bcl-2 was observed to be localised in the epithelial cells at the base of the crypts where the stem cells and the proliferating cells reside, which is consistent with Han et al on Bcl-2 expression in colorectal cancers. In contrast to the expression of Beclin 1, Bcl-2 expression was absent in most of the adenocarcinoma cases but in only 2 cases that showed low expression. However, in tubular adenomas, Bcl-2 expression was mostly moderate, similar to Goussia et al who reported that higher proportion of adenomas (30.8%) than carcinomas (16.7%) expressed Bcl-2. The loss of Bcl-2 expression was also supported by studies from Poincloux et al and Han et al. They reported both negative and low expression for Bcl-2 in colorectal cancers. Considering the moderate expression of Bcl-2 in tubular adenoma cases and the subsequent loss in adenocarcinoma cases, Bcl-2 expression may be an early event in tumour development, confirming the general hypothesis that Bcl-2 might be up-regulated in early stages of the development of colorectal adenomas but is lost in invasive cancer.

A limited number of studies have assessed the intracellular localization of p62 in colorectal tumours. In this study, we observed a moderate cytoplasmic p62 expression pattern in colorectal adenomas, in contrast with a moderate to high nuclear and cytoplasmic expression in colorectal adenocarcinomas. A previous study demonstrated a similar finding of both cytoplasmic and nuclear localisation of p62 but in breast cancers [20]. However, studies by Qian et al. and Su et al. reported only cytoplasmic expression of p62 in colorectal adenocarcinomas. For the first time, we demonstrate the expression of p62 in both cytoplasmic and nuclear of colorectal adenocarcinomas. Since p62 overexpression has been linked with poor prognosis in breast and human lung cancer [20,21]. It may be postulated that nuclear p62 expression signals a poor prognosis of colorectal cancer development. Nonetheless, more research work needs to be done to understand the significance of p62 role in nucleocytoplasmic transport in cell proliferation, invasion and chemotheraphy response in colorectal adenocarcinomas [22].

Conclusion

Beclin 1 and p62 expression levels were increased in both colorectal tubular adenomas and adenocarcinomas. This pattern of expression suggests an up-regulation of Beclin 1 and p62 may occur in the development of colorectal adenomas to invasive cancer. Additionally, the expression pattern of Bcl-2 was low in adenocarcinomas and moderately expressed in tubular adenomas, suggesting that Bcl-2 might also contribute to adenoma-adenocarcinoma transition sequence. We provide evidence for the first time of the loss of nuclear expression for p62 in colorectal adenomas but increase expression in colorectal adenocarcinomas.

Ethics Approval

Ethical approval from the Research Ethics sub-Committee of the University of Westminster, in compliance to the Human Tissue Act (HTA) was given for this study.

Consent for Publication

Archival human tissue samples were assigned anonymous identifiers using SNOMED coding system, thus this manuscript does not contain any personal data of the human histological samples that were used in this study.

Availability of Data and Materials

All the data (mainly from images of H&E and immunohistochemical staining) from which this study's conclusion was made have been presented in this manuscript.

Competing Interest

We confirm that, regarding this paper; there is no conflict of interest among the authors. Further the work is original and has not been accepted for publication nor is concurrently under investigation elsewhere and will not be published elsewhere without the permission of the Editor.

Authors' Contributions

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