Autophagy Flux Correlates with Upregulation of AKT-1 in RAS Mutated Colon Cancer Cells

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Abstract

Background: The AKT/PKB (protein kinase B) kinase is the main regulator of autophagy in mammalian cells, which consists of three isoforms, including AKT-1, AKT-2, and AKT-3. Rat sarcoma viral oncogene homolog (RAS), known as the most frequently mutated oncogene in colorectal cancers, is one of the major activators of AKT signaling. However, the relationship between AKT isoforms expression and autophagy level in RAS-driven cancer cells has not been fully investigated.

Method: In this experimental in vitro study, RAS mutated colon cancer cell lines (HCT116, SW480, and LS180) and HT29 cells, which are the wild type of RAS, were cultured and real-time polymerase chain reaction (RT-PCR) was utilized to determine the mRNA level of AKT-1, AKT-2, and autophagy markers, including microtubule-associated protein 1 light chain-3B (LC3B) and p62/sequestosome-1 (p62). In addition, Western blotting was performed to assess the protein expression of p62 and LC3B lipidation.

Results: We found that RAS mutated colon cancer cells up-regulate basal autophagy. Moreover, highly expressed AKT-1 was observed in RAS mutated colon cancer cells. However, no significant differences were found in AKT-2 expression between RAS-driven cells and HT29 cells.

Conclusion: Our obtained data suggested that RAS-driven colon cancer cells regulated the autophagy machinery, possibly, through the upregulation of AKT-1 isoform.

Keywords: Colorectal neoplasms, RAS, Oncogenes, Autophagy, AKT/PKB kinase
Introduction

Cancer is believed to be the main cause of mortality worldwide and the global fatality due to cancer is on a rising trend.\(^1\) Colorectal cancer (CRC) is the third commonly diagnosed malignancy accounting for virtually one million deaths annually.\(^2\) Activation of the rat sarcoma viral oncogene homolog (RAS) is shown to be involved in the pathogenesis of 20 to 30% of human cancers, and is overexpressed in more than 53% of the CRC cases.\(^3,4\) Thus, RAS oncogene is considered as a potential target in cancer therapy; however, despite continuous efforts, it has not been possible to directly target the RAS oncogene. Alternatively, researchers have shown an increased interest in targeting the downstream RAS signalling pathways as a way to foster the development of new antitumour drugs.\(^5\)

RAS-driven tumours are usually associated with modulated levels of autophagy.\(^6\) As a tightly programmed mechanism, autophagy plays a crucial role in the cell homeostasis machinery and is found to be involved in cancer formation and progression.\(^7\) Microtubule-associated protein 1A/1B-light chain 3 (LC3B) is a ubiquitously expressed protein with a fundamental role in autophagy machinery.\(^8\) The modified LC3B, conjugated to phosphatidylethanolamine, is referred to as LC3B-II and is involved in autophagosome membrane expansion and fusion events. During autophagy, LC3B-II is degraded in the lysosomes, and the lysosomal turnover of LC3B-II reflects the induction of autophagy flux.\(^9\) Sequestosome 1 (p62/SQSTM1) is an adaptor protein involved in selective recognition of autophagy substrates. This protein also facilitates the engulfment of the substrates into autophagosomes through interacting with ubiquitin-like modifiers.\(^10\) On the other hand, p62 could be accumulated once autophagy flux is impaired. Given that LC3B and p62 proteins play a pivotal role in autophagy mechanism, these two proteins are employed as excellent markers for the detection of autophagy flux in the cells.\(^11\)

Depending on several factors, such as the cell type and context, autophagy could act as an anti- or pro-tumorigenic pathway.\(^12\) The regulatory effect of RAS oncogene on autophagy is described as multifaceted crosstalk, which is mediated through different signalling pathways. For instance, it has been shown that RAS induces autophagy through Raf-1/MEK1/2/ERK pathway; however, the inhibitory effects are exerted through PI3K/AKT/mTOR1 pathway.\(^13\) The existing crosstalk between mutated RAS and autophagy indicates that in most RAS-mutated tumours, activated autophagy flux contributes to tumour cells survival, allowing them to overcome the treatment.\(^14\) Therefore, identifying molecular pathways that regulate autophagy in RAS-mutated cancer cells could be therapeutically beneficial.

AKT pathway is a well-known mechanism that negatively regulates autophagy processes.\(^15\) AKT or serine/threonine protein kinase B is involved in a wide range of important cellular pathways, such as energy metabolism,\(^16\) cell proliferation and migration,\(^17\) apoptosis, and cell survival.\(^18\) Additionally, AKT is known as a highly activated effector in the oncogenic landscape.\(^19\) Functionally distinctive but structurally similar, 80% homology, AKT family consists of three isoforms, named as AKT-1, AKT-2, and AKT-3.\(^20\) Even though a lot of research has been carried out on autophagy regulation in RAS mutated cancer cells, far too little attention has been paid to the relationship between AKT isoforms expression and autophagy level in RAS-driven cancer cells.

The present study, therefore, was conducted to investigate the basal autophagy level and determine the basal expression of two AKT isoforms, including AKT-1 and AKT-2, in three RAS mutated colon cancer cell lines (HCT116, SW480, and LS180) and HT29 cell line, which is a wild type for RAS.

Materials and Methods

Cell culture

In the current experimental in vitro study, human colon carcinoma cell lines, HCT116, LS180, SW480, and HT29 were obtained from...
National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™; Cat #: 16000044) and 1% penicillin/streptomycin (Gibco, USA) at 37°C with 5% CO2 in a humidified atmosphere.

The Ethics Committee of Shiraz University of Medical Sciences approved the study (Ethics code: IR.SUMS.REC.1397.306).

Quantitative real-time polymerase chain reaction (PCR)

The total RNA was extracted from the cultured cells, using the BIOZOL-RNA, RNA extraction reagent (BSC51M1, Zhejiang, China). The Fermentase cDNA Synthesis Kit (USA) was utilized for synthesizing single-stranded cDNA from the total RNA. Real-time PCR was done on 7500 Real-time PCR system (Applied Biosystems, USA), using SYBR Green. The relative amounts of mRNA of the AKT-1, AKT-2, p62, and LC3B genes were normalized to the endogenous control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and calculated with the 2-ΔΔCt formula. Table 1 represents the primer pairs used for quantitative real-time PCR.

Western blot analysis

Western blotting was performed using the following antibodies for the analysis: rabbit polyclonal anti-LC3B antibody (L7543, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-p62 antibody (88588S, Cell Signaling Technology, Beverly, MA, USA), and mouse monoclonal anti-GAPDH antibody (sc-47724, Santa Cruz Biotechnology, California, USA). The cells were lysed in NP40 buffer [40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors and phosphatase inhibitors. The protein concentrations were determined with the BCA protein assay kit (Novagen, San Diego CA, USA). Proteins (20 µg of lysate) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL). After blocking with 5% non-fat dry milk in Tris-buffered saline for 1h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: p62 (diluted 1:1000), LC3B (diluted 1:2000), and GAPDH (diluted 1:2000). The membranes were incubated with appropriate antirabbit or antimouse monoclonal IgG conjugated to horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) (DAKO; Santa Cruz Biotechnology) for 1 h at room temperature. The proteins were detected via incubation with ECL reagent. All the proteins and proteins of interest were acquired and visualized employing ChemiDoc TM MP System.

Table 1. The primers sequence used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers sequence</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5′-CGACCACTTTGTCAAGCTCA-3′</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>R: 5′-AGGGGTCTACATGGCAACTG-3′</td>
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</tr>
<tr>
<td>AKT-1</td>
<td>F: 5′-TTGTATGTATTAGTTGGTTCA-3′</td>
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<td></td>
<td>R: 5′-AAGTGCTACCGTGAGAG-3′</td>
<td></td>
</tr>
<tr>
<td>AKT-2</td>
<td>F: 5′-CCCTAAACAACTCCTAGCA-3′</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GCAGGCAGCGTATGACAAA-3′</td>
<td></td>
</tr>
<tr>
<td>p62</td>
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<tr>
<td></td>
<td>R: 5′-AGTGGAGGACTTTTTTGTTGG-3′</td>
<td></td>
</tr>
<tr>
<td>LC3B</td>
<td>F: 5′-AACGGGTCTTGAGAAAAC-3′</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>R: 5′-AGTGGAGGACTTTTTTGTTGG-3′</td>
<td></td>
</tr>
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GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; AKT: Also called protein kinase B; p62: Also known as sequestosome-1; LC3B: Microtubule-associated protein 1 light chain-3B; bp: Base pair; F: Forward; R: Reverse
analysis, the bands were selected and quantified using Image Lab software (BioRad, Cressier, Switzerland) and the data were transformed and normalized to GAPDH.

Statistical analysis

We determined the statistical significance of the experiments using GraphPad Prism, version 6 (GraphPad Software). The comparisons were made with one-way ANOVA followed by Dunnett test. \( P < 0.05 \) was considered to be statistically significant.

Results

Up-regulated basal autophagy in RAS-mutated colon cancer cells

Real-time PCR was performed in order to determine the mRNA levels of LC3B and p62, as well-known autophagy markers in three RAS mutant colon cancer cell lines (HCT116, SW480, and LS180) versus HT29 cells, which is the wild type for RAS. All RAS mutant colon cancer cell lines significantly over-expressed the LC3B and p62 mRNA compared with HT29 cells (Figure 1 A, B). Subsequently, we performed western blot analysis to assess the protein level of p62 and proteolytic processing of endogenous LC3B-I to LC3B-II. As shown in figures 2 A and B, the LC3B lipidation was significantly higher in the RAS mutated cells. However, these cell lines presented a significant decrease in the protein level of p62 compared with HT29 cells. Overall, our data indicated that RAS mutated CRC cells displayed functional basal autophagy.

High expression of AKT-1 in RAS mutated colon cancer cell lines

AKT is the main regulator of autophagy in mammalian cells which consist of three isoforms. To find out which isoform might play an important role in autophagy regulation in RAS mutated colon cancer lines, we performed real-time PCR to assess the gene expression of AKT-1 and AKT-2 isoforms. All RAS mutant colon cancer cell lines significantly over-expressed the AKT-1 mRNA compared to HT29 cells (Figure 3A). However, neither of RAS mutated cell lines and HT29 cells showed apparent differences in AKT-2 mRNA levels (Figure 3B).

Discussion

In the current study, we revealed that RAS-mutated colon cancer cell lines increased autophagy at the basal level, which is parallel
with the upregulation of AKT-1. Herein, it was concluded that basal autophagy flux, which is associated with upregulation of AKT-1, sustains proliferation in RAS mutated CRC cells.

In this study, we analyzed the expression of mRNA and protein level of LC3B-II, as a well-known autophagy marker, using real-time PCR and western blotting techniques in RAS-driven colon cancer cells, including HCT116, LS180, and SW480. The results were compared to HT29 cells, which is the wild type for RAS. We found that RAS-mutated colon cancer cells highly express LC3B-II both at mRNA and protein level compared with HT29 cells. We also depicted that the mRNA level of p62, as another marker of autophagy, in RAS-mutated cells was significantly higher than that of HT29 cells. Conversely, the protein level of p62 was significantly lower in RAS-mutated cells compared with HT29 cells. During autophagy, cytoplasmic components were targeted, isolated, and finally delivered to the lysosomes by double layer membranes known as autophagosome. It has been indicated that the level of autophagosomes formation is directly associated with the level of a membrane-bound protein named LC3B-II. Additionally, the balance between the autophagosome’s formation and degradation could be indicated by the level of LC3B-II and p62. LC3B and p62 are the proteins fused into the autophagosomes and are

Figure 2. Expression of LC3B and p62 at the level of protein in colon cancer cell lines. (A) The amounts of LC3B and p62 proteins were determined employing Western blot. GAPDH was used as a loading control. (B, C) Density analysis of the Western blot band of LC3B-II and p62. The data are expressed as the mean ± S.D. from three independent experiments. One-way ANOVA with Dunnett’s multiple comparison test was used to analyze the statistical significance in B and C; **p < 0.01; ***p < 0.001; ****p < 0.0001. LC3B: microtubule-associated protein 1 light chain-3B; p62: also known as sequestosome-1.
S.D.: Standard deviation; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
degraded during the autophagosome’s degradation.\textsuperscript{21} Inhibition of autophagy using pharmacological inhibitors leads to accumulation of these proteins, which implies the basal level of autophagy.\textsuperscript{23} Although the accumulation of p62 protein is considered as a marker of autophagy flux impairment, its accumulation also happens in a correctly activated autophagy flux.\textsuperscript{24} This is because of the induced levels of transcription and translation of p62 by autophagy stimulus.\textsuperscript{24} Hypothetically, it seems that the increase in the expression level of p62 is a compensatory mechanism for the loss of the p62 protein during lysosomal degradation. All in all, these findings suggested that the autophagy machinery is functionally activated in RAS-mutated CRC cells. Our findings are in agreement with Guo’s (2011) results which showed that RAS-driven cancer cell lines upregulated the basal autophagy to maintain mitochondrial metabolism that is needed for their robust growth.\textsuperscript{6} Kim et al. demonstrated that oncogenic K-RAS and autophagy were tightly involved in malignant transformation, in a way that autophagy is essential for oncogenic K-RAS-induced malignant cell transformation.\textsuperscript{25} In another study, Alves et al. reported that in RAS-mutated colon cancers, starvation induced autophagy, which contributed to the cell survival and growth.\textsuperscript{26} They also showed that RAS-induced autophagy proceeds via activation of the MEK/ERK pathway. These findings indicate that RAS-mutated cancer cells are dependent on increased basal autophagy levels for normal growth and survival.

Increased level of autophagy in RAS-driven tumor cells is an adaptive response through sustaining mitochondrial homeostasis and recycling materials to provide the proper environment needed for rapid cell proliferation.\textsuperscript{6,13} Moreover, they also activate signaling pathways, such as AKT pathway, to keep autophagy machinery at the basal level.\textsuperscript{13,27} The AKT pathway acts as a negative regulator of autophagy and is involved in oncogenic signals.\textsuperscript{28} Activated AKT pathway inhibits the aberrant activation of autophagy flux in the cells, which could lead to autophagic cell death.\textsuperscript{29} Activation of AKT is frequently observed in RAS-driven tumours.\textsuperscript{30} Although all the three AKT members, AKT-1, AKT-2, and AKT-3, are expressed in the colon cancer cells, AKT-1 and AKT-2 are dominant.\textsuperscript{31} Based on the results of AKT isoforms mRNA expression analysis, we observed a significant increase in the AKT-1 mRNA level in RAS-
mutated CRC cells compared with HT29 cells. However, we did not observe any differences in AKT-2 expression between RAS-driven cells and HT29 cells. Our result seems to be consistent with that of previous studies, indicating that RAS-induced oncogenic signaling pathways is mainly mediated through AKT-1.32 Similarly, Hollander et al. demonstrated that mutant K-RAS leads to lung tumor initiation and progression through the upregulation of AKT-1.33 Furthermore, Chen et al. revealed that AKT-1 significantly increased the proliferation rates and contributed to colony formation in the RAS mutant HCT116 CRC cell line.34 All in all, these results imply that AKT-1 is the most important isoform of AKT, which transmits oncogenic signals of RAS.

The differences in the expression level and involvement among different tumors indicate that AKT-1 and AKT-2 possibly exert their effects via different signaling pathways. AKT-2 is mainly involved in insulin-related pathways, and it has been reported that selective AKT-1 inhibition prevents cytotoxic consequences of inhibited insulin signaling.32,35 A number of studies have demonstrated that AKT-1 regulated cell proliferation, survival, and tumor formation.36-38 On the other hand, AKT-1 acts as an inhibitor of chaperone-mediated autophagy (CMA). Yang et al. found that AKT-1 inhibited the process of autophagy by down-regulation of ultraviolet irradiation resistance-associated gene (UVRAG), which is a key factor in autophagosome formation and autophagosome maturation.39 Moreover, AKT-1 has been reported to be the most important isoform for regulating autophagy in the cardiomyocyte cells.40 Taken together, these findings demonstrate that AKT-1 plays an important role in regulating autophagy in different cells.

Certain limitations should be noted in current study. Primarily, AKT1 and AKT2 were not detected at the level of proteins and no inhibitors were used to evaluate the exact correlation between these two pathways. However, in view of these findings, it is probable, therefore, that RAS-driven colon cancer cells modulate autophagy machinery through the upregulation of AKT-1 isoform. To the best of our knowledge, this is the first study to report a link between AKT-1 isoform and autophagy in RAS-mutated CRC cells. However, further studies are needed to investigate the possible role of AKT-1 isoform on autophagy machinery in RAS mutated cells.

**Conclusion**

In conclusion, we shed light on the fact that RAS-mutated colon cancer cell lines increased autophagy at the basal level, which is parallel with the upregulation of AKT-1. Hence, inhibition of AKT-1 could be regarded as a novel target to be utilized in the treatment of RAS-driven tumor cells, such as colon cancer cells which are dependent on autophagy for survival.

**Acknowledgements**

The present article was extracted from the thesis by Hamid Behrouj and was financially supported by Shiraz University of Medical Sciences, (Grant No. 96-15414). This project was also supported by the office of the President and Vice-president for Science and Technology, Iran National Science Foundation (INSF) (Grant No. 96015616).

**Conflict of Interest**

None declared.

**References**


27. Furuta S, Hidaka E, Ogata A, Yokota S, Kamata T. Ras is involved in the negative control of autophagy through the class I PI3-kinase. *Oncogene.* 2004;23(22):3898-904. doi:10.1038/sj.onc.1207539.


32. Kim IA, Bae SS, Fernandes A, Wu J, Muschel RJ, McKenna WG, et al. Selective inhibition of Ras, phoshoinositide 3 kinase, and Akt isoforms increases the...
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