

## Chitinase-3 Like-Protein-1 (YKL-40) Promotes Anorectal Mucosal Melanoma Progression via the PI3K-AKT Signaling Pathway

Li C, Wang Y, Ren X and Li Y\*

Department of Colorectal and Anal Surgery, Shanxi Provincial People's Hospital, Shanxi, China

### \*Corresponding author:

Yaoping Li,  
Department of Colorectal and Anal Surgery,  
Shanxi Provincial People's Hospital, Shanxi, China

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### Keywords:

YKL-40; PI3K-AKT; Anorectal mucosal melanoma

## 1. Abstract

**1.1. Objective:** To investigate whether chitinase-3-like protein-1 (YKL-40) promotes the development of anorectal mucosal melanoma through the PI3K-AKT signaling pathway.

**1.2. Methods:** Perianal cells from healthy volunteers and melanoma cells from patients with early, middle and advanced anorectal melanoma were obtained. Western blotting was performed to detect the protein expression of PI3K, AKT, and the downstream proteins mTOR, p-mTOR, ERK, and p-ERK, respectively. Subsequently, we constructed knockout and overexpression of YKL-40 melanoma cell lines, then used western blot assay to test for YKL-40, PI3K and AKT protein expression.

**1.3. Results:** A significant increase in the expression of PI3K, AKT, and the downstream proteins mTOR, p-mTOR, ERK, and p-ERK was observed in melanoma cells, and the expression of these proteins increased with the development of melanoma. After YKL-40 was knocked out, PI3K and AKT expression decreased in melanoma cells in patients with advanced melanoma. On the contrary, PI3K and AKT protein expression increased significantly after YKL-40 overexpression.

**1.4. Conclusion:** There is a positive correlation between the expression levels of PI3K, AKT, mTOR, p-mTOR, ERK, and p-ERK and the stage of tumor development. The PI3K-AKT signaling pathway promotes the progress of anorectal mucosal melanoma. Chitinase-3-like protein-1 (YKL-40) regulates the progression of anorectal mucosal melanoma through the PI3K-AKT signaling pathway.

## 2. Introduction

Melanoma is one of the most aggressive diseases and has a very poor overall survival rate [1,2]. Despite significant progress in treating malignant melanoma, particularly with new targeted therapies and improved immunotherapy, there is still a low chance of survival in patients with metastatic melanoma [3]. It is therefore crucial to investigate the mechanisms participated in the melanoma development.

YKL-40, also known as Chitinase-3-Like-1 and Human Cartilaginous Glycoprotein, belongs to the Chitinase-3 family [4,5]. YKL-40 is named after the one letter code of its three N-terminal amino acids and molecular weight (40 kDa) [6]. This glycoprotein is primarily secreted by tumor cells and tumor associated macrophages, arthritic chondrocytes, activated neutrophils, macrophages during a late differentiation state [7-9], differentiated vascular smooth muscle cells [10], and fibroblast-like synovial cells [11]. While its exact function is not fully understood, it is reported to be participate in multifarious pathophysiology including inflammation, cell proliferation and tissue remodeling [12-14]. YKL-40 is a connective tissue cell growth factor, and it has been shown to have synergistic effects with insulin-like growth factor 1 (IGF-1) [15,16]. YKL-40 activates a cascade of MAP kinases (MAP kinases) and PI-3K signaling in fibroblasts, leading to extracellular signaling regulating kinases (ERK) -1/2 MAP kinases and AKT phosphorylation, which are related to the regulation of mitogenesis [17,18]. In addition, it inhibited cell migration through inhibition of apoptosis and cell cycle progression. These findings indicate that YKL-40

participates in tissue remodeling and anti-apoptotic.

High concentrations of YKL-40 in serum can lead to all kinds of diseases, including cardiovascular disease, diabetes [19], cancer, and idiopathic pulmonary fibrosis [20,21]. Several studies have shown that YKL-40 is also significantly associated with cancer progression, such as incidence of metastasis, reduced relapse-free survival, and reduced overall survival [22]. Increases in serum YKL-40, have been demonstrated to be an independent prognostic factor for low survival in colorectal, ovarian, renal, and gastric cancers [23,24].

We know that the PI3K-AKT signaling pathway is also extensively activated in tumorigenesis, especially the high frequency mutation of PIK3CA, PIK3R1, AKT and other genes, which is tightly linked to tumorigenesis, development and drug resistance [25,26]. Research suggests that high levels of YKL-40 in human connective tissue may accelerate cell proliferation through PI3K-AKT pathway. And YKL-40 can undergo a PI3K-AKT cascade reaction leading to phosphorylation of AKT and thus activating this pathway in glioma cells, suggesting that YKL-40 is closely linked to the PI3K-AKT pathway and influences the biologic behavior of tumour cells [27].

However, the expression of YKL-40, PI3K and AKT in anorectal mucosal melanoma and their interrelationships remain unclear. Therefore, we attempted to reveal the relationship between YKL-40 and the PI3K-AKT pathway during the progression of anorectal mucosal melanoma. Our findings showed that PI3K and AKT expression increased in melanoma cells compared to normal cells, and their expression gradually increased as melanoma progressed. After the YKL-40 knockout, the expression of PI3K and AKT decreased in advanced melanoma cells. Overexpression of YKL-40 had opposite effects. YKL-40 regulates the development and progression of anorectal mucosal melanoma through the PI3K-AKT signaling pathway.

### 3. Materials and Methods

#### 3.1. Cell Culture

We take perianal cells from healthy volunteers and melanoma cells from patients with early, intermediate, and advanced anorectal melanoma. Written informed consent was obtained from each patient, and the project received approval from the local ethics committee. Cells were cultured in medium (Hyclone, Cat.No.SH30809.18) in a 5% CO<sub>2</sub> atmosphere at 37. The medium was added with 10% fetal bovine serum (Excell Bio, China) and 1% mixture of penicillin and streptomycin (MACKLIN, China). The medium was changed every two days. The cells were passaged at 80% of the confluence.

#### 3.2. Cell Transfection

The PLKO.1 plasmid vector was used to construct cell lines of advanced melanoma patients with knockdown YKL-40, and the pLVX plasmid vector was used to construct cell lines of advanced melanoma patients with overexpression of YKL-40. These were

synthesized and packed in lentivirus by Shanghai Sangon Biotech. Cells from advanced melanoma patients were seeded in 6-well plates at a density of  $1 \times 10^6$ /mL. When cell fusion reaches 80%, the optimal vector lipid complex and overexpressed plasmid vector were added to the cells using transfection reagents according to the manufacturer's guidelines. After 48 hours, the transfer efficiency was observed with inverted fluorescence microscope, and the cells were collected for further analysis.

#### 3.3. Western Blot Analysis

The total protein sample was extracted in accordance with the kit specification (Beyotime, China), followed by quantification using the BCA Protein Assay (NCM Biotech, China). Cell protein lysates was separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto PVDF (Sigma-aldrich, USA) membranes. The membranes were blocked with 5% skimmed milk powder for 2 h at room temperature. The primary antibody was then applied at 4°C overnight with gentle shaking. After washing the membranes three times, the HRP-conjugated second antibody was used at room temperature for 2 hours with gentle shaking. An ECL assay kit (NCM Biotech, China) was used to assess the protein levels. An exposure instrument (JP-K6000 chemiluminescence instrument) was used to visualize protein expression. Western blot data were quantified with Gel-pro software. Primary antibodies included GAPDH (Bioss, bsm-33033M), PI3 Kinase p110 Alpha (proteintech, 67071-1-Ig), AKT (proteintech, 60203-2-Ig), mTOR (proteintech, 66888-1-Ig), Phospho-mTOR (Ser2448) (p-mTOR, proteintech, 67778-1-Ig), ERK1 (Abcam, ab32537), Anti-ERK1 (phospho T202) + ERK2 (phospho T185) antibody (p-Erk1/2, Abcam, ab201015), YKL-40 (proteintech, 12036-1-AP). Secondary antibodies included horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) (Bioss, bs-0295G-HRP), horseradish peroxidase-labeled goat anti-mouse IgG (Bioss, bs-0296G-HRP).

#### 3.4. Statistical Analysis

Graphpad Prism 9 (Version 9.4.0) was used for all analyses. Data were recorded as mean  $\pm$  SD. The statistical significance between groups was determined by T-Test. ANOVA was used for comparison between multiple groups.  $P < 0.05$  was considered significant.

### 4. Results

#### 4.1. PI3K and AKT expression were upregulated in melanoma cells

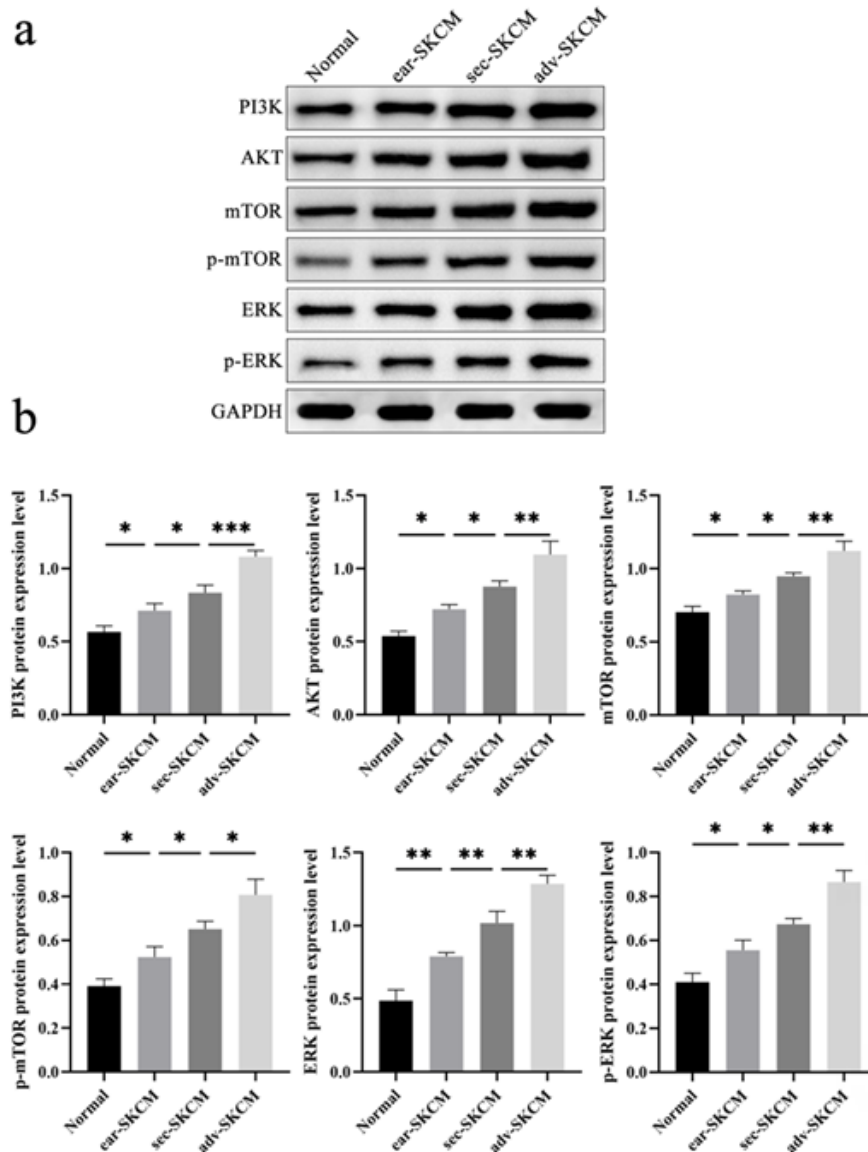
Expression of PI3K PI3K AKT in perianal and melanoma melanoma cells was investigated by western blotting assay. In Figure 1a, PI3K and AKT were expressed higher in melanoma groups, and mTOR, pmTOR, ERK, and pERK were also higher in melanoma groups than those in the normal group. In addition, we found that the expression of PI3K, AKT, mTOR, pmTOR, ERK and pERK increased with the severity of anorectal melanoma. These results indicate that The PI3K-AKT signaling pathway is positively correlated with the progression of tumor development and may promote the progression of anorectal melanoma.

#### 4.2. Knockdown of YKL-40 inhibited PI3K-AKT pathway activation

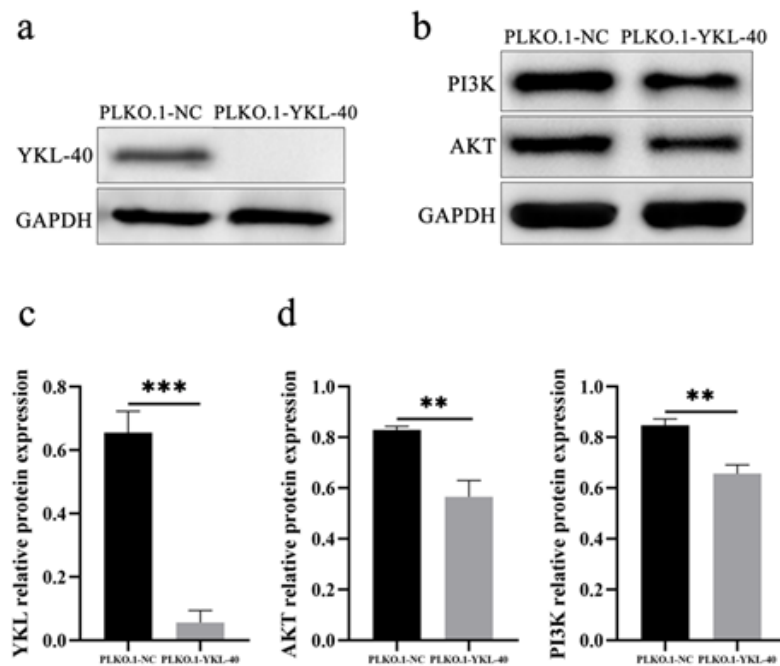
YKL-40 has been shown to be activated by the PI3K-AKT pathway via a cascade reaction. In melanoma cells, we knocked out the YKL-40 gene and detected the expression of the associated protein. As demonstrated in Figure 2a, the results showed that there was no expression of YKL-40 after YKL-40 knockout. In addition, PI3K and AKT expression decreased significantly following YKL-40 knockout (Figure 2b). Our results indicate that YKL-40 may regulate the PI3K-AKT pathway in melanoma cells.

#### 4.3. YKL-40 overexpression activates PI3K-AKT pathway to promote melanoma progression

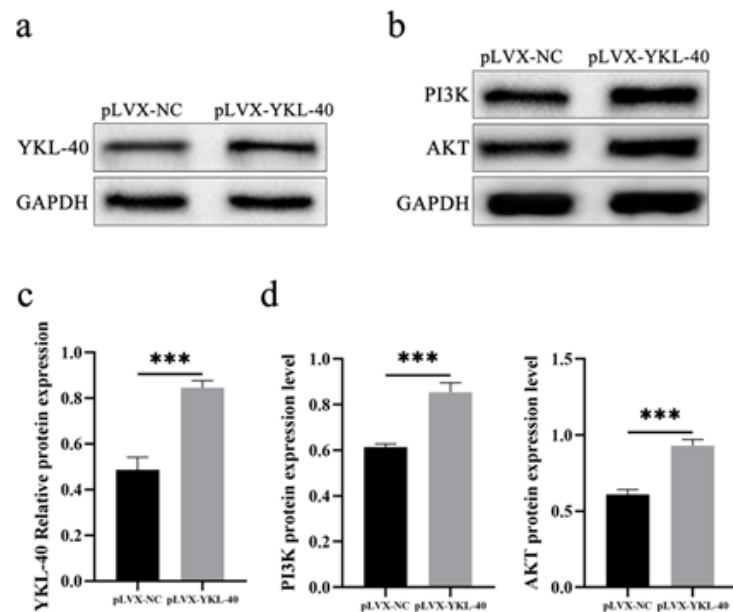
Taking into account that knockdown or over-expression of YKL-40 may have different functions in melanoma cells, we then constructed an over-expression plasmid of YKL-40. We can observe the successful construction of the highly expressed YKL-40 plasmid (Figure 3a). In addition, we investigated the PI3K and AKT expression following the over-expression of YKL-40. As shown in Figure 3b, PI3K and AKT protein expression increased markedly after YKL-40 was overexpressed. These results suggest that over-expression of YKL-40 may activate PI3K-AKT pathway and regulate the progression of melanomas.



**Figure 1:** (a) Western blot assay was performed to assess the expressions of proteins related with PI3K-AKT/mTOR signaling pathways. (b) Quantitative results of proteins related with PI3K-AKT/mTOR signaling pathways. Normal: perianal cells from healthy volunteers; ear-SKCM: melanoma cells from early anorectal melanoma patients; sec-SKCM: melanoma cells from mid-stage anorectal melanoma patients; adv-SKCM: melanoma cells from advanced anorectal melanoma patients. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 2:** (a) Protein expression of YKL-40 in PLKO.1-NC and PLKO.1-YKL-40 cells. (b) Protein expression of PI3K and AKT in PLKO.1-NC and PLKO.1-YKL-40 cells. (c) Quantitative results of YKL-40. (d) Quantitative results of PI3K and AKT. groups; PLKO.1-NC group: melanoma cells from advanced anorectal melanoma patients without YKL-40 gene knockout, PLKO.1-YKL-40 group: melanoma cells from advanced anorectal melanoma patients with YKL-40 gene knockout. (\*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .)



**Figure 3:** (a) Protein expression of YKL-40 in pLVX-NC and pLVX-YKL-40 cells. (b) Protein expression of PI3K and AKT in pLVX-NC and pLVX-YKL-40 cells. (c) Quantitative results of YKL-40. (d) Quantitative results of PI3K and AKT. pLVX-NC: melanoma cells from advanced anorectal melanoma patients; pLVX-YKL-40: melanoma cells from advanced anorectal melanoma patients with YKL-40 overexpression. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

## 5. Discussion

Our findings suggest that YKL-40 is significantly increased in melanoma cells from anorectal mucosal melanoma patients than in perianal cells from healthy volunteers, and it is associated with the severity of melanoma. Moreover, YKL-40 regulates the progression of anorectal melanoma via PI3K-AKT signaling. For all

we know, this is the first time to examine the role of YKL-40 and PI3K-AKT signaling pathways in anorectal mucosal melanoma.

The incidence of melanoma has been increasing for many years in several countries, including the United States [28]. While the incidence of all cancer sites combined is declining, that of melanoma continues to increase [29]. As one of the cancers with the highest

case fatality rate, melanoma has gradually become a major public health problem, with a huge economic and social burden [30]. In the treatment of melanoma, the main treatment is a combination of radiotherapy and chemotherapy, as well as biomolecular targeted therapy [3]. However, in the existing research on melanoma, there is still a lack of biomolecules for grading melanoma, and the mechanism of its occurrence and development still needs to be further explored.

YKL-40 may be participate in the cancer cells' proliferation and differentiation, metastasis, anti-apoptosis, angiogenesis stimulation, and regulation of extracellular tissue remodeling [31]. This likely causal role has led to research into targeting YKL-40 as a therapeutic option in cancers [32,33]. Previous research indicated that YKL-40 was increased in all kinds of cancers [31,32,34]. Serum level of YKL-40 was also determined to be an independent prognostic biomarker in patients with metastatic NSCLC and melanoma [35,36]. We believe that YKL-40 may be a prognostic factor in cancer development and patient response to applied therapy [23]. Treatment with YKL-40-neutralising antibodies or ionizing radiation in a xenografted glioblastoma model resulted in inhibition of the growth of the cancer and improved the survival of the mice [37]. Furthermore, in a latest study involving mice injected with a melanoma cell line, knocking down the YKL-40 gene reduced the size and quantity of metastatic melanomas in lung tissues of the mice [38]. Elevated serum levels of YKL in cancer patients might be related to angiogenesis, proliferation and migration of tumor cells, and the mechanism may be due to the action of YKL through FAK and PI3K-AKT signaling pathway [39].

The PI3K-AKT signaling pathway plays an important role in regulating cell proliferation and apoptosis [40]. Activation of PI3K-AKT signaling pathways can promote proliferation of multiple cell types including melanoma cells by regulating the expression of growth factors [41-43]. Our study found that compared with healthy people, the expressions of PI3K-AKT pathway related proteins were increased in melanoma patients, and the protein expression levels were gradually increased with the progression of anorectal mucosal melanoma. Previous researches have shown that elevated YKL-40 serum levels in tumor patients may be caused by angiogenesis, proliferation of tumor cells which regulated by FAK and PI3K-AKT signaling. Therefore, we assumed that YKL-40 regulates the development and progression of anorectal mucosal melanoma by targeting the PI3K-AKT signaling pathway. We constructed YKL-40 knockout and overexpression plasmid vectors and transferred them into melanoma cells from advanced melanoma patients. The results demonstrated that PI3K and AKT were reduced after YKL-40 knockout, while PI3K and AKT were increased in the presence of over-expression. Therefore, it was verified that YKL-40 regulates the progression of anorectal mucosal melanoma through the PI3K-AKT signaling pathway. We know the PI3K-AKT signaling pathway is a vital pathway in regulating

cell proliferation and apoptosis [44], so it is necessary to further investigate whether YKL-40 can influence cell apoptosis and proliferation by the PI3K-AKT signaling pathway, thus regulating the progression of anorectal mucosa melanoma. One of the limitations of this study is the lack of relevance in vivo experiments. Our next step is to further test it out in animals. To sum up, we have shown that YKL-40 regulates the progression of anorectal melanoma via PI3K-AKT signaling.

## 6. Author Contributions

Yaoping Li conceived and designed the experiments. Chaoyi Li, Yonggang, Wang, Xiaodong Ren performed the experiments. Chaoyi Li, Yonggang, Wang, Xiaodong Ren analyzed the data. Chaoyi Li, Yonggang, Wang, Xiaodong Ren drafted the manuscript.

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