ASXL2 promotes colorectal cancer tumorigenesis by inducing cell proliferation

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Abstract

Background: Colorectal cancer is one of the most common malignant tumors worldwide. ASXL2 is an enhancer of trithorax and polycomb gene, which have been proved to act in many tumor types. The role of ASXL2 in the occurrence and development of tumors have been extensively studied in recent years. However the relationship between ASXL2 and the prognosis of CRC is still unclear.

Methods: In this study, quantitative real-time polymerase chain reaction (qRT-PCR), Western blot analysis and immunohistochemistry (IHC) were used to examine the expression of ASXL2 in CRC tissues. Cells were transfected with siRNAs or lentivirus to regulate the expression of ASXL2. The effects of ASXL2 on proliferation of CRC cells were determined by CCK8 assay.

Results: This study demonstrated that ASXL2 was significantly more expressed in CRC specimens relative to the normal adjacent tissues. The upregulation of ASXL2 was related to advanced clinical stages. Patients who exhibited high expression levels of ASXL2 had poorer overall survival, whereas those with low expression of ASXL2 survived longer. Multivariate Cox regression analysis revealed ASXL2 expression could be considered as an independent prognostic factor for CRC. Inhibition or overexpression of ASXL2 markedly influenced the proliferation of CRC cells.

Conclusion: These results showed that ASXL2 could induce cell proliferation which is associated with poor prognosis of CRC patients and might be a new therapeutic target for CRC.

1. Background

Based on the latest statistics, colorectal cancer (CRC) is ranked the third most common lethal malignancies and the second leading cause of cancer-related mortality globally (1). Although the diagnosis and treatment options for CRC have been improved, the disease outcomes have largely remains unsatisfactory, particularly in patients with lymph node or distant metastasis(2, 3). Tumor invasion and metastasis are the major causes of mortality in colorectal cancer(4) . Therefore, it is important to analyze the factors related to the metastasis and prognosis of colorectal cancer. As the research progresses, several etiologic factors related to CRC progression have been reported (5-8). Therefore, understanding the underlying molecular mechanisms involved in CRC progression and identifying novel biomarkers for evaluating the prognosis is of great significance.

Additional sex combs-like 2 (ASXL2) genes belonging to the ASXL gene family is located on chromosome 2p23.3, with length 144,722 bp. It contains 12 exons and encodes a nuclear protein with a length of 1,435 amino acids. Notably, homologous gene, ASXL1 and ASXL2 are positive regulators of trithorax and Polycomb(ETP) genes. Both of which encode essential transcriptional and epigenetic regulatory proteins in the course of important developmental stages(9-13). Compared with ASXL1, there is less research on the function of ASXL2. Studies have shown that the potential roles of ASXL2 in cardiac function, adipogenesis and osteoclastogenesis(14, 15). Besides, ASXL2 was found to be related to the occurrence and development of tumors by involving in transcriptional activation and repression in a context-

dependent manner(16, 17). Park *et al.* found that ASXL2 could be potentially be used as a biomarker for predicting the disease outcomes of breast cancer patients. The gene has been shown to facilitate breast cancer cell proliferation via linking ERα to histone methylation(18). Meanwhile, existing evidence indicates that ASXL2 are associated with poor prognosis in many solid tumors, but the function of ASXL2 in colon cancer has not been fully elucidated(19-21).

In this study,

In this study, we compared the transcription and expression of ASXL2 between CRC tissue and normal adjacent tissues to explore the relationship between ASXL2 expression level and clinicopathological features of patients with colon cancer. Survival analysis was performed to explore whether ASXL2 is a critical factor in the prognosis of colon cancer. Besides, we performed the in-vitro experiments to further confirm the impact of ASXL2 expression on the proliferative ability of CRC cell lines. Finally, we concluded that ASXL2 is related to poor prognosis of colorectal carcinoma via induction of cell proliferation.

2. Materials And Methods

2.1. Tissue samples

The samples were taken from 182 cases of CRC tissues obtained from colorectal surgery at the Renji Hospital, Shanghai Jiao Tong University School of Medicine from April 2003 to November 2010. The ethics committee of the hospital provided approval for the execution of the current study. The duration of the follow-up covered the period between the day of surgery to the death of a patient or the last day of follow-up. These tissues were made into the tissue microarrays (TMA). Furthermore, the specimens used for qRT-PCR were derived from 52 cases of CRC tissues and paired normal adjacent tissues obtained from the surgery at Renji Hospital from April 2017 to November 2018. None of the patients received radiotherapy or chemotherapy before surgery. The clinical stages and histology were categorized based on the American Joint Committee on Cancer (AJCC) guidelines(22). We received signed informed consent from all patients.

2.2. Gene expression assay

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from the tissues and cell lines according to the manufacturer's instructions. Next, the PrimeScript RT-PCR kit (Takara, Japan) was used for reverse transcription. We performed real-time quantitative PCR (RT-qPCR) using a 7500 real-time PCR system (Applied Biosystems, Inc. USA). The following primer sequences were used for ASXL2 detection: forward: 5'-GGA AAA GGG ACG TAG GAA GAA G-3'; reverse: 5'-ACT CAT GGG TGT ATT GGG GTA-3'. GAPDH served as internal control. The relative expression levels of mRNA were calculated using the $2^{-\Delta Ct}$ method. RNA was examined by PCR array kit (Boster, Shanghai, China).

2.3. Western blot (WB)

Refer to kit instructions for the protein extraction. Transfer the protein to polyvinylidene difluoride membrane after electrophoresis. Blocked with milk, incubated with primary and secondary antibodies. Bands were detected by enhanced chemiluminescence (ECL) kit.

2.4. Immunohistochemistry (IHC)

Tissue microarray (TMA) of this study was constructed by Suzhou Xinxin. Immunohistochemical (IHC) staining was performed by the avidin-biotin-peroxidase method. We used immunohistochemistry to detect the expression of ASXL2 expression in CRC. Firstly, deparaffinized the slides, remove the endogenous peroxidase with a 3% hydrogen peroxide solution. Then after blocked with serum, the slides were incubated with anti-ASXL2 antibody (1:200, Abcam, USA) overnight at 4°C. Normal goat serum instead of primary antibody as a negative control. Finally, incubate with secondary antibody for 1 hour at room temperature, observe the color reaction of immunohistochemical staining section under microscope. The score of IHC was based on the intensity of staining and the percentage of positive cells(23). The intensity of staining can be divided into: negative : 0; weak staining : 1; moderate staining : 2; strong staining : 3, and the scoring standards of the percentage of positive cells was: 0-4 (<5% : 0; 5%-30% : 1; 30%-50% : 2; 51%-75% : 3; >75% : 4). The final score is the product of the positive cell score and the percentage of positive cells, and the samples were divided into low and high expression groups based on whether the final score is 6 or more.

2.5 Cell culture and transfection

The human CRC cell lines (SW480, SW1116, Caco-2, HT29) were all from the Shanghai Cancer Institute. The cells were maintained in a DMEM medium augmented with fetal bovine serum (10%) and antibiotics (1%) at 37 ° C in an incubator with 5% carbon dioxide. The transfections were performed by Lipofectamine 2000 (Invitrogen, USA) as per the manufacturer's instructions. The plasmids, used for constructing the ASXL2 over-expressing CRC cells, were procured from the Genearray Biotechnology (Shanghai, China). ASXL2 silencing CRC cells were constructed by transfecting with si-ASXL2 (GeneTech).

2.6. Cell proliferation assay

The Cell Counting Kit 8 (CCK-8, Dojindo) was used to assess cell viability. In brief, the cells (treated and control) were seeded (7000 cells/well) into 96-well plates. Subsequently, CCK-8 solution (10μL) was added in every well at each time point, then incubated at 37°C for 2 h in a CO2 incubator. A microplate reader was used to determine the absorbance rate at 450 nm. Each experiment was repeated thrice.

2.7. Statistical analysis

The analyses were performed using GraphPad Prism 8 and SPSS 22.0 software. We applied the Student's t-test to determine the difference in ASXL2 expression between CRC tissues and normal adjacent tissues. Chi-square test was used to analyze the relationship between ASXL2 expression and the clinicopathological features of CRC patients. The Kaplan-Meier method and Log-rank test was employed

to produce the survival curve and compare the survival rate between groups. Cox proportional hazard model was used to conduct univariate and multivariate analyses. Statistical significance was set at $P < 0.05$. The analyses were performed using SPSS 22.0 and GraphPad Prism 8 software.

3. Results

3.1. ASXL2 is significantly elevated in CRC patients and is related to the degree of differentiation

Based on the qRT-PCR assay, the levels of ASXL2 mRNA in CRC tissues were remarkably higher than in normal adjacent tissues ($P < 0.001$) (Fig. 1A). We also investigated the data from the TCGA database. Among 524 participants, the expression level of ASXL2 was higher in those with tumor, which echoes our results (Fig. 1C). Furthermore, with the improvement of tumor differentiation, ASXL2 levels were gradually decreased (Figure 1B).

3.2. ASXL2 is expressed diversely in CRC at protein level.

We performed IHC analysis to detect the expression of the ASXL2 in TMAs. There were 182 cases of CRC and matched normal adjacent tissues in TMAs. Based on the results, ASXL2 was downregulated in 97 (53.3%) of CRC specimens and upregulated in the remaining 85 (46.7%) samples (Fig. 2).

3.3. Relationship between ASXL2 expression and clinicopathological features of CRC patients

The overexpression of ASXL2 in CRC patients was significantly related to AJCC staging ($p = 0.020$), T classification ($p = 0.038$), and N classification ($p = 0.022$). No considerable difference was observed between ASXL2 expression levels and other features, including patient's age, gender, tumor size and location and M classification ($P > 0.05$, Table 1).

3.4. ASXL2 overexpression is an independent risk factor for poor prognosis in CRC

Patients who had high ASXL2 expression exhibited a lower overall survival rate than patients with lower expression of the gene (Figure 3A, log-rank test, $P < 0.001$). Furthermore, the clinicopathological features of CRC patients were considered for the univariate analysis. The results suggested that the AJCC stage, classification of TNM and the expression level of ASXL2 were risk factors of colorectal cancer prognosis. Also, multivariate Cox regression analysis revealed that ASXL2 upregulation was an independent risk factor for poor colorectal cancer prognosis (Table 2).

Besides, the one, three, and five-year survival rates of patients with lymph node metastasis were lower than those in the non-lymph node metastasis group. Among them, the five-year survival rate decreased most significantly. The subgroup analysis among the patients with lymphatic metastasis revealed that high ASXL2 expression was more likely to confer a poor prognosis. (Fig. 3C).

3.5. Effect of ASXL2 on CRC Cell Proliferation in Vitro

To examine the ASXL2 expression in vitro, the level of ASXL2 expression was assessed in CRC cell lines (Caco-2, HT29, SW480, SW1116) of the same source with different colorectal cancer (Fig. 4A), and divided the cell lines into two groups based on ASXL2 expression levels.

Subsequently, Caco-2 and SW1116 were classified into the high expression group, whereas SW480 and HT29 were put in the low expression group. We inhibited ASXL2 expression by transfecting siRNAs-ASXL2 plasmids into the cell lines in the high expression group (Caco-2, SW1116). Cell proliferation was considerably reduced with a decrease in ASXL2 expression (Fig. 4B-4C). For the cell lines in low expression group. We transfected the plasmid overexpressing ASXL2 into HT29 and SW480 to overexpress ASXL2. Relative to the control group (empty vector), overexpression of ASXL2 levels led to a remarkable increase in cell proliferation. (Figures 4E-4F).

Also, we measured the Ki-67 expression because it has been associated with cell proliferation. As we expected, the mRNA levels of Ki-67 had significantly increased with the overexpression of ASXL2 and vice versa, which confirmed our findings. (Fig. 4G).

4. Discussion

CRC, like most forms of cancer, has biologically and epidemiologically heterogeneity which has an impact on the prognosis(24). In recent years, how to improve the prognosis of patients with CRC has become a problem to be solved. Therefore, understanding the underlying molecular mechanisms involved in the progression of CRC and identifying new biomarkers are of great importance for the prognostic evaluation and clinical management of CRC patients.

ASXL2, located on chromosome 20q11, is one of the enhancers of trithorax and polycomb gene(25). With deeper study into tumor in recent years, ASXL2 has attracted much attention for its functional roles in tumor. Studies show that ASXL2 acts as an epigenetic regulator by recruiting a multicomb repressor complex (PRC), which activate the DUB and regulate the cell proliferation(10). Nowadays, abnormal expression of ASXL2 has been reported in more and more different types of tumors, and ASXL2 is known to cause poor prognosis in tumor patients(9-11). However, the prognostic value and clinical significance of ASXL2 in CRC have not been reported at home and abroad.

Herein, we firstly revealed that ASXL2 was upregulated in CRC tissues relative to normal adjacent tissues through RT-qPCR. In addition, we observed a remarkable decrease in the levels of ASXL2 mRNA in patients with less tumor differentiation and early clinical stages of CRC, indicating that ASXL2 upregulation was related to aggressive CRC phenotype. In addition, compared with adjacent normal colonic mucosa, mRNA levels of ASXL2 in CRC tissues were significantly overexpressed ($P < 0.001$). Collectively, these findings suggest that ASXL2 may be an oncogene for CRC.

Subsequently, we determined the link between the level of ASXL2 expression and clinicopathological features. In this article, we described a phenomenon where the upregulation of ASXL2 in CRC was significantly related to the AJCC stage, T classification, and N classification. Univariate analysis indicated

that the over-expression of ASXL2 in CRC patients was closely related to the incidence of OS. Multivariate analysis revealed that over-expression of ASXL2 in CRC patients was an independent predictor of poor disease outcomes. In general, these results indicate that ASXL2 expression can act as a biomarker for CRC and has the potential to become a new prognostic indicator for CRC patients.

Herein, we revealed that the levels of ASXL2 mRNA were higher in poorly differentiated CRC cell lines. Meanwhile, by overexpressing the ASXL2 gene in CRC cell lines, we found that over-expression of ASXL2 can significantly promote the proliferation of CRC cells. According to other literature, in human breast cancer cell line, over-expression of ASXL2 was found to promote MCF7 cell proliferation and MCF7-derived tumor growth(9). CCK-8 also found that low expression of ASXL2 could result in the decrease of cell proliferation, compared with the control group (empty vector). However, However, the exact molecular mechanism by which ASXL2 affects the growth and differentiation of colorectal cancer cells is still unknown.

In general, our data suggest that ASXL2 over-expression is related to poor prognosis of colorectal carcinoma by inducing cell proliferation. These results may offer new insights into the clinical management of CRC patients and the development of new therapeutic targets for CRC treatment. However, further studies on related signaling pathways are needed to fully elucidate the precise role of ASXL2 in CRC pathogenesis and how it can be exploited for the development of new therapeutic targets for CRC treatment. Moreover,

5. Conclusion

Collectively, we elucidated the critical role of ASXL2 in human CRC progression, where it elevated the proliferation. The discovery of the ASXL2 will aid in further CRC investigation and in developing therapeutic strategies against CRC.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

RC and MY conceived and designed the study. YW and LY contributed to carry out the experiments. MY and MZ provided clinical samples and clinical information. RC wrote the manuscript. BC supervised the research. All authors read and approved the final manuscript.

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Availability of data and material

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, and informed consent was obtained from all patients before enrolling in the research program.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Relationship between ASXL2 expression and clinicopathological features in 182 colorectal cancer patients

Characteristics	ASXL2 expression		P-value
	Low (N =97)	High (N =85)	
Age			
<65	56 (59.6%)	38 (40.4%)	0.079
≥65	41 (46.6%)	47 (53.4%)	
Gender			
Male	57 (50.4%)	56 (49.6%)	0.323
Female	40 (58.0%)	29 (42.0%)	
Tumor size			
≤5 cm	68 (55.7%)	54 (44.3%)	0.347
>5 cm	29 (48.3%)	31 (51.7%)	
Tumor location			
Rectum	72 (53.3%)	63 (46.7%)	1.000
Colon	25 (53.2%)	22 (46.8%)	
AJCC Stage			
I	34 (70.8%)	14 (29.2%)	0.020
II	23 (52.2%)	21(47.7%)	
III	31 (41.9%)	43 (58.1%)	
IV	9 (56.2%)	7 (43.8%)	
T classification			
T1	16 (76.2%)	5 (23.8%)	0.038
T2	25 (56.8%)	19 (43.2%)	
T3	30 (55.6%)	24 (44.4%)	
T4	26 (41.3%)	37 (58.7%)	
N classification			
N0	59 (62.8%)	35 (37.2%)	0.022
N1	20 (47.6%)	22 (52.4%)	
N2	18 (39.1%)	28 (60.9%)	
M classification			

M0	88 (53.0%)	78 (47.0%)	0.805
M1	9 (56.2%)	7 (43.8%)	

The bold number represents the P-values with significant differences.

Table 2 Univariate and multivariate analyses showing the overall survival in colorectal cancer

Variable	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
ASXL2	2.782(1.791, 4.321)	<0.001	2.150(1.350, 3.423)	0.001
Age	0.920(0.608, 1.391)	0.692	-	-
Gender	1.175(0.770, 1.794)	0.455	-	-
Size	0.937(0.606, 1.446)	0.768	-	-
Location	1.244(0.793, 1.951)	0.343	-	-
AJCC Stage	2.475(1.890, 3.242)	<0.001	-	-
T classification	2.405(1.820, 3.178)	<0.001	2.075(1.431, 3.009)	<0.001
N classification	2.457(1.914, 3.154)	<0.001	2.108(1.359, 3.270)	0.001
M classification	4.548(2.432, 8.505)	<0.001	5.932(1.928,18.252)	0.002

HR: hazard ratio; CI: confidence interval. The bold number represents the P-values with significant differences.

Figures

Figure 1

ASXL2 overexpression in CRC specimens detected by qRT-PCR analyse. (A) The mRNA levels of ASXL2 in 104 CRC tissues and paired normal mucosae were determined by qRT-PCR. (B) The mRNA levels of ASXL2 in CRC patients with different tumor differentiation were analyzed. (C) The mRNA levels of ASXL2 in 275 CRC tissues and 349 paired normal mucosae from TCGA database. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 2

Representative immunohistochemistry staining of ASXL2 in CRC tissues and normal colon mucosae. (A) (B) high expression of ASXL2. (C)(D) low expression of ASXL2. Representative images are shown at 50× and 400× magnification respectively.

Figure 3

The prognostic significance of ASXL2 for CRC patients assessed via Kaplan-Meier analysis. The patients with lower ASXL2 expression had better OS (A). Significant difference was observed between high and low ASXL2 expression groups in both CRC patients with and without lymphatic metastasis (B-C).

Figure 4

Effect of ASXL2 on CRC Cell Proliferation in Vitro. (A) The ASXL2 protein level in Caco-2, HT29, SW480 and SW1116 cells. (B-C) siRNAs-ASXL2 decreased cell proliferation measured by CCK8 assays. (E-F) ASXL2 over-expressing plasmids increased cell proliferation measured by CCK8 assays. (D) Quantitative real time PCR analysis of the mRNA levels of proliferation associated gene (Ki-67) in cells with empty vectors and cells with siRNAs-ASXL2 plasmids in Caco-2, SW1116. (G) Quantitative real time PCR analysis of the mRNA levels of proliferation associated gene (Ki-67) in cells with empty vectors and cells with ASXL2 over-expressing plasmids in HT29 and SW480. All data are representative of three independent experiments. Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, compared with the controls.