ORIGINAL RESEARCH

Elevated Expression of ASXL2 is Associated with Poor Prognosis in Colorectal Cancer by Enhancing Tumorigenesis and Inducing Cell Proliferation

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

Materials and 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 the proliferation of CRC cells were determined by CCK8 assay.

Results: This study demonstrated that ASXL2 was significantly more highly expressed in CRC specimens than in normal adjacent tissues. The upregulation of ASXL2 was related to advanced clinical stage. 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 that ASXL2 expression could be considered 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 was associated with poor prognosis of CRC patients, suggesting that ASXL2 might be a new therapeutic target for CRC.

Keywords: ASXL2, colorectal cancer, cell proliferation, prognosis marker

Background

Based on the latest statistics, colorectal cancer (CRC) is ranked as the third most common lethal malignancy 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 remained 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 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 prognosis is of great significance.

The additional sex combs-like 2 (ASXL2) gene, belonging to the ASXL gene family, is located on chromosome 2p23.3 and has a length of 144,722 bp. It contains 12 exons and encodes a nuclear protein with a length of 1435 amino acids. Notably, as homologous genes, ASXL1 and ASXL2 are positive regulators of trithorax and Polycomb (ETP) genes, both encode essential transcriptional and epigenetic regulatory proteins involved in important developmental stages.[9–13] Compared with that of ASXL1, ASXL2 function has been less studied. Studies have shown the potential roles of ASXL2 in cardiac function, adipogenesis and osteoclastogenesis.[14,15] In addition, ASXL2 was found to be related to the occurrence and development of tumors by being involved in transcriptional activation and repression in a context-dependent manner.[16,17] Park et al found that ASXL2 could 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 by linking ERα to histone methylation.[18] In addition, existing evidence indicates that ASXL2 is 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, we compared the transcription and expression of ASXL2 between CRC tissues and adjacent normal tissues to explore the relationship between ASXL2 expression 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. In addition, we performed in vitro experiments to further confirm the impact of ASXL2 expression on the proliferative ability of CRC cell lines. Ultimately, we conclude that ASXL2 is related to the poor prognosis of colorectal carcinoma via induction of cell proliferation.

Materials and Methods
Tissue Samples

All specimens were histopathologically confirmed by the pathologists and obtained with informed consent. 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. This study was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (RA-2020-001). 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.

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′. 18S RNA served as an 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).

Western Blot (WB)

Treated cells were lysed in RIPA buffer containing protease inhibitor (Beyotime, Nanjing, China). The BCA Protein Assay kit (Beyotime, Nanjing, China) was used to determine protein concentrations. Transfer the protein to polyvinylidene difluoride membrane after electrophoresis. Blocked with milk, membranes were incubated with primary and secondary antibodies. Bands were detected by enhanced chemiluminescence (ECL) kit using the image-forming system of Amersham Imager 600.

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 the immunohistochemical staining section under a 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 H-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. The H-score analysis was carried out independently by two experienced pathologists who were blinded to the final clinical diagnosis of all cases studied.

Cell Culture and Transfection

This study was approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (RA-2020-001). The human CRC cell lines (SW480, SW1116, Caco-2, HT29) were purchases from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and 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). The following primer sequences were used for negative si-RNA detection: 3′-GCTTCAGGCTACGCATTTA-5′.

Cell Proliferation Assay

Proliferation was measured using CCK8 assay. Cells were seeded into 96-well plates for 24 h. CCK8 (10μg/mL) was added into the plates, and then the absorbances at 450 nm were recorded (Biotek Instruments Inc., Winooski, VT, USA). Replicate experiments were performed. Cell survival was then calculated.

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 were 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.

Results

ASXL2 Was Significantly Elevated in CRC Patients

Based on the qRT-PCR assay, the levels of ASXL2 mRNA in CRC tissues were remarkably higher than those in normal adjacent tissues (P < 0.001) (Figure 1A). We also investigated data from the TCGA database. Among 524 subjects, the expression level of ASXL2 was higher in those with tumors than in normal individuals, which agrees with our results (Figure 1C). Furthermore, with increasing tumor differentiation, ASXL2 levels gradually decreased (Figure 1B). We next performed IHC analysis to detect the expression of ASXL2 in TMAs. There were 182 cases of CRC and matched normal adjacent tissues in the TMAs. Based on the results, we divided the cases into two groups according to the median expression of ASXL2. ASXL2 was downregulated in 97 (53.3%) CRC specimens and upregulated in the remaining 85 (46.7%) samples (Figure 2).

Relationship Between ASXL2 Expression and Clinicopathological Features of CRC Patients

To assess the clinical significance of ASXL2, the chi-square test was used to analyze the correlation between the expression of ASXL2 and clinicopathological parameters in 182 CRC patients (Table 1). Overexpression of ASXL2 in CRC patients was significantly related to AJCC stage (p = 0.020), T stage (p = 0.038), and N stage (p = 0.022). No considerable relationship was observed between ASXL2 expression level and other features,
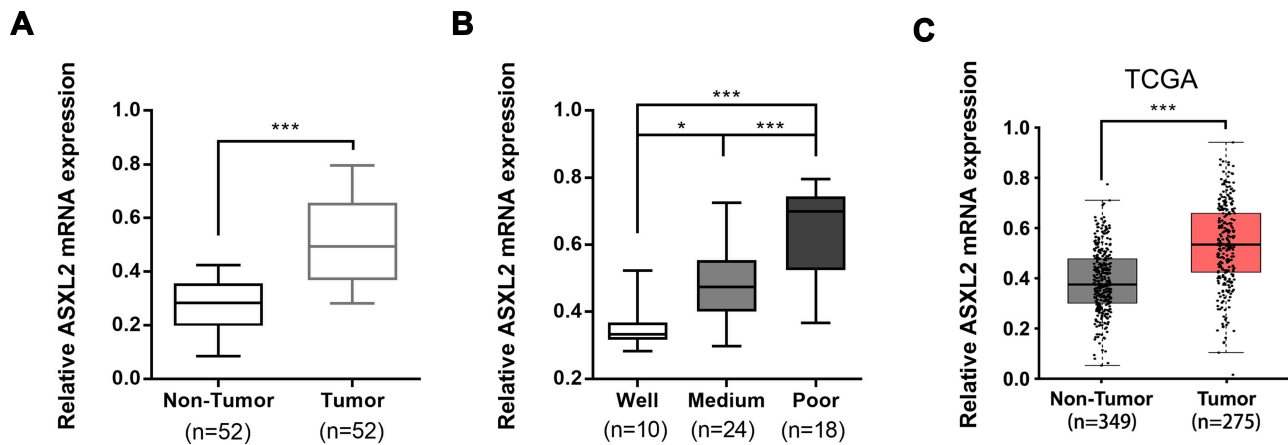
Figure 1 ASXL2 overexpression in CRC specimens detected by qRT-PCR analyse. (**A**) The mRNA levels of ASXL2 in 104 CRC tissues and paired normal mucosae were determined by qRT-PCR. (**B**) The mRNA levels of ASXL2 in CRC patients with different tumor differentiation were analyzed. (**C**) The mRNA levels of ASXL2 in 275 CRC tissues and 349 paired normal mucosae from TCGA database. (*P < 0.05; ***P < 0.001).
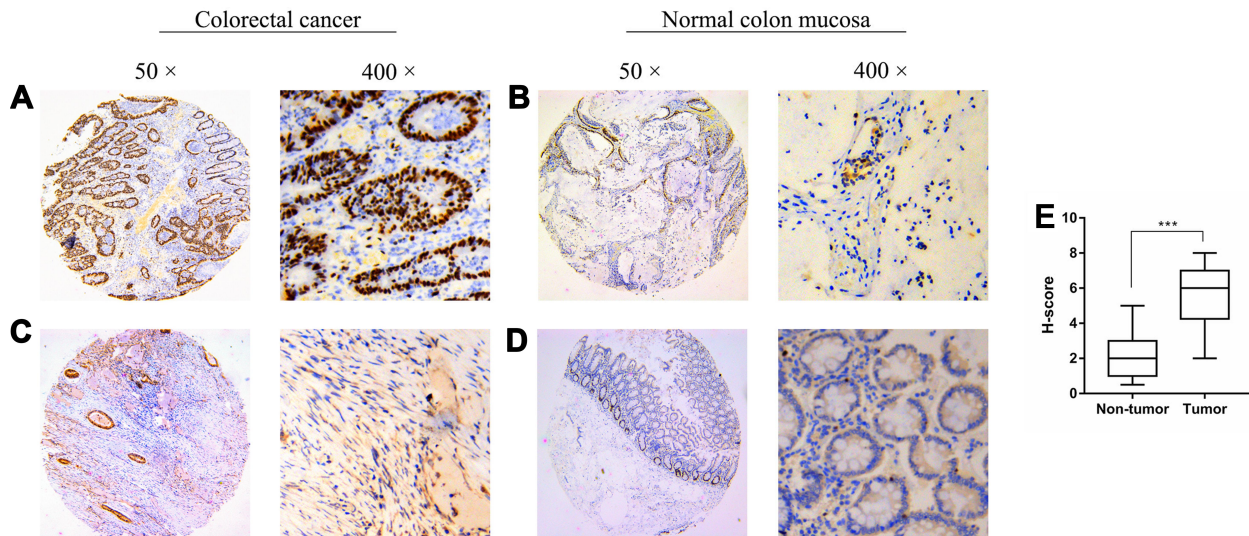

Figure 2 Representative immunohistochemistry staining of ASXL2 in CRC tissues and normal colon mucosae. (**A** and **B**) high expression of ASXL2. (**C** and **D**) low expression of ASXL2. Representative images are shown at 50× and 400× magnification respectively. H-score of Non-tumor and Tumor immunoreactivity (**E**). Data were presented as median (interquartile range). (***P<0.001).

including patient age, sex, tumor size and location and M stage (P > 0.05).

ASXL2 Overexpression Was an Independent Risk Factor for Poor Prognosis in CRC

Patients who had high ASXL2 expression exhibited a lower overall survival rate than patients with low 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, TNM stage and

expression of ASXL2 were risk factors for colorectal cancer prognosis. Additionally, the multivariate Cox regression analysis revealed that ASXL2 upregulation was an independent risk factor for poor colorectal cancer prognosis (Table 2).

In addition, 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 those patients with lymph node metastasis, the five-year survival rate was the most significantly decreased. The subgroup analysis among the patients with lymphatic metastasis revealed that high ASXL2 expression was likely to confer a poor prognosis (Figure

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%)	

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

3B and C). We also studied the data from the TCGA database. The higher ASXL2 expression and overall survival rate at mRNA levels showed a lower overall survival rate (Figure 3D).

Effect of ASXL2 on CRC Cell Proliferation in vitro

To examine ASXL2 expression in vitro, the level of ASXL2 expression was assessed in human CRC cell lines (Caco-2, HT29, SW480, and SW1116) with different types of colorectal cancers (Figure 4A), and the cell lines were divided into two groups based on ASXL2 expression

levels. Further experiments confirmed the degree of over/underexpression of AXSL2 in Caco-2 and SW480 (Figure 4B).

Subsequently, Caco-2 and SW1116 cells were classified into the high expression group, whereas SW480 and HT29 cells were classified into the low expression group. We inhibited ASXL2 expression by transfecting siRNA-ASXL2 plasmids into the cell lines in the high expression group (Caco-2 and SW1116). Cell proliferation was considerably reduced with a decrease in ASXL2 expression (Figure 4C and D). Into the cell lines in the low expression group (HT29 and SW480), we transfected plasmids overexpressing ASXL2 to induce overexpression of ASXL2. Relative to the control group (empty vector), the group with overexpression of ASXL2 levels had a remarkable increase in cell proliferation (Figure 4E and F).

Additionally, we measured the Ki-67 level because it is known to be associated with cell proliferation. As we expected, the mRNA levels of Ki-67 were significantly increased with the overexpression of ASXL2 (and significantly decreased with knockdown of ASXL2), which confirmed our findings (Figure 4G and H).

Discussion

CRC, like most forms of cancer, has biological and epidemiological heterogeneity, which have an impact on prognosis.[24] In recent years, research has attempted to uncover how to improve the prognosis of patients with CRC. Therefore, understanding the underlying molecular mechanisms involved in the progression of CRC and identifying new biomarkers is 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 genes.[25] With the deeper study of tumors in recent years, ASXL2 has attracted much attention for its functional roles. Studies show that ASXL2 acts as an epigenetic regulator by recruiting a polycomb repressor complexes (PRCs), which activate DUBs and regulate cell proliferation.[10] Recently, abnormal expression of ASXL2 has been reported in an increasing number of 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 to date.

Herein, we first revealed that ASXL2 was upregulated in CRC tissues relative to normal adjacent tissues
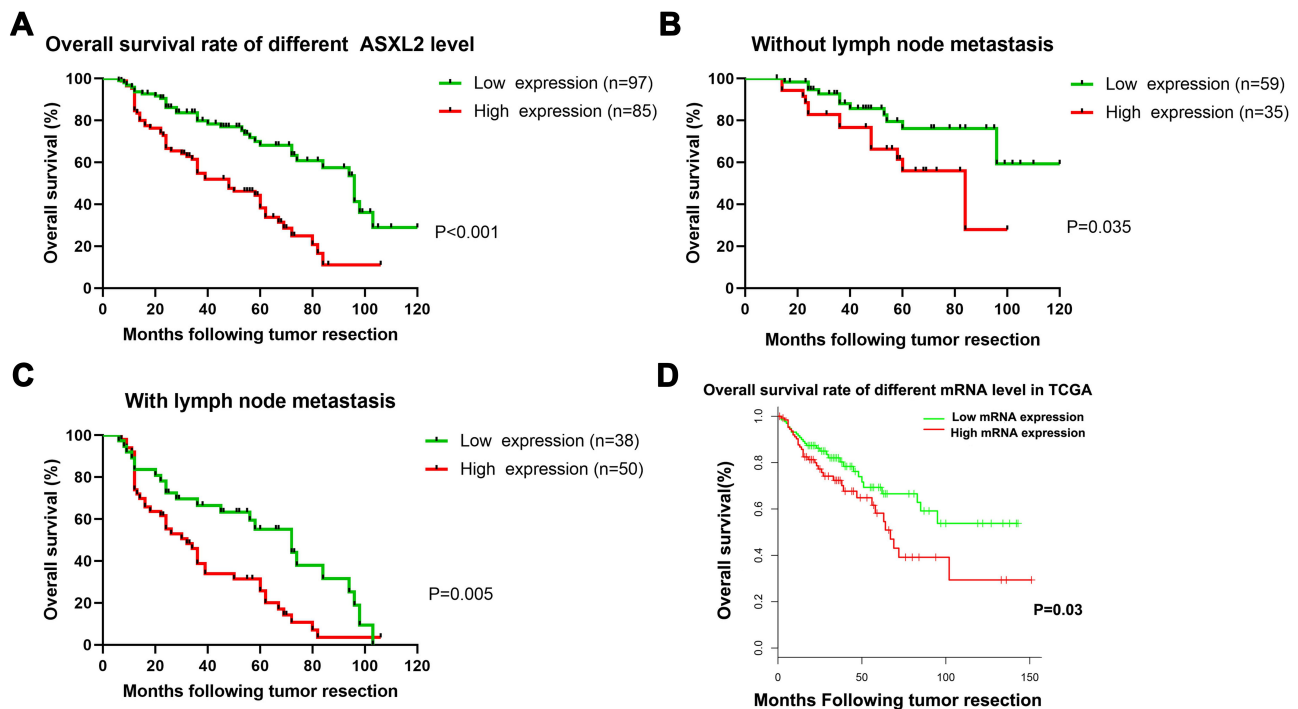
Figure 3 The prognostic significance of ASXL2 for CRC patients assessed via Kaplan–Meier analysis. The patients with lower ASXL2 expression had better OS (**A**). Significant difference was observed between high and low ASXL2 expression groups in both CRC patients with and without lymphatic metastasis (**B** and **C**). The patients with lower ASXL2 mRNA expression had better OS in TCGA database (**D**).

through RT-qPCR. In addition, we observed a remarkable decrease in the levels of ASXL2 mRNA in patients with low tumor differentiation and early clinical stages of CRC, indicating that ASXL2 upregulation is related to an aggressive CRC phenotype. In addition, compared with those in the adjacent normal colonic mucosa, the 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 examined the relationship between ASXL2 expression and clinicopathological features. We found that the upregulation of ASXL2 in CRC was significantly related to the AJCC stage, T stage, and N stage. The univariate analysis indicated that the overexpression of ASXL2 in CRC patients was closely related to OS. The multivariate analysis revealed that overexpression 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

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**

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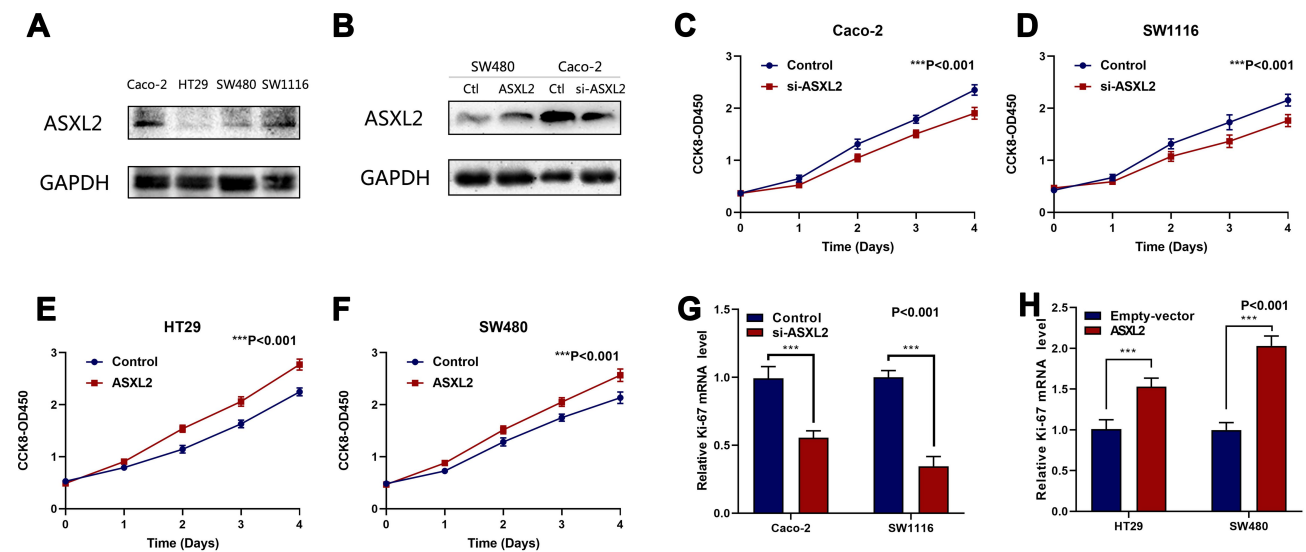**Abbreviations:** HR, hazard ratio; CI, confidence interval.

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**) degree of over/underexpression of AXSL2 in Caco-2 and SW480. (**C** and **D**) siRNAs-ASXL2 decreased cell proliferation measured by CCK8 assays. (**E** and **F**) ASXL2 over-expressing plasmids increased cell proliferation measured by CCK8 assays. (**G**) 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. (**H**) 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. (***$P<0.001$)

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 than in well-differentiated CRC cell lines. In addition, by over-expressing the ASXL2 gene in CRC cell lines, we found that overexpression of ASXL2 could significantly promote the proliferation of CRC cells. According to other literature, in a human breast cancer cell line, overexpression of ASXL2 was found to promote MCF7 cell proliferation and MCF7-derived tumor growth.[9] The CCK-8 assay results also showed that low expression of ASXL2 resulted in a decrease in cell proliferation compared with that seen in the control group (empty vector). 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 overexpression induces poor prognosis in colorectal carcinoma by enhancing 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.

Conclusion

Collectively, we elucidated the critical role of ASXL2 in human CRC progression, in which it was found to elevate proliferation. The discovery of the role of ASXL2 in CRC progression will aid in further CRC investigations and in developing therapeutic strategies against CRC.

Data Sharing Statement

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 (RA-2020-001), and informed consent was obtained from all patients before enrolling in the research program.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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