

Deletion of Setd2 Aggravates Gastric Adenoma Induced by c-Myc Overexpression Involving PI3K/AKT Signaling Pathway

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Abstract: Globally, gastric cancer (GC) is an urgent health concern, necessitating an understanding of its genetic and epigenetic regulation. The tumor suppressor Setd2, H3K36me3 methyltransferase, has been associated with malignancies. The underlying signaling mechanisms and the function of Setd2 in GC aggravation are yet unclear. To figure out this question, we utilized a mouse model with c-Myc overexpression and Setd2 knockout in gastric parietal cells to conduct histological and molecular analysis. Our results demonstrated that deletion of Setd2 exacerbated gastric adenoma induced by c-Myc overexpression. Moreover, we revealed that PI3K/AKT signaling pathway contributed to the development of gastric adenoma, offering GC patients a potentially effective treatment approach.

1. Introduction

About 769,000 fatalities of gastric cancer (GC) were reported in 2020, ranked as the fourth most cancer deaths in 2020.^[1] Although there has been a reduction in the occurrence of GC over the last few decades, it nevertheless remains a serious worldwide health issue, particularly in East Asian countries.^[2, 3] Therefore, it is crucial to investigate the function and mechanism of GC-related genes.

The development and course of GC are significantly influenced by epigenetic regulation.^[4] One of the epigenetic changes linked to the silence of important tumor suppressor genes is histone methylation.^[5] Histone methyltransferases (HMTs) add methyl group(s) to a lysine or arginine residue through the methylation process.^[6] Setd2 is one of the HMTs that tri-methylates histone H3 at lysine-36 (H3K36me3), which participates in several biological mechanisms like transcriptional control, and the differentiation of embryonic stem cells.^[7] Mutation or reduced expression of Setd2 causes methylation and demethylation imbalance, leading to tumorigenesis in various malignancies.^[8] Previous studies also reported that GC patients exhibiting reduced SETD2 expression experienced notably diminished 5-year survival rates in contrast to those with elevated SETD2 expression.^[9] It's also proved that overexpression of Setd2 in GC cell lines results in significant suppression of cell proliferation, migration, and invasion.^[10] However, how Setd2 deficiency functions in GC aggravation remains unknown.

Cancer relies on a constant activation of transcription factors (TFs) to thrive. For example, hypoxia-inducible factors (HIFs), ETS-1, and β -catenin are crucial TFs that can govern numerous other TFs engaged in various cellular processes.^[11] C-Myc is an important TFs, playing a vital role in pro-oncogenic processes including differentiation, and the progression of the cell cycle.^[12] Dysregulation of C-Myc leads to various cancer types including GC.^[13] Over 40% of GC had elevated c-Myc expressions.^[14] Further studies have shown that the progression of GC is affected by the amplification of c-Myc.^[15] Moreover, it has been proved that stomach-specific c-Myc overexpression could induce gastric adenoma by the activation of AKT/mTOR pathway in mice.^[16]

Many signaling pathways have been identified as frequently genetically altered in GC.^[17] Among them, the PI3K/AKT pathway holds significant importance for promoting the proliferation and survival of gastric adenoma. It regulates GC progression via processes like apoptosis, autophagy, and metastasis.^[18] An increasing amount of research has elucidated that the epigenetic mechanisms govern the pathway in GC regulation.^[19] Nevertheless, the relationship between Setd2 and PI3K/AKT pathway is not fully understood.

To evaluate the significance of Setd2, we employed an animal model in which stomach parietal cells exhibited high levels of c-Myc, and Setd2 was deleted. We identified that these mice exhibited much more severe gastric adenoma traits than c-Myc overexpression mice. Our research demonstrated that the PI3K-AKT pathway is activated by loss of Setd2, suggesting a putative target for GC treatment.

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2. Materials and Methods

2.1. Mice

Atp4b-cre; *Myc^{OE/+}*; *Setd2^{F/F}* (*Myc^{OE}*; *Setd2^{KO}*) mice, referred to as AMS mice, were generated by crossing *Atp4b-cre*; *Myc^{OE/+}*; *Setd2^{F/+}* mice with *Setd2^{F/+}* mice. *Atp4b-cre*; *Myc^{OE/+}*; *Setd2^{+/+}* (*Myc^{OE}*; *Setd2^{WT}*) mice, referred to as AM mice, were also selected as control. Every mouse was produced using the C57BL/6 background and both female and male mice were used. Shanghai Biomodel Organism Co. produced the *Setd2^{F/F}* mice, while The Jackson Laboratory supplied the *Myc^{OE}* mice. The Animal Ethics Committee of our center authorized all experiment techniques. Table 1 contains a collection of genotyping primers.

Table 1. Primers for Genotyping

Primer name	Sequence (5'-3')
H-Setd2-F	GTA AAG TAG TAT TAT GCC AAG GCC C
H-Setd2-R	TAT TTA AAC TCT CTC TGG GGG TGG
M-Atp4b-cre-F	GCC TGC ATT ACC GGT CGA TGC AAC GA
M-Atp4b-cre-R	GTG GCA GAT GGC GCG GCA ACA CCA TT
H-c-Myc-F1	CCA AAG TCG CTC TGA GTT GTT ATC
H-c-Myc-R1	GAG CGG GAG AAA TGG ATA TG
H-c-Myc-F2	CCA AGA GGG TCA AGT TGG A
H-c-Myc-R2	GCA ATA TGG TGG AAAATA AC

2.2. RNA extraction and RT-qPCR

The Biotek RNA extraction kit was used to extract total RNA. Reverse transcription was performed by the RT reagent kit Takara. cDNAs were subjected to SYBR Green-based qPCR. Table 2 displays the primers used in the qPCR.

Table 2. Primer Sequence for qPCR

Gene name	Forward (5'-3')	Reverse (5'-3')
mGapdh	AGGTCGGTGTGAA CGGATTTG	TGTAGACCATGTA GTTGAGGTCA
mFasL	TTGCCTTGGTAGG ATTGG	ATGGACCTTGAG TTGGACTT
mLama4	ATGAGCTGCAAGG AAAACATATCC	CTGTTTCGTTGGC TTCCTGA
mThbs3	ATGGAGAAGCCG GAACCTTTGG	AGTGAGTAAAGC TGTCCGAATCT

2.3. Histology, haematoxylin and eosin (H&E), and immunohistochemistry (IHC) staining

Mice were sacrificed at 12 weeks. The stomachs of the mice were removed, the larger curvature was cut through, and PBS rinsed. After being dried and fixed for 24 hours in 4% poly-formaldehyde, tissues were embedded in paraffin. Sections were rehydrated after being deparaffinized for IHC. Blocking was conducted with 5% BSA for one hour. After that, sections were treated with an anti-Ki67 primary antibody (Abcam, ab6526, 1:1000) at 4°C overnight. After incubation, DAB and hematoxylin were used for staining.

2.4. Immunofluorescence (IF)

Sections were deparaffinized, rehydrated, and then antigen retrieved for IF. After that, sections were permeabilized using Triton X-100, blocked with 5% BSA, and left overnight at 4°C to be incubated with the primary antibody anti-p-AKT (CST, #4060-). After that, secondary antibody incubation lasted for one hour. DAPI was used as a counterstain for nuclei.

2.5. Statistics

At least three replications of each experiment were conducted. GraphPad Prism 6 was used to display the data as average ± SEM and to determine the P-value using the Student t test. P < 0.05. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 were defined.

3. Results

3.1. Disruption of *Setd2* aggravates gastric adenoma induced by c-Myc overexpression

Firstly, we knocked out *Setd2* in mice of c-Myc overexpression, which has been documented to induce GC. Both *Setd2* deletion and c-Myc overexpression were limited in *Atp4b⁺* gastric parietal cells. *Atp4b-cre*; *Myc^{OE/+}*; *Setd2^{F/+}* mice were crossed with *Setd2^{F/+}* mice. AMS mice and AM mice were selected by genotyping for histology and qPCR analysis (Figure 1A).

To examine the histology impact of *Setd2* deficiency, H&E and IHC staining were performed. H&E staining showed that 12-week-old AM mice had a minor reduction of chief and parietal cells and a modest elongation of epithelium, while 12-week-old AMS mice had more severe atrophy and hyperplasia, along with a significantly increased nuclear-cytoplasmic (N-C) ratio. IHC staining of Ki67 also confirmed that 12-week-old AMS mice had more cell proliferation than 12-week-old AM mice (Figure 1C). Altogether, our findings showed that in mice, c-Myc overexpression-induced stomach adenoma was exacerbated by *Setd2* loss.

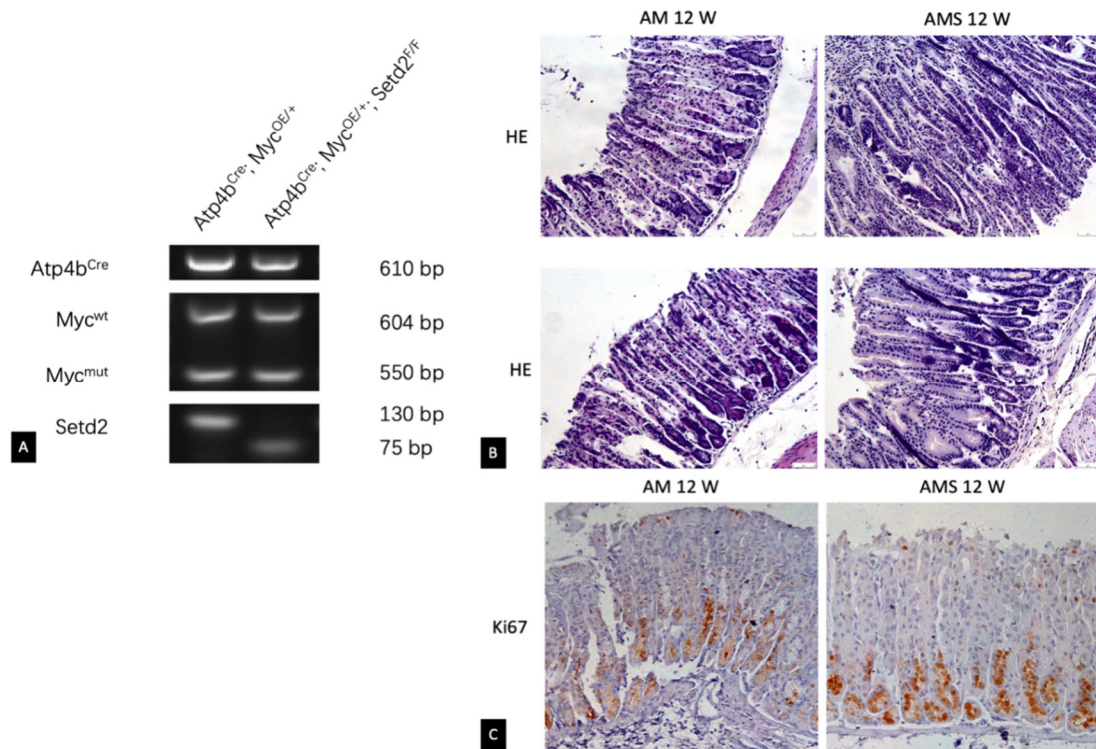


Figure 1. Setd2 deletion aggravates gastric adenoma induced by c-Myc overexpression (A) Identification of AM and AMS mice by genotyping. (B) H&E staining shows that AMS mice have an increased nuclear-cytoplasmic (N-C) ratio, more severe loss of parietal cells and chief cells, and an elongation of epithelium compared to AM mice. (C) IHC staining of Ki67 shows that AMS mice have a higher cell proliferation rate than AM mice.

3.2. Deletion of Setd2 promotes tumorigenesis by impacting PI3K/AKT signaling pathway

To understand the mechanism through which the loss of Setd2 aggravates gastric adenoma, the well-known cancer-related signaling pathway PI3K/AKT was

examined. RT-qPCR analysis demonstrated that FasL was upregulated, while Lama4 and Thbs3 were downregulated by Setd2 deficiency (Figure 2B). To validate the activation of PI3K/AKT pathway, IF staining was performed. The level of p-AKT in AMS mice was greater in AM mice (Figure 2A). These results illustrated that Setd2 deletion aggravated gastric adenoma by impacting PI3K/AKT signaling pathway.

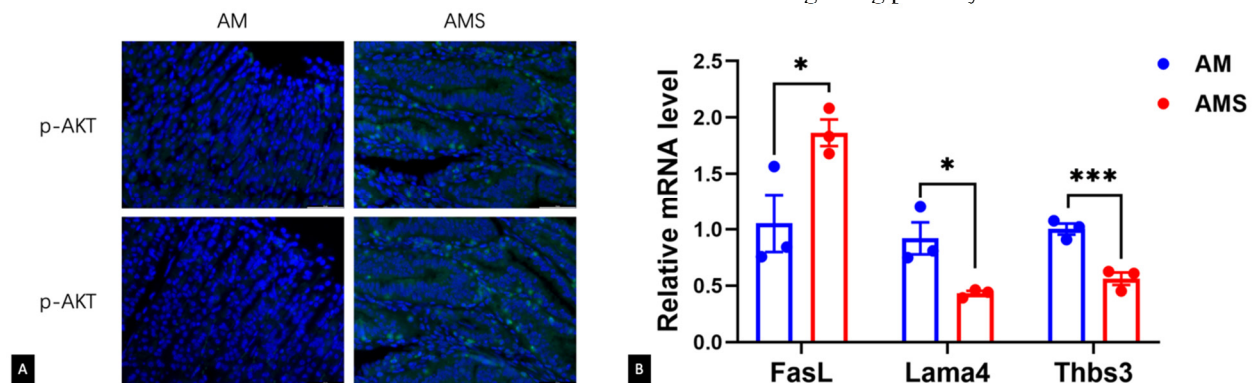


Figure 2. Setd2 disruption promotes tumorigenesis by impacting PI3K/AKT signaling pathway (A) Immunofluorescence staining shows a higher p-AKT protein expression level in AMS mice than in AM mice. (B) RT-qPCR analysis of PI3K/AKT-related genes shows that FasL is upregulated, while Lama4 and Thbs3 are downregulated.

4. Discussion

The significance of Setd2 in GC was identified by the Setd2 knockout and c-Myc overexpression mouse model. The atrophy and hyperplasia occurred in AMS mice at a younger age than AM mice, and AMS mice showed a

higher cell proliferation rate than AM mice at the same age, according to the results. In other words, Setd2 deficiency accelerated the progression of tumorigenesis and aggravated gastric adenoma. This could lead to new therapeutic approaches such as making Setd2 a target of GC treatments.

Histone modification may contribute to the malignant change of various cancers. Previous research has shown a correlation between Setd2 and GC, with patients with low Setd2 expression having a much poorer 5-year survival rate than those without.^[14] However, the mechanism by which low expression of Setd2 causes GC aggravation remains unknown. Our RT-qPCR and immunofluorescence results for the first time highlighted that Setd2 disruption aggravated the gastric adenoma and expedited the progression of tumors involving PI3K/AKT signaling pathway. Patients with Setd2 disruption and heightened PI3K/AKT signaling might benefit from tailored treatments aimed at modulating this pathway.

PI3K/AKT signaling pathway was proved to be activated by the deletion of Setd2, but it was beyond the scope of this study to answer whether gastric adenoma aggravation depends on this mechanism. To investigate if PI3K/AKT signaling pathway is sufficient to promote GC, avenues for future research include treating the Setd2 and C-myc double mutant mice with PI3K-specific inhibitors to observe its effect on GC.

Taken together, we proved that Setd2 deficiency aggravated gastric adenoma by impacting PI3K/AKT signaling pathway. Our study sheds light on epigenetic regulation behind the development of GC. In terms of clinical translation, it presents a possible treatment method for suppressing carcinogenesis in GC patients.

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