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Research article

A novel lnc-LAMC2-1:1 SNP promotes colon adenocarcinoma progression by targeting miR-216a-3p/HMGB3

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ABSTRACT

Single nucleotide polymorphisms (SNPs) was associated with altering the secondary structure of long non-coding RNA (lncRNA). Increasing reports showed that *lnc-LAMC2-1:1 SNP* played an important role in cancer development and invasion. This study is to elucidate the molecular function of *lnc-LAMC2-1:1 SNP rs2147578* promoting tumor progression in colon adenocarcinoma (COAD). In this study, we found that the *lnc-LAMC2-1:1 SNP rs2147578* was upregulated in COAD cell lines. Furthermore, *lnc-LAMC2-1:1 SNP rs2147578* promoted colon cancer migration, invasion, and proliferation. Interestingly, *lnc-LAMC2-1:1 SNP rs2147578* positively regulated *HMGB3* expression via *miR-216a-3p* in colon cancer cells. Functional enrichment analysis showed that targeting genes of m*iR-216a-3p* were enriched in regulating the pluripotency of stem cells, MAPK signaling pathway, TNF signaling pathway, neurotrophin signaling pathway, relaxin signaling pathway, and FoxO signaling pathway. Tumor Immune Estimation Resource (TIMER) database revealed that there was a significantly positive correlation between *HMGB3* expression and the infiltration of $CD8^+$ T cells, B cells, neutrophils, macrophages, and $CD4^+$ T cells. Finally, *HMGB3* overexpression was validated in external data. In conclusions, *lnc-LAMC2-1:1 SNP rs2147578* was involved in promoting COAD progression by targeting *miR-216a-3p/HMGB3*, and this study will provide a novel molecular target for COAD.

1. Introduction

Colorectal cancer (CRC) is a frequent malignancy that ranks third in terms of incidence, but second in terms of mortality in 2021 [1]. Colon adenocarcinoma (COAD) is a common malignant tumor with the highest incidence and the leading causes of cancer death in CRC. Although surgery, radiotherapy, chemotherapy, and immunotherapy have developed in recent years, the overall survival rate of COAD patients is still unsatisfactory [2]. This needs to explore effective therapies and identify novel prognostic biomarkers for COAD patients. Over 100 CRC risk loci have been identified by genome-wide association studies (GWASs) [3]. Single nucleotide polymorphisms (SNPs) usually consist of intronic and exonic sites. However, most of the susceptibility loci are located in noncoding regions [4]. Long non-coding RNA (lncRNA) is an RNA transcript with a length of more than 200 nucleotides and no protein coding ability [5]. LncRNA SNP plays an important role in tumor growth [6]. Increasing recent evidence has revealed that SNPs can influence susceptibility to disease by changing the expression of lncRNAs [7]. Previous studies have proved that *lnc-LAMC2-1:1 SNP rs2147578* affected tumor invasion and progression [8, 9, 10]. However, the role and molecular mechanism of *lnc-LAMC2-1:1 SNP rs2147578* in COAD have not been elucidated.

In this study, we investigated the role of *lnc-LAMC2-1:1 SNP rs2147578* promoting the proliferation, migration, and invasion in COAD. Our results showed that *lnc-LAMC2-1:1 SNP rs2147578* could positively regulated high-mobility group-box 3 (HMGB3) expression via binding *miR-216a-3p* in COAD. Moreover, *miR-216a-3p*-mediated regulatory network was constructed. *HMGB3* overexpression was validated in external data. Besides, we explored the correlation of the expression of

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HMGB3 with tumor immune infiltrating cells. This study revealed a novel mechanism of *lnc-LAMC2-1:1 SNP* promoting tumor behavior and suggested a potential new molecular target in COAD.

2. Materials and methods

2.1. Network construction of miR-216a-3p-mRNAs

To ananlyzed the mechanism of *miR-216a-3p*, firstly, the target genes of miR-216a-3p were predicted from the miRDB, miRTarBase and TargetScan databases using the Perl (5.26.3.0000) software program. Then, the regulatory network of miRNA-mRNA and hub genes was constructed using Cytoscape software 3.6.0 [11].

2.2. Functional enrichment analysis of target genes of miR-216a-3p

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using R (v3.6.3) and Perl (5.26.3.0000) software. The packages "colorspace" "dose" biocLite ("DOSE") "clusterProfiler" and "pathview" were installed. The selection criterion for significant GO and KEGG pathway terms was $P < 0.05$.

2.3. Cell culture and transfection

The SW480 cell line was obtained from COSMOBIO Company in China and was grown in DMEM supplemented with 10% FBS (GIBCO) and 1% antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) in a humidified atmosphere of 5% CO2 at 37 °C. For transfection assays, cells were seeded in 96-well plates and simultaneously transfected with the psiCHECK-2 vector and miRNA mimics using Lipofectamine 3000 (Invitrogen). All experiments were independently performed in triplicates.

2.4. Real-time qRT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and transcribed into cDNA using M-MLV reverse transcriptase (TaKaRa Bio, Japan) following the manufacturer's instructions. The primer sequences were as follows:

lncRNA LAMC2-wt:
5′-CATAGTCCCTCAGTGTGGGTCATTTTCATTAG-3′
lncRNA LAMC2 SNP:
5′-CATAGTCCCTCACTGTGGGTCATTTTCATTAG-3′
β-actin-S: 5′-CGTGACATTAAGGAGAAGCTG-3′
β-actin-AS: 5′-CTAGAAGCATTTGCGGTGGAC-3′
HMGB3-S: 5′-ATTCGGAATTCCGTATCTGGCCTTTTGAC-3′
HMGB3-AS: 5′-CGGTTACTCGGCTTACGCTTGGACTG-3′

2.5. Luciferase activity assay

StarBase online predicted the potential targets of *miR-216a-3p*. The wild-type (wt) or mutant (MUT) *HMGB3*-binding *miR-216a-3p* was subcloned into the pGL3 Basic vector (Promega). A total of SW480 cells were seeded in 24-well plates for 48 h. Mimics or inhibitors of *miR-216a-3p* (RiboBio, Guangzhou, China) were cotransfected with 10 μg pLUC-wt-HMGB3 or p LUC-MUT- HMGB3. The same procedure was used to assess the combined effect of *HMGB3* and *miR-216a-3p*. Luciferase reporter system (Promega) was used to assess the luciferase activity in SW480 cells.

2.6. TIMER database

TIMER database could provide the association between immune cells infiltrating and clinical factors, including gene expression, clinical outcomes, somatic mutations, and somatic copy number alterations [12]. We comprehensively investigated molecular characterization of tumor-immune interactions using TIMER database.

2.7. Validation of HMGB3 expression in The Human Protein Atlas database

The Human Protein Atlas (HPA; http://www.proteinatlas.org/), GSE8671, GSE9348, online databases TCGA, GEPIA and The Human Protein Atlas Database were used to validate the expression of *HMGB3* in COAD samples [13].

2.8. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Student's t-test and one-way analysis of variance were carried out to evaluate significant differences. P values <0.05 were considered to indicate statistical significance.

3. Results

3.1. Lnc-LAMC2-1:1 SNP rs2147578 could promote the progression and invasion in COAD

Accumulating evidences showed that *lnc-LAMC2-1:1 SNP rs2147578* increased the risk of ovarian cancer [9]. To evaluate the role of *lnc-LAMC2-1:1 SNP rs2147578*, transwell and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays were performed. Here, we found that *lnc-LAMC2-1:1 SNP rs2147578* significantly promoted proliferation in SW480 cells (Figure 1A). Moreover, the result showed that *lnc-LAMC2-1:1 SNP rs2147578* markedly increased the migration and invasion in SW480 cells (Figure 1B and 1C). These results showed that *lnc-LAMC2-1:1 SNP rs2147578* could promote the tumor development in COAD.

3.2. The lnc-LAMC2-1:1 SNP is a sponge of miR-216a-3p

A recent study revealed that *lncRNA SNPSNP rs140618127* contained a binding site for *miR-539-5p* promoting cell proliferation and tumor progression [14]. Previous study showed that *lnc-LAMC2-1:1 SNP rs2147578* can interact with *hsa-miR-128-3p*, *hsa-miR-216a-3p*, and *hsa-miR-368-3p* [10]. In this study, we revealed that the expression of *miR-216a-3p* was the most significantly downregulated. Moreover, *miR-216a-3p* was significantly in the *lnc-LAMC2-1:1 SNP rs2147578* group compared to the *lnc-LAMC2-1:1-wt* group (Figure 2A). To explore whether *lnc-LAMC2-1:1 SNP rs2147578* bind with *miR-216a-3p*, StarBase database online was used to predict the binding site between *lnc-LAMC2-1:1 SNP rs2147578* and *miR-216a-3p*. To validate this relationship, *lnc-LAMC2-1:1-wt* and *lnc-LAMC2-1:1-SNP rs2147578* were constructed and transfected into SW480 cells. As shown in Figure 2B, overexpression of *miR-216a-3p* led to obvious loss of luciferase activity in the *lnc-LAMC2-1:1-SNP rs2147578* group in the two cell lines, while it did not affect those in the *lnc-LAMC2-1:1-wt* group.

3.3. Regulatory network of miR-216a-3p

miRNAs post-transcriptionally suppress the target mRNA expression, mostly through interaction with the 3′ UTR [15]. To find the target gene of *miR-216a-3p*, twenty-six target genes of *miR-216a-3p* were predicted using miRDB, miRTarBase, and TargetScan by Perl software. Investigating the interaction among genes, we used Cytoscape [16] (version 3.6.1) to construct the regulatory network, as shown in Figure 3A. The PPI network was constructed using the STRING database [17] (http://string-db.org) with a total of 26 target genes of *miR-216a-3p*. After removing the isolated and partially connected nodes, a complex network of target genes was identified as the top 10 hub genes using the cytoHubba [18] (Figure 3B).

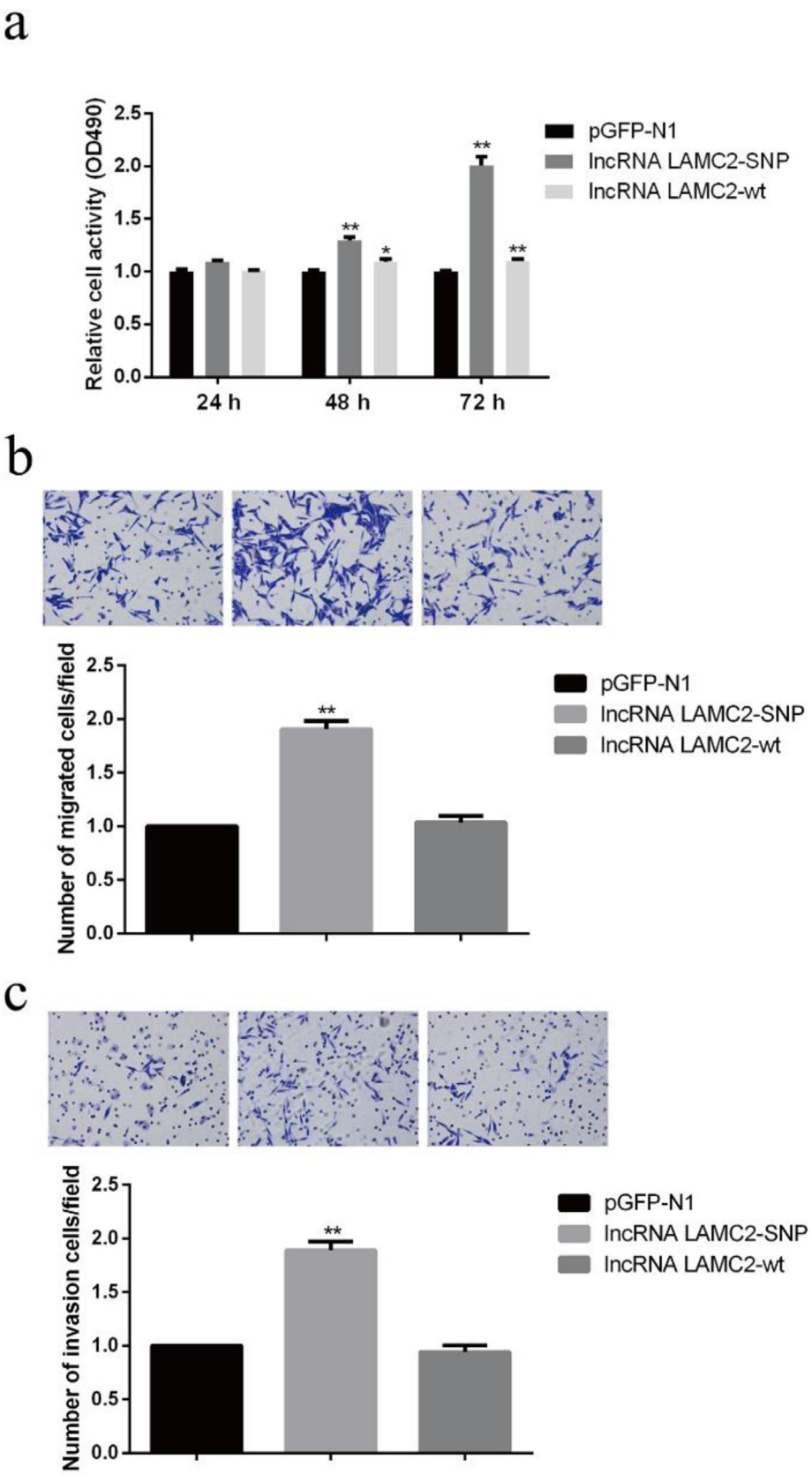

Figure 1. The *lnc-LAMC2-1:1 SNP* is increased and promotes colon cancer progression. Knockdown of *lnc-LAMC2-1:1 SNP* inhibits (a) proliferation, (b) migration, and (C) invasion in COAD. *P < 0.05, **P < 0.01 compared with the indicated control group.

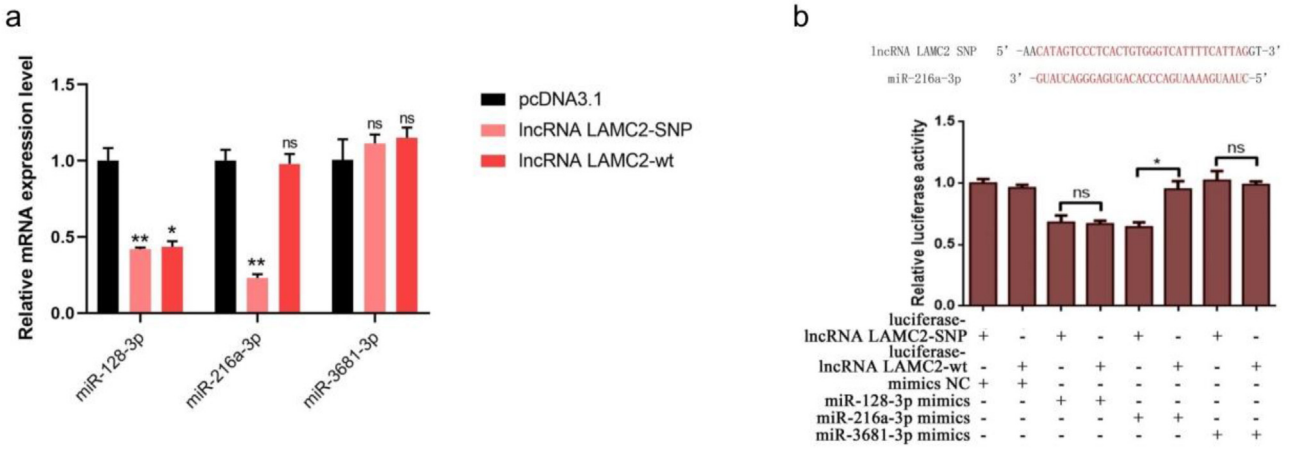

Figure 2. The *lnc-LAMC2-1:1 SNP* is a sponge of *miR-216a-3p*. Bar graphs of (a) and (b) show the relative luciferase activity of vectors containing SW480. A dual-luciferase reporter assay was performed, and the co-transfection of *lnc-LAMC2-1:1 SNP* and *miR-216a-3p* reduced the luciferase activity.

3.4. Functional enrichment analysis

In the present study, GO functional enrichment and KEGG pathway analyses were performed to explore the molecular function of the target genes of *miR-216a-3p*. Functional enrichment analysis with a P-value of 0.05 was obtained. The results were shown in Figure 4A. We found that the phospholipid transporter activity of the target gene was the most abundant. KEGG pathways analysis were mainly enriched in pathways regulating the pluripotency of stem cells, MAPK signaling pathway, TNF signaling pathway, neurotrophin signaling pathway, relaxin signaling pathway, and FoxO signaling pathway. These results were shown in Figure 4B.

3.5. The lnc-LAMC2-1:1 SNP positively regulates HMGB3 by sponging miR-216a-3p

High-mobility group box 3 (HMGB3), a member of the high-mobility group box (HMGB) family, was reported to be over-expressed in cancers [19, 20]. StarBase online predicted the targets of *miR-216a-3p*, and the results showed *HMGB3* had potential complementary sequences of *miR-216a-3p* (Figure 5A). qRT-PCR assay displayed that *HMGB3* was negatively regulated by *miR-216a-3p* (Figure 5B). In addition, this result showed that the expression of *HMGB3* level in SW480 cells was positively regulated by *lnc-LAMC2-1:1 SNP rs2147578* (Figure 5C). To validate the relationship of *miR-216a-3p* with *HMGB3*, the luciferase reporter assay showed that *miR-216a-3p* upregulation significantly led to reduction in luciferase activity in the *HMGB3-WT* group, while its efficacy was lost when the binding sites were mutated (Figure 5D).

3.6. Correlation of the expression of HMGB3 with immune cell infiltration

Tumor immune cell was closely associated with COAD progression. In this study, we explored the correlation between the expression of *HMGB3* and immune cell infiltration using the TIMER database. There was a significantly positive correlation between *HMGB3* expression and the infiltration of $CD8^+$ T cells (Cor = 0.12, p = 1.57e − 2; Figure 6A), $CD4^+$ T cells (Cor = 0.162, p = 1.1e − 3; Figure 6B), macrophages (Cor = 0.185, p = 1.79e − 4; Figure 6C), neutrophils (Cor = 0.122, p = 1.47e − 2; Figure 6D), and B cells (Cor = 0.122, p = 1.42e − 2; Figure 6F). However, dendritic cells did not show significant expression of *HMGB3* (Cor = 0.088, p = 7.71e − 2; Figure 6E).

3.7. Validation of HMGB3 expression in external data

To test *HMGB3* expression in the present study, a total of 64 samples in GSE8671 including 32 COAD samples and 32 normal samples were screened out. The results revealed that COAD patients had markedly high expression of *HMGB3* (Figure 7A). Moreover, we found

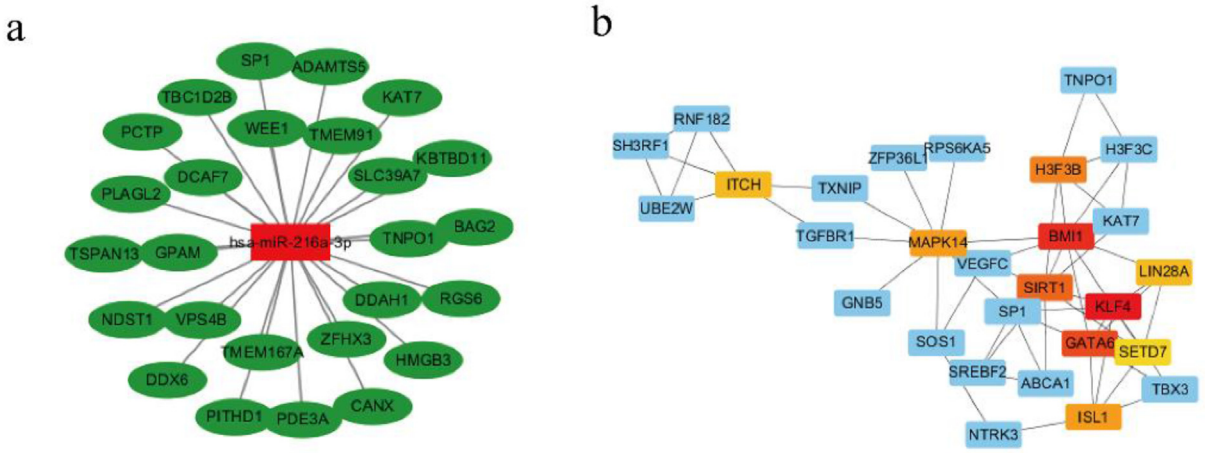

Figure 3. The regulatory network of *miR-126-3p* and PPI (a) The network of *miR-126-3p* target genes. Red indicates *miR-126-3p*, and green indicates genes. (b) protein-protein interaction of target genes.

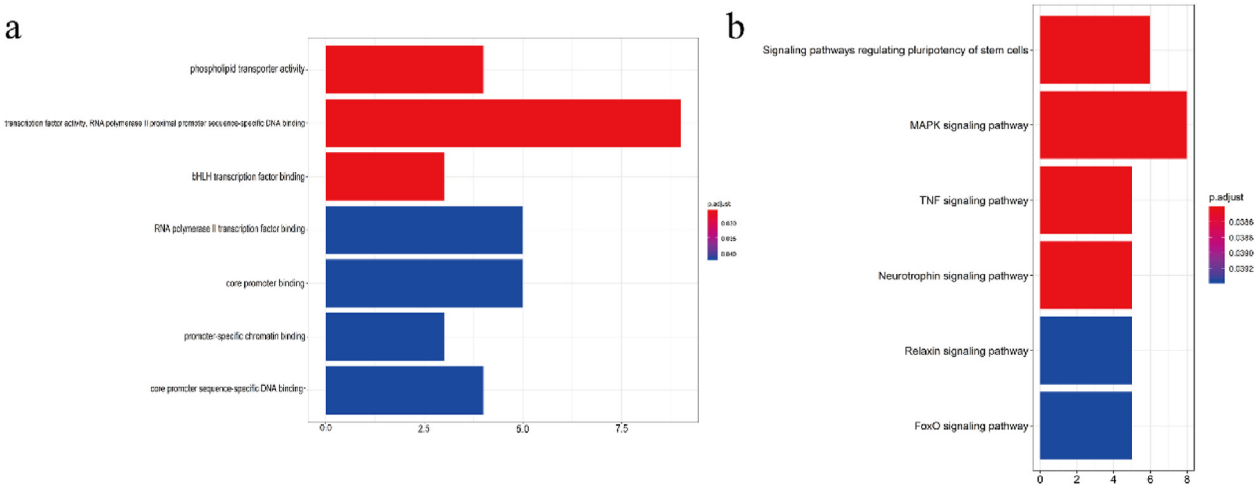

Figure 4. GO and KEGG functional enrichment analysis (a) GO enrichment significance items (b) Gene ratio and KEGG pathway items. Red indicates upregulated genes, and green indicates downregulated genes.

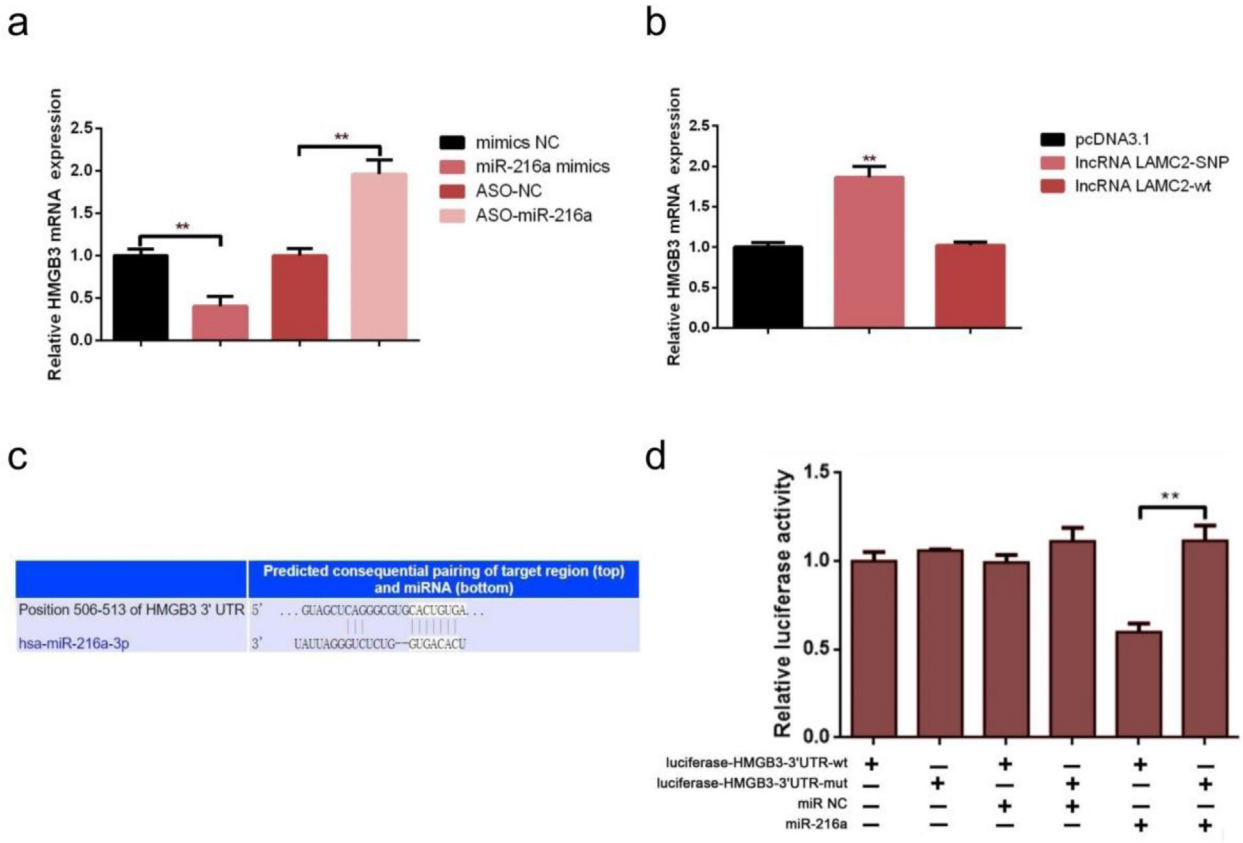

Figure 5. The *lnc-LAMC2-1:1 SNP* positively regulates *HMGB3* by sponging *miR-126-3p* (a) mRNA expression of *HMGB3* in SW480 cells. Cells were transfected with mimics of NC, *miR-126-3p* mimics, ASO-NC, and ASO-miR-126-3p. (b) mRNA expression of *HMGB3* in SW480 cells. Cells were transfected with pcDNA3.1, *lnc-LAMC2-1:1 SNP*, and *lnc-LAMC2-1:1-wt*. (c) Complementary sequences of *HMGB3* and *miR-126-3p* in the StarBase database. (d) A dual-luciferase reporter assay was performed, and the co-transfection of *miR-126-3p* and *HMGB3* reduced the luciferase activity.

that *HMGB3* expression was upregulated in tumor samples compared to that in normal samples of GSE9348 (Figure 7B). This result was also found that *HMGB3* expression was validated in 286 tumor samples compared to 41 normal samples in online TCGA database (Figure 7D). We used the GEPIA online database to validate the expression of *HMGB3*, and we found that the results were consistent with those mentioned above (Figure 7C). Immunohistochemistry showed that *HMGB3* was performed based on the HPA database, and the results revealed that *HMGB3* was overexpressed in tumor samples than in normal samples (Figure 7E-F).

4. Discussion

LncRNAs are regulators of transcription and are increasingly recognized to play a role in cancer biology [21]. For example, Zhong et al found that LINC00636 also promotes lymph node metastasis and carcinogenesis

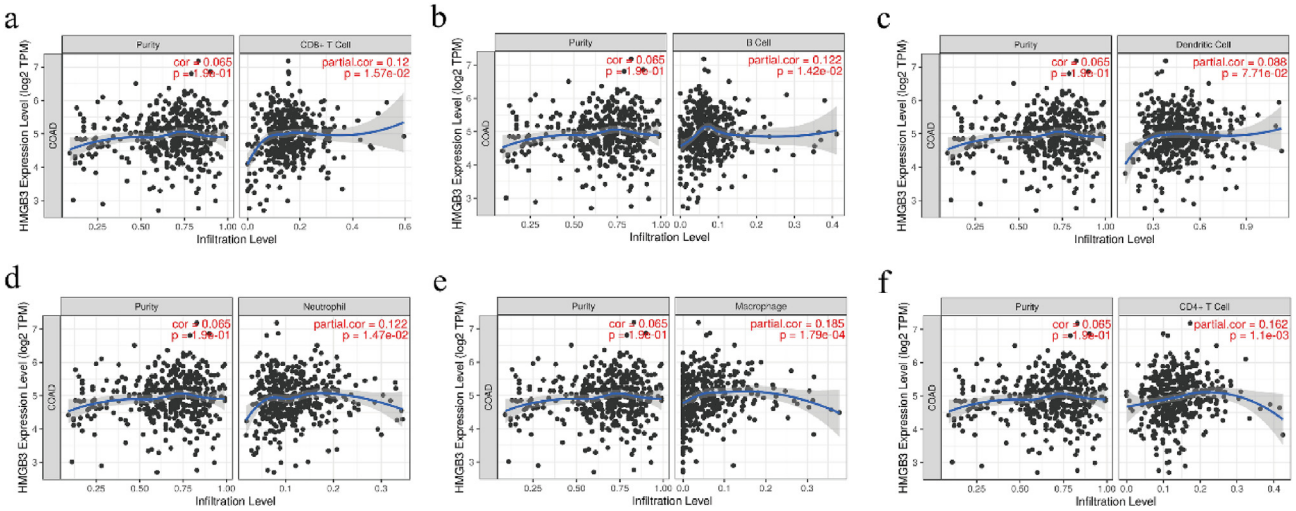

Figure 6. Correlation of *HMGB3* expression with immune cell infiltration. Association of the expression of (a) $CD8^+$ T cells, (b) $CD4^+$ T cells, (c) macrophages, (d) neutrophils, (e) dendritic cells and (f) B cells with *HMGB3* expression were shown in this study.

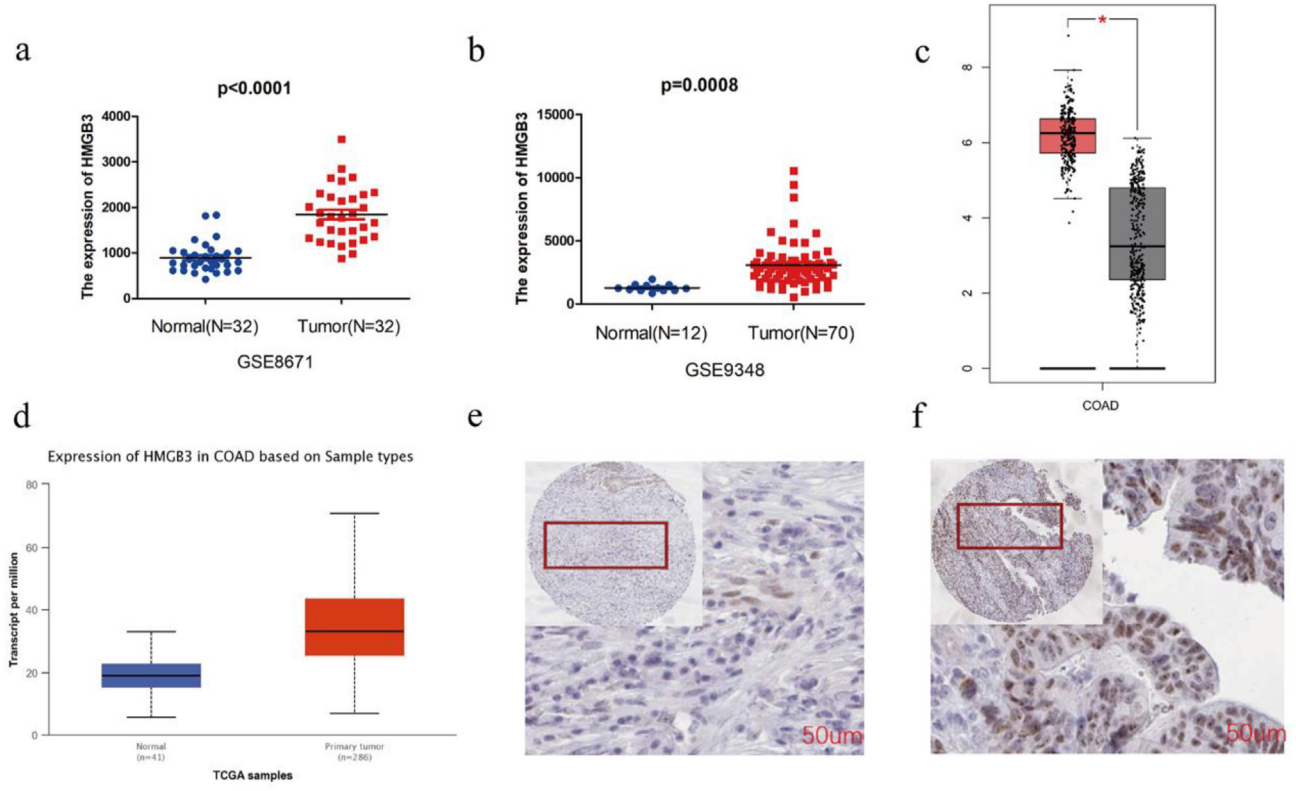

Figure 7. External data validation of HMGB3 expression. *HMGB3* expression was validated in (a) GSE8671, (b) GSE9348, and (c) GEPIA database and (d) TCGA database. (e) and (f) show the expression of *HMGB3* in COAD tissues on immunohistochemistry staining.

by regulating NM23 [21]. Meanwhile, the *lnc-LAMC2-1:1 SNP rs2147578* plays essential roles in many diseases [9, 22, 23]. To the best of our knowledge, no study has investigated the mechanism of *lnc-LAMC2-1:1 SNP rs2147578* in colorectal cancer. In this study, we found that *lnc-LAMC2-1:1 SNP rs2147578* could promote colon cancer proliferation, migration, and invasion. This study aimed to analyze the mechanism of *lnc-LAMC2-1:1 SNP rs2147578* in COAD and explore the novel ceRNA network of *lnc-LAMC2-1:1 SNP rs2147578/miR-216a-3p/HMGB3*.

There are abundant miRNA binding sites in LncRNA molecules, which act as sponges, counteracting the effect of miRNA on its target genes and increasing the expression level of target genes [24]. We studied the association of miRNAs in COAD affected by *lnc-LAMC2-1:1 SNP rs2147578*. A previous study reported that *lnc-LAMC2-1:1 SNP rs2147578* may affect *miR-128-3p*, *hsa-miR-216a-3p*, and *hsa-miR-368-3p* binding and confer a high risk of colorectal cancer [10]. Hence, we selected these three miRNAs for our study. We used a luciferase reporter assay to validate the target association between *lnc-LAMC2-1:1 SNP rs2147578* and *miR-216a-3p*. The results showed that *lnc-LAMC2-1:1 SNP rs2147578* significantly repressed the expression of *miR-216a-3p* compared to that in the *lnc-LAMC2-1:1-wt* group. *MiR-216a-3p* has been reported as a tumor suppressor in colon cancer [24], gastric cancer [25, 26], breast cancer [27] and pancreatic ductal adenocarcinoma [28]. In order to verify the

biological relationship between *lnc-LAMC2-1:1 SNP rs2147578* and *miR-216a-3p* in COAD, we carried out functional studies. The activity, invasion and proliferation of SW480 cells transfected with *lnc-LAMC2-1:1SNP rs2147578* were significantly enhanced. Therefore, *lnc-LAMC2-1:1 SNP rs2147578* acts as a sponge to regulate COAD by combining with *miR-216a-3p*.

Previous studies have suggested that *HMGB3* is an oncogene in multiple cancers, including breast cancer [20, 29, 30], gastric cancer [31, 32], colon carcinoma [19], esophageal squamous cell carcinoma [33], ovarian cancer [34], non-small cell lung cancer [35, 36], liver cancer [37, 38, 39], thyroid carcinoma [40] and prostate cancer [41]. We predicted that *HMGB3* had *miR-216a-3p*-binding sites in the StarBase database. In this study, we found that *HMGB3* was downregulated in the *miR-216a-3p* mimics group, and the luciferase activity showed that *HMGB3* could bind to *miR-216a-3p*. In our experiment, the RT-qPCR assay showed that *lnc-LAMC2-1:1 SNP rs2147578* could promote *HMGB3* expression, suggesting the potential ceRNA of *lnc-LAMC2-1:1 SNP rs*2147578/miR-*216-a-3p/HMGB3*. We also investigated that *HMGB3* was highly expressed in COAD, which is also in agreement with the data of GSE8671, GSE9348, TCGA database, the GEPIA database and the HPA database online, indicating that high expression of HMGB3 might contribute to COAD development. To sum up, the role of *lnc-LAMC2-1:1 SNP rs2147578* in COAD may be related to the expression of *miR-216a-3p/HMGB3*.

Moreover, previous studies have suggested that *HMGB3* is associated with immune regulation [42, 43, 44, 45]. In the current study, we also found that *HMGB3* affected the expression of $CD8^+$ T cells, B cells, neutrophils, macrophages and $CD4^+$ T cells, which is expected to be explored in future studies.

5. Conclusion

In conclusion, our study highlighted that *lnc-LAMC2-1:1SNP rs2147578* promoted the progression of COAD by regulating *miR-216a-3p/HMGB3* axis as a ceRNA. This study elucidated a new mechanism for development of COAD and indicated a novel target for treatment of COAD. Furthermore, future studies are required to investigate the molecular mechanism, biomarker value and therapeutic significance of lnc-LAMC2-1:1SNP rs2147578 in COAD.

Declarations

Author contribution statement

Daqing Sun and Jianming Wei: Contributed reagents, materials, analysis tools and data.

Fulong Ji and Zhiwei Yao: Conceived and designed the experiments; Wrote the paper.

Chunxiang Liu and Lushun Ma: Performed the experiments.

Siqi Fu, Bingbing Ren and Yong Liu: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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