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LETTER TO THE EDITOR

Long non-coding RNA MILNR1 retards colorectal cancer growth by inhibiting c-Myc

Dear Editor,

Colorectal cancer (CRC) is a prevalent subtype of carcinoma which accounts for about 10% of all cancer diagnosis and is the third leading cause of cancer-related deaths globally [1].However, a great number of pathogenic factors associated with CRC development are still elusive and need further investigation. In the last several years, long non-coding RNAs (lncRNAs) were deemed to be a critical driving force for the progression of CRC [2-6], and c-Myc was discovered to be a functional partner of lncRNAs [7]. Nucleoporin 88 (NUP88) is a component of nucleoporins, which is upregulated in tumor tissues including CRC [8]. It is reported that NUP88 interacts with vimentin and protects its serine residue (Ser83) from dephosphorylation, thereby promoting cell proliferation [9]. Another form of vimentin phosphorylation, namely the phosphorylation at Ser39, results in the development of cell migration and tumor metastasis [10]. Here we report a lncRNA MILNR1, which was found to be down-regulated in CRC cells, that could regulate *NUP88 in cis* by interacting with c-Myc and inhibit vimentin phosphorylation and CRC growth.

We first analyzed the expression levels of lncRNAs from both colon and rectum adenocarcinoma patients' samples and their adjacent normal colorectal tissues from The Cancer Genome Atlas (TCGA) database. We found that a non-coding transcript AC087500.2 was down-regulated in colon and rectum adenocarcinoma samples, suggesting its potential tumor-suppressive role, which was chosen for further study (Figure 1A). The lncRNA was then designated as MILNR1 (c-Myc interacting long non-coding RNA 1), by us, because it was demonstrated to be interacted with c-Myc. Consistent with this finding, we also discovered that the expression of MILNR1 was lower in 10 surgically resected CRC samples than their adjacent normal colorectal tissues (Figure 1B).

Next, we determined the subcellular distribution of MILNR1 and found that MILNR1 was predominantly localized in the nucleus of HCT116 CRC cells (Figure 1C). We then selected the genes adjacent (< 150k bp) to MILNR1 on the Human Chromosome 17, including *NUP88, RABEP1, SCIMP, USP6, ZNF232 and ZNF594*, and examined whether their expressions were affected by MILNR1. We discovered that *NUP88*, rather than any other adjacent genes, was repressed by MILNR1 overexpression (Figure 1D), suggesting that MILNR1 modulates *NUP88* expression *in cis*. Consistently, the mRNA level of *NUP88* was up-regulated when we silenced MILNR1 using its antisense oligonucleotide (ASO) (Figure 1E). The protein level of NUP88 was also decreased in MILNR1 overexpressed cells and was elevated in MILNR1 deficient cells (Figure 1F and G). Furthermore, to avoid the off-target effect of ASO, we constructed ASO resistant MILNR1 overexpression plasmid by which the protein level of NUP88 in MILNR1 silenced cells was rescued, indicating that *NUP88* was indeed suppressed by MILNR1 (Figure 1H).

Subsequently, we explored the underlying mechanisms by which MILNR1 regulates *NUP88*. After scanning the genomic sequence upstream of *NUP88*, we found a c-Myc response element sequence (CACGTG) at the -411 to -406 site of the *NUP88* promoter region (Figure S1A). We thereby investigated whether *NUP88* was regulated by c-Myc. We found that when c-Myc was knocked down by short hairpin RNA (shRNA), both the mRNA and protein levels of *NUP88* were decreased (Figure S1B and C). On the contrary, when exogenous c-Myc was overexpressed, the mRNA and protein levels of *NUP88* were upregulated

Abbreviations: ASO, antisense oligonucleotide; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; COAD, Colon Adenocarcinoma; CRC, Colorectal cancer; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic acid; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GENT, Gene Expression database of Normal and Tumor tissues; KD, knocked-down; lncRNA, long non-coding RNA; MILNR1, c-Myc interacting long non-coding RNA 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NUP88, nucleoporin 88; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; RIP, RNA immunoprecipitation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; TCGA, The Cancer Genome Atlas

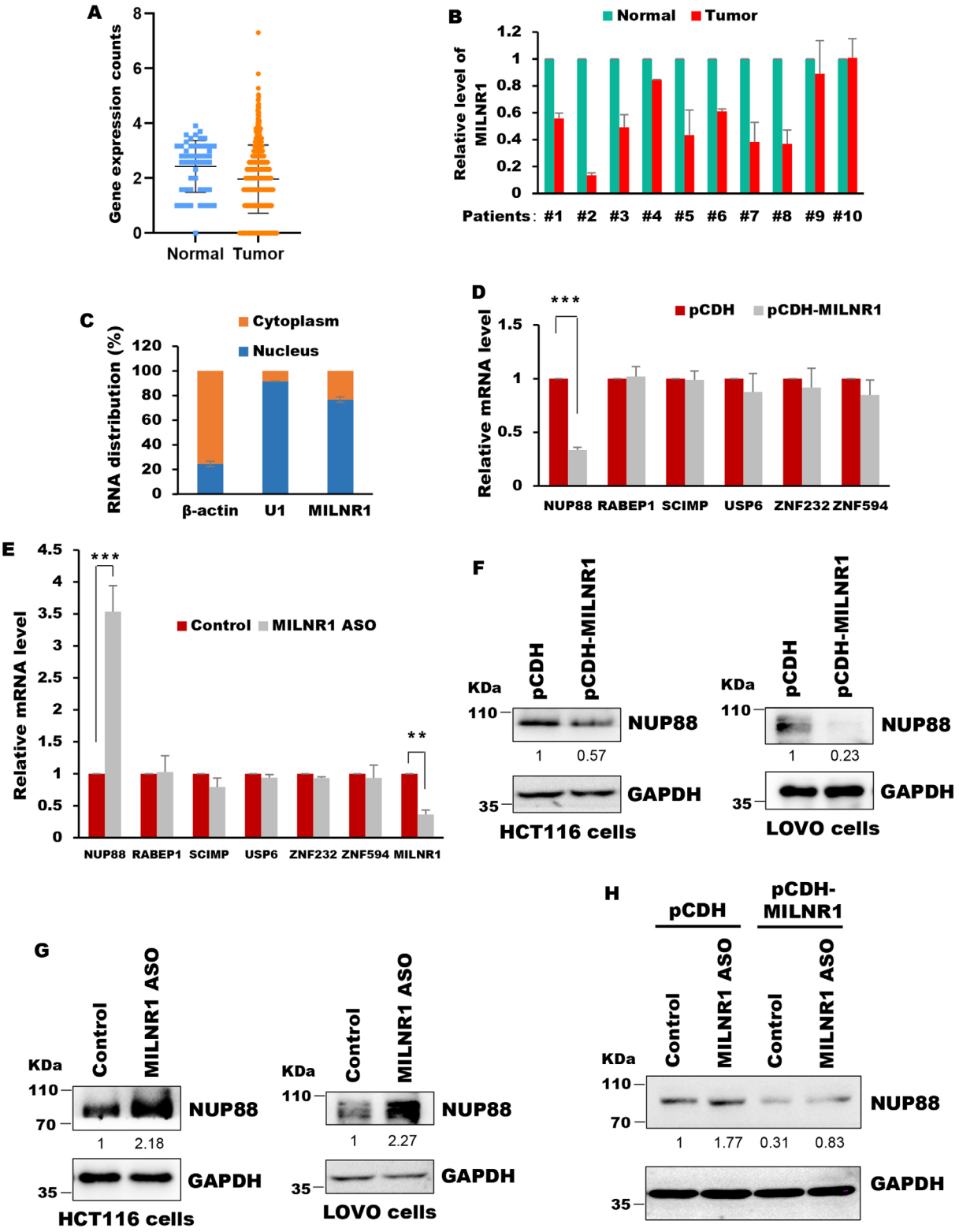

FIGURE 1 **MILNR1 inhibits NUP88 in CRC cells.** (A) Gene expression counts of MILNR1 in colorectal adenocarcinoma and adjacent normal tissues from RNA-seq data in the TCGA database. (B) qPCR analysis of MILNR1 expression in cancerous and normal colorectal tissues from clinical CRC samples. (C)Subcellular distribution of MILNR1 in HCT116 cells, and RNA levels of MILNR1, U1 (small nuclear RNA) and *β*-actin mRNA were analyzed by qPCR. HCT116 refers to a colorectal cancer cell line. (D) qPCR analysis of MILNR1 adjacent genes in MILNR1 overexpressed HCT116 cells. pCDH refers to a plasmid expressing gene of interest under CMV promoter. (E) qPCR analysis of MILNR1 adjacent genes in MILNR1 knocked-down HCT116 cells. (F) Western blot analysis of NUP88 in MILNR1 overexpressed cells. HCT116 and LOVO refers to colorectal cancer cell lines. (G) Western blot analysis of NUP88 in MILNR1 knocked-down cells. (H) Western blot analysis of NUP88 in MILNR1 knocked-down and/or ASO resistant MILNR1 overexpressed HCT116 cells. Abbreviations: qPCR: quantitative real-time PCR

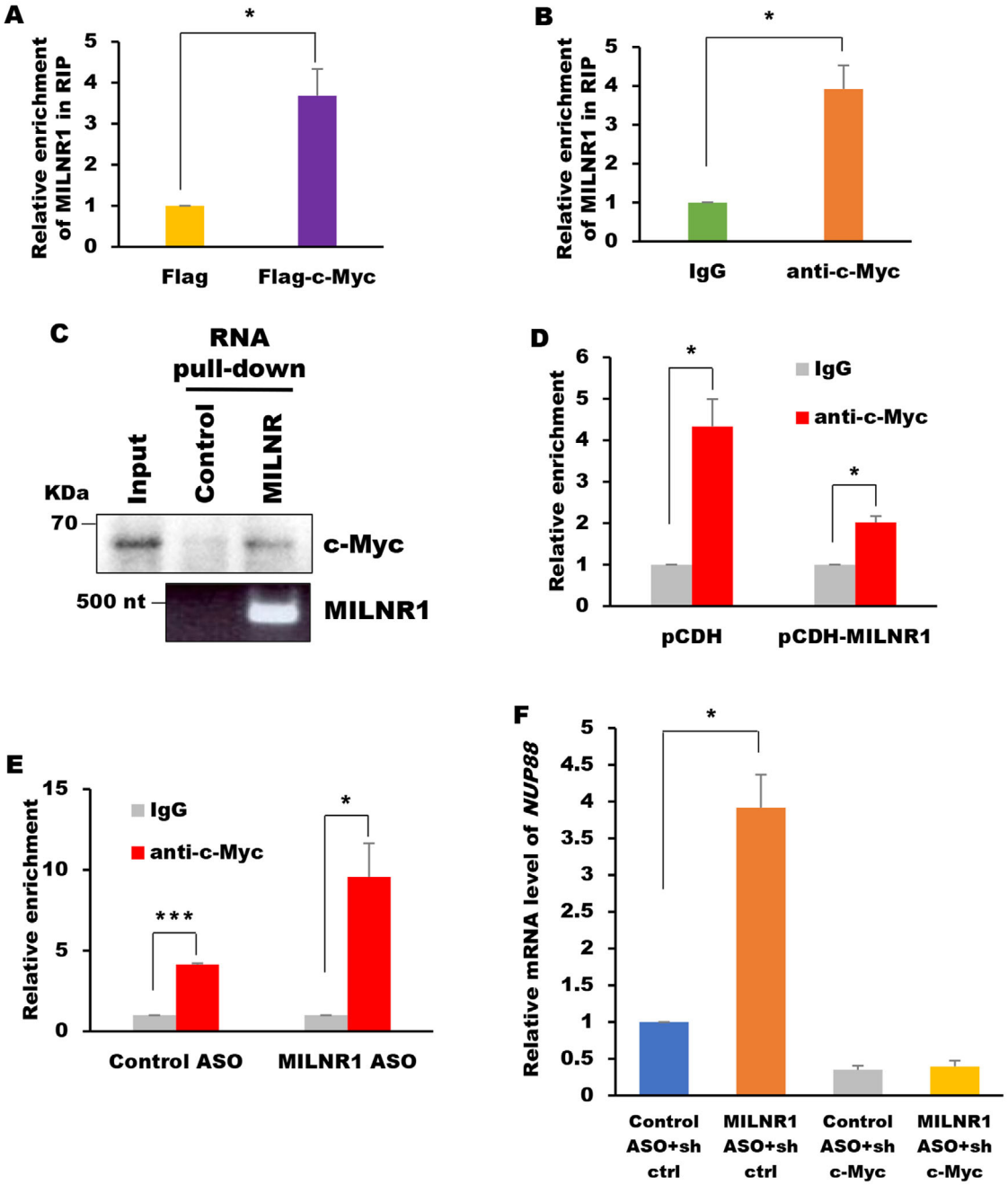

FIGURE 2 **MILNR1 binds to c-Myc and inhibits its transcription**. (A) RIP assays with Flag antibody. (B) RIP assays with c-Myc antibody or IgG. (C) RNA pull-down assay with *in vitro* transcribed biotin-labeled MILNR1. (D) ChIP assays with c-Myc antibody or IgG in MILNR1 overexpressed HCT116 cells. (E) ChIP assays with c-Myc antibody or IgG in MILNR1 knocked-down HCT116 cells. (F) qPCR analysis of *NUP88* in MILNR1 and/or c-Myc knocked-down HCT116 cells

Abbreviations: RIP: RNA immunoprecipitation; ChIP: chromosome immunoprecipitation.

(Figure S1D and E). To determine whether the above-discussed putative c-Myc binding site responded to c-Myc, we performed a chromatin immunoprecipitation (ChIP) assay, and our findings confirmed that the promoter region of *NUP88* gene containing the E-box sequence indeed interacted with the c-Myc protein (Figure S1F). Furthermore, we constructed luciferase reporter plasmids containing the *NUP88* promoter region with the potential c-Myc binding site or its mutant. We found that the reporter activity was enhanced by exogenous c-Myc but was abolished when mutations were introduced into the c-Myc response element mentioned above (Figure S1G). Taken together,

these data indicated that c-Myc mediated the transcriptional activation of *NUP88*.

The regulation of *NUP88* expression by both MILNR1 and c-Myc led us to further analyze the potential interaction between them at the *NUP88* promoter. We found that both ectopic and endogenous c-Myc co-precipitated with MILNR1 by carrying out RNA immunoprecipitation (RIP) assays (Figure 2A and B). Moreover, we confirmed that c-Myc protein could be pulled down by *in vitro* transcribed MILNR1 (Figure 2C), indicating that MILNR1 could bind to c-Myc protein.

To further assess whether MILNR1 could inhibit the transcriptional activity of c-Myc, we performed ChIP assay in MILNR1 overexpressed or knocked down cells. We found that MILNR1 overexpression reduced the c-Myc binding ability at the *NUP88* promoter (Figure 2D), and consistently, a higher occupancy of c-Myc protein on the same site was detected when MILNR1 was deficient (Figure 2E). These results demonstrated that MILNR1 repressed c-Myc binding to the *NUP88* promoter and thereby reduced *NUP88* expression *in cis*. We then sought to determine whether MILNR1 would inhibit *NUP88* expression via interaction with c-Myc. We found that the *NUP88* mRNA alterations in MILNR1 knocked-down (KD) cells could be reversed by *c-Myc* shRNA (Figure 2F), demonstrating that c-Myc acted as a mediator for MILNR1 to regulate *NUP88* expression.

Next, as an upstream regulator of NUP88, we examined whether MILNR1 would affect vimentin phosphorylation. First, we overexpressed MILNR1 and found that the phosphorylation of vimentin at Ser39 and Ser83 were both reduced, whereas the phosphorylation at Ser56 exhibited no significant change ($P = 0.80$, Figure S2A and B). On the contrary, when MILNR1 was silenced, the phosphorylation on vimentin, except for Ser56, were elevated (Figure S2C and D). Furthermore, if NUP88 was knocked down in MILNR1 silencing cells, the phenotype of vimentin phosphorylation could be rescued, indicating that NUP88 could indeed mediate the effect of MILNR1 on vimentin phosphorylation (Figure S2E). As vimentin phosphorylation at Ser39 was discovered to be involved in tumor cell metastasis, we continued our investigation by performing wound-healing assays and Transwell invasion assays to evaluate the potential role of MILNR1 in CRC cell migration and invasion. However, neither the overexpression nor knockdown of MILNR1 exhibited a strong effect on the speed of cell migration and invasion (Figure S3A-D), which led us to investigate the potential function of MILNR1 in CRC cell proliferation via vimentin phosphorylation at Ser83.

We then assessed whether MILNR1 could inhibit CRC cell proliferation by regulating *NUP88* expression. We discovered that the acceleration of tumor cell proliferation rate when MILNR1 was knocked down could be partially rescued by silencing *NUP88* in anchorage-dependent cell proliferation assays (Figure S4A-C). Our hypothesis was further validated by cell viability comparison between MILNR1 KD and MILNR1 and NUP88 double KD cells in MTT assays (Figure S4D and E). Next, we examined the MILNR1 expression in normal and tumoral colorectal cells. Analysis of several colorectal cell lines showed that cancer cells, such as HCT116, LOVO, and RKO cells, expressed lower levels of MILNR1 than normal cells (Figure S4F). These results suggested that the MILNR1-NUP88 axis played a tumor-suppressive role through the inhibition of cancer cell proliferation (Figure S5). However, Kaplan-Meier analysis revealed that the level of neither NUP88 nor MILNR1 in colon cancer tissues were significantly associated with patient prognoses ($P = 0.22$ and 0.97, respectively, Figure S6A and B), although colon cancer patients with lower NUP88 or higher MILNR1 expression showed a overall better survival probability, which is consistent with our hypothesis. We also found that NUP88 expression was elevated in tumoral tissues including colon cancer in Gene Expression database of Normal and Tumor tissues (GENT) database (Figure S6C).

Taken together, in this study we uncovered a lncRNA MILNR1 which was involved in CRC growth. We found that MILNR1 could suppress tumor cell proliferation by interacting with c-Myc *in cis*, inhibited its transcriptional activity, regulated *NUP88* expression and vimentin phosphorylation. Our findings further showed the MILNR1-c-Myc-NUP88 pathway in CRC development and the MILNR1-NUP88 axis provided potential targets for CRC treatment.

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Not applicable.

DECLARATIONS

AUTHOR CONTRIBUTIONS

H.G., Y.L. and WL.H. designed this study. H.G. performed most of the experiments with the help of Y.Xia., LL.G., ZF.W., S.W., Y.Xu., YQ.Z., and J.H., and analyzed the data with Y.L. and WL.H. H.G. and WL.H. wrote the manuscript. All authors approved the final version manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The institutional review boards of Anhui Medical University approved the study. Written informed consent was obtained from each colorectal cancer patient.

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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