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Research Article
Significance of LncRNA NEAT1 Alterations During Treatment of Nonalcoholic Fatty Liver Disease and its Association with Gut Microbiota

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Abstract

Background and Objective: Nonalcoholic fatty liver disease can directly lead to decompensated cirrhosis and may affect the progression of other chronic liver diseases. To investigate the significance of alterations of LncRNA NEAT1 during treatment of nonalcoholic fatty liver disease (NAFLD) and its relationship with gut microbiota (GMB). **Materials and Methods:** Sixty-five diabetic patients with NAFLD (observation group, OG) and 51 patients with simple diabetes (control group, CG) admitted to Linyi People's Hospital between March, 2019 and January, 2020 were selected. The NEAT1 expression in both cohorts was detected. Patients in OG were followed up for 1 year to analyze the connection between NEAT1 and NAFLD recurrence. Additionally, SD rats were purchased for NAFLD modelling and adenovirus with silenced NEAT1 expression was used for intervention to observe the alterations of liver function, inflammatory cytokines (ICs), oxidative stress (OS) and GMB. **Results:** Serum NEAT1 was higher in OG than in CG and decreased gradually during treatment. The NEAT1 was also higher in relapsed patients compared with those without recurrence. And in *in vitro* experiments, NEAT1 showed elevated expression in NAFLD rats, while under the intervention of NEAT1-silenced adenovirus, the liver function, inflammatory reaction and OS of NAFLD rats were alleviated, with decreased aerobic bacteria and increased anaerobic bacteria in GMB. **Conclusion:** The NEAT1 keeps at a high level in NAFLD, with a close connection with the disease progression of NAFLD. Silencing NEAT1 expression can effectively inhibit the inflammatory reaction and OS in NAFLD and regulate the imbalance of GMB.

Key words: LncRNA NEAT1, nonalcoholic fatty liver disease, gut microbiota, inflammatory factors, oxidative stress, anaerobic bacteria, genetic susceptibility

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) refers to a pathological syndrome of excessive deposition of fat in hepatocytes caused by factors rather than alcohol and other definite liver damaging factors, which is closely related to insulin resistance and genetic susceptibility[1]. Investigations show that NAFLD has become the main cause of chronic liver disease in many developed countries with the global prevalence of obesity and its related metabolic syndrome[2]. In the general adult population, the prevalence of NAFLD is already as high as 10-30%, of which more than 10% will eventually lead to hepatic cirrhosis[3]. Clinically, NAFLD is not only the prime reason for decompensated cirrhosis and hepatocellular carcinoma but also a key factor affecting the progression of other chronic liver diseases[4]. The NAFLD kills more than 300,000 patients worldwide every year with an ever-higher mortality rate[5]. In clinical practice, NAFLD has been defined as a new challenge in the contemporary medical field and researchers are constantly exploring new diagnoses and treatment schemes for the disease.

In recent years, molecular pathogenesis research has gradually become a hot spot in modern medical research, among which LncRNA, as a genetic RNA substance in the human body, is essential in a wide spectrum of diseases[6,7]. For NAFLD, there are also many studies confirming the role of LncRNA in it[8,9]. We found that LncRNA NEAT1 was closely related to liver function[10], which can accelerate the occurrence of liver fibrosis via targeting miR-148a-3p and miR-22-3p[11]. Therefore, we speculate that NEAT1 is also closely related to NAFLD. Moreover, gut microbiota (GMB) is known to be linked to NAFLD and NEAT1 has also been pointed out to be closely related to flora infection[12]. Accordingly, this research project analyzed the connection between NEAT1 and GMB in NAFLD to further elucidate the potential of NEAT1 in NAFLD diagnosis and treatment, to provide reliable reference and guidance for clinical practice.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of the Infection Control Centre, Linyi People's Hospital from March 2019-January 2020.

Patient data: This study enrolled 65 diabetic patients with NAFLD (observation group, OG) and 51 simple diabetes patients (observation group, OG) admitted to Linyi people's hospital between March, 2019 and January, 2020.

Eligibility criteria: To be included in OG, patients should meet the following criteria: Age 18-80, following the diagnostic standard of NAFLD, with a confirmed diagnosis of NAFLD by liver function and ultrasound examinations in hospital, with diabetes as the basic disease, with NAFLD-related treatment after admission. In contrast, patients with other cardio-cerebrovascular diseases, autoimmune defects, mental diseases and organ dysfunction were excluded, as well as those with viral hepatitis or drug-induced fatty liver, drug allergies and intestinal infections. Patients in CG were included based on the criteria as follows: Age 18-80, simple diabetes, no other major medical history in the past, normal examination results.

Treatment: After admission, patients were treated according to NAFLD treatment guidelines[13] and reasonable energy intake, diet structure adjustment, moderate aerobic exercise and correction of unhealthy lifestyles and behaviours were formulated. In addition, metformin and thiazolidinediones were used to improve insulin resistance and control blood glucose. For patients with hepatitis and liver fibrosis, targeted anti-inflammatory and anti-fibrosis treatment was given. The treatment period was 2 months.

Human sample collection and examination: Four millilitres of fasting venous blood were collected, left indoors for 30 min and centrifuged to obtain the serum for the quantification of NEAT1 by qRT-PCR. The sampling time was at the time of admission (T_0) in CG and at T_0, as well as 1 month (T_1) and 2 months after treatment (T_2) in OG. The TRIzol-isolated total RNA was then reverse transcribed into cDNA according to kit recommendations, followed by amplification. The PCR reaction conditions: Pre-denaturation (95/10 min), denaturation (95/0 sec), annealing (50/30 min) and extension (70/10 min), for 40 cycles. Primer sequences were constructed by Thermo Fisher Scientific and NEAT1 expression relative to GAPDH was obtained using the formula $2^{-\Delta\Delta CT}$.

Prognostic follow-up: Patient prognosis in OG was followed up for one year via hospital reexamination and telephone follow-up and NAFLD recurrence was recorded to calculate the recurrence rate.

Animal data: Fifteen 2-3 weeks old SPF SD rats supplied by Shenzhen TOP Biotechnology (SYXK [Guangdong] 2020-0230) were allowed free access to chow and water for 2 weeks of adaptive feeding.

NEAT1 intervention: Rats were randomized into three groups: One treated by tail vein injection of 1 mL NEAT1-silenced adenovirus, once a day for 4 weeks, as the intervention group, one used as the model group treated with the same amount of normal saline through tail vein injection and one normally fed as the normal group.

NAFLD modeling: Rats in intervention and model groups were injected with 100 m kg^{-1} streptozotocin intraperitoneally and their blood glucose was detected 3 days later. Random blood glucose $\geq$16.7 mmol L^{-1} for 3 days was considered successful DM modelling. Then the diabetic rats were fed a high-fat diet. After 9 weeks, the rats were killed to observe the pathological conditions of liver tissue to confirm whether the NAFLD model was successfully built.

Collection and detection of rat specimens: After modelling, the intact liver tissue of rats was obtained and tissue homogenate was made. The levels of aspartate-oxaloacetate aminotransferase (AST) and alanine-pyruvic aminotransferase (ALT) were detected with the use of an automatic biochemical analyzer. The NEAT1 expression in liver tissue was detected by PCR with the same method as above. Measurement of inflammatory cytokines (ICs) IL-1, IL-1β, IL-6 and TNF-α in liver tissue employed ELISA. In addition, rat fresh faeces were collected, sterilized, diluted and dropped into the culture medium. The logarithmic value of colony-forming units of aerobic bacteria (*Enterobacter*, *Staphylococcus*) and anaerobic bacteria (Bifidobacterium, Lactobacillus) in 1g faeces was examined.

Statistical processing: The statistical analysis of this study used SPSS22.0. The comparison of categorical data (%) adopted the Chi-square test, while differences in quantitative data ($\bar{x}\pm s$) were identified via independent samples t-test, one-way ANOVA and Bonferroni post-hoc test. The ROC curves were plotted for diagnostic predictive value analysis and Spearman correlation coefficients were used for correlation analysis. The $p<0.05$ indicated the presence of statistical significance.

RESULTS

NEAT1 Expression in NAFLD: According to the detection results, serum NEAT1 at T0 was (4.28±0.15) in OG, higher than that of (3.77±0.43) in CG ($p<0.05$, Fig. 1a), indicating highly expressed NEAT1 in NAFLD. Then, ROC analysis of NEAT1 level in OG and CG at T0 showed that when NEAT1 >4.025 in serum, its sensitivity and specificity for diagnosing NAFLD in diabetic patients were 95.38 and 78.43%, respectively ($p<0.001$, Fig. 1b), with a predictive AUC of 0.880 and a 95% CI of 0.809-0.952, showing an excellent effect.

Changes in NEAT1 during NAFLD treatment: After detection, it was found that serum NEAT1 was lower at T_1 (4.18±0.24) than T_0 in OG ($P<0.05$), while it further decreased to (3.95±0.23) at T_2, lower than that at T_1 ($p<0.05$, Fig. 2a). According to Spearman correlation coefficient analysis, NEAT1 in OG patients was negatively correlated with treatment time ($p<0.05$, Fig. 2b), that is, the longer the treatment time, the lower the NEAT1 level.

Prognostic significance of NEAT1 in NAFLD: In the prognostic follow-up, we successfully tracked all patients in OG and found 12 cases of NAFLD recurrence, with a 1 year recurrence rate of 18.46%. Comparing NEAT1 levels between recurrent and non-recurrent patients, it can be seen that NEAT1 was higher in relapsed patients at T_0, T_1 and T_2 compared with those without recurrence ($p<0.05$, Fig. 3a-c). Moreover, through ROC analysis of NEAT1 levels in relapsed patients and non-relapsed patients at each time point, we found that NEAT1 at T_0-T_2 had an excellent predictive value for patients' prognostic recurrence ($p<0.05$, Fig. 3d-e), among which the value at T_2 was the most effective. When NEAT1 at T_2 was >3.965, its sensitivity and specificity to predict NAFLD recurrence within one year were 91.67 and 62.26%, respectively, with an AUC of 0.811 and 95% CI of 0.702-0.919 ($p<0.05$, Fig. 3f).

NEAT1 expression in NAFLD rats: Pathological examination showed that all NAFLD rats were successfully modelled. Similarly, by detecting NEAT1 levels in rats of each group, we found that NEAT1 in the model group was (5.00±0.21), significantly higher than that in the normal group ($p<0.05$), which again verified high NEAT1 expression in NAFLD. In addition, NEAT1 in the intervention group was (3.79±0.17), which was lower than that in the model group but still higher compared with the normal group ($p<0.05$, Fig. 4a), indicating that the NEAT1-adenovirus intervention was successful. And as indicated by the liver function test results, the AST and ALT of the normal group were the lowest among the three groups, while the AST and ALT of the intervention group were statistically lower compared with the model group ($p<0.05$, Fig. 4b).

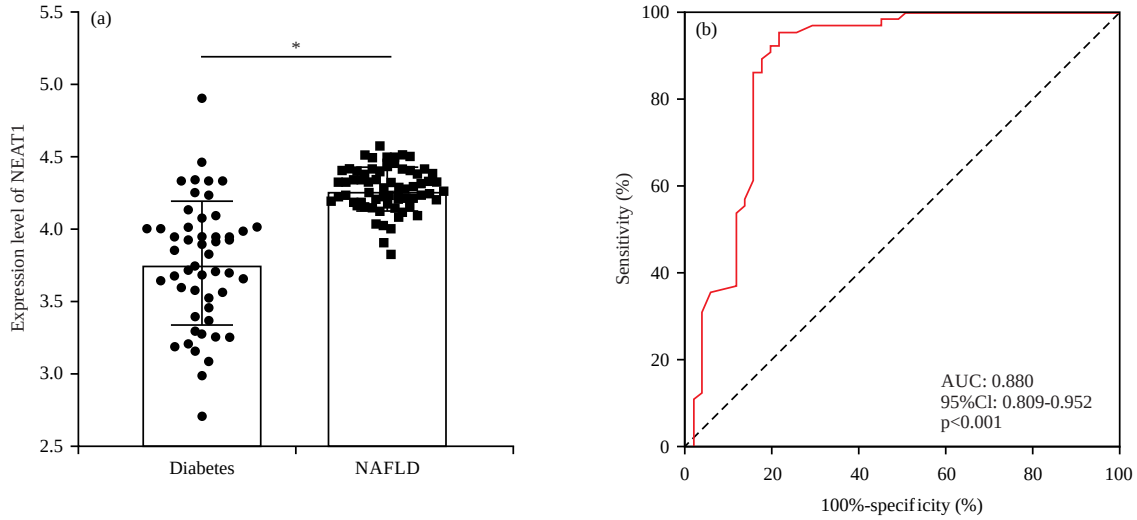

Fig. 1(a-b): NEAT1 expression in NAFLD, (a) Comparison of serum NEAT1 expression between OG and CG at T0 and (b) ROC curve of NAFLD in diabetic patients diagnosed by NEAT1
*p<0.05

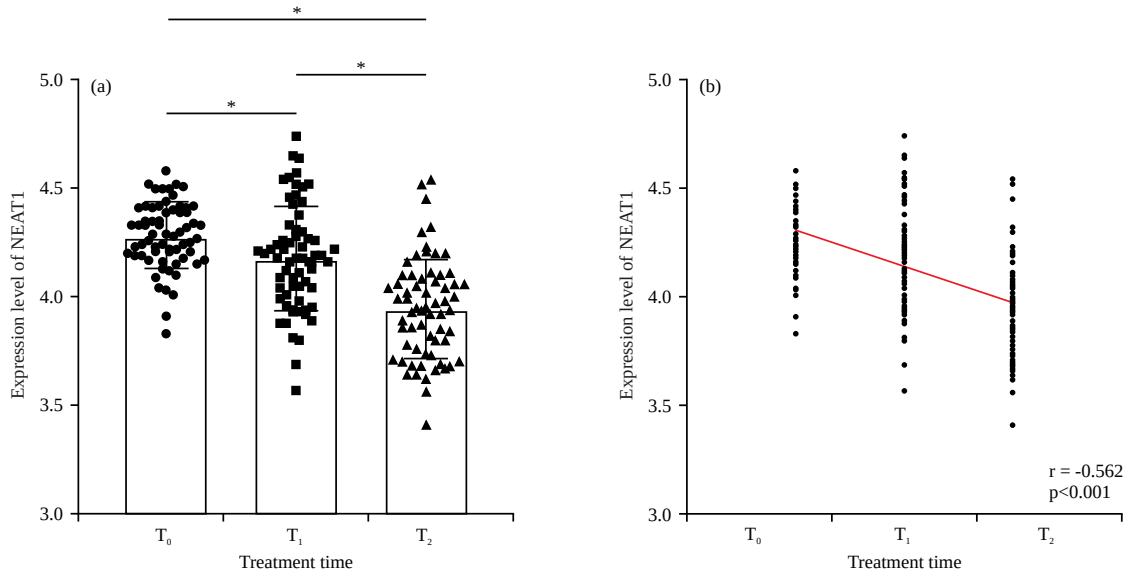

Fig. 2(a-b): Changes of NEAT1 during NAFLD treatment, (a) Changes of NEAT1 expression in OG patients during T_0-T_2 and (b) Correlation analysis between NEAT1 and treatment time in OG patients
*p<0.05

Impacts of NEAT1 on ICs and oxidative stress (OS) in NAFLD rats: The detection results of ICs determined notably higher IL-1β, IL-6 and TNF-α in the model group compared with the normal group ($p<0.05$), suggesting the presence of obvious inflammatory responses in NAFLD. While in the intervention group, the levels of IL-1 β, IL-6 and TNF-α were 42.69±3.04, 53.88±6.77 and 66.08±8.72 ng L^{-1}, respectively, significantly lower than those in the model group ($p<0.05$, Fig. 5a), indicating that NEAT1 inhibition can alleviate the inflammatory reaction in NAFLD. The results of OS reaction in the three groups showed that the model group had the lowest SOD and the highest MDA ($p<0.05$). The SOD in the intervention group was higher than that in the model group but lower than that in the normal group, while MDA was lower than that in the model group and higher than that in the normal group ($p<0.05$, Fig. 5b). It suggests that OS response in NAFLD rats is significantly intensified and NEAT1 can inhibit this process.

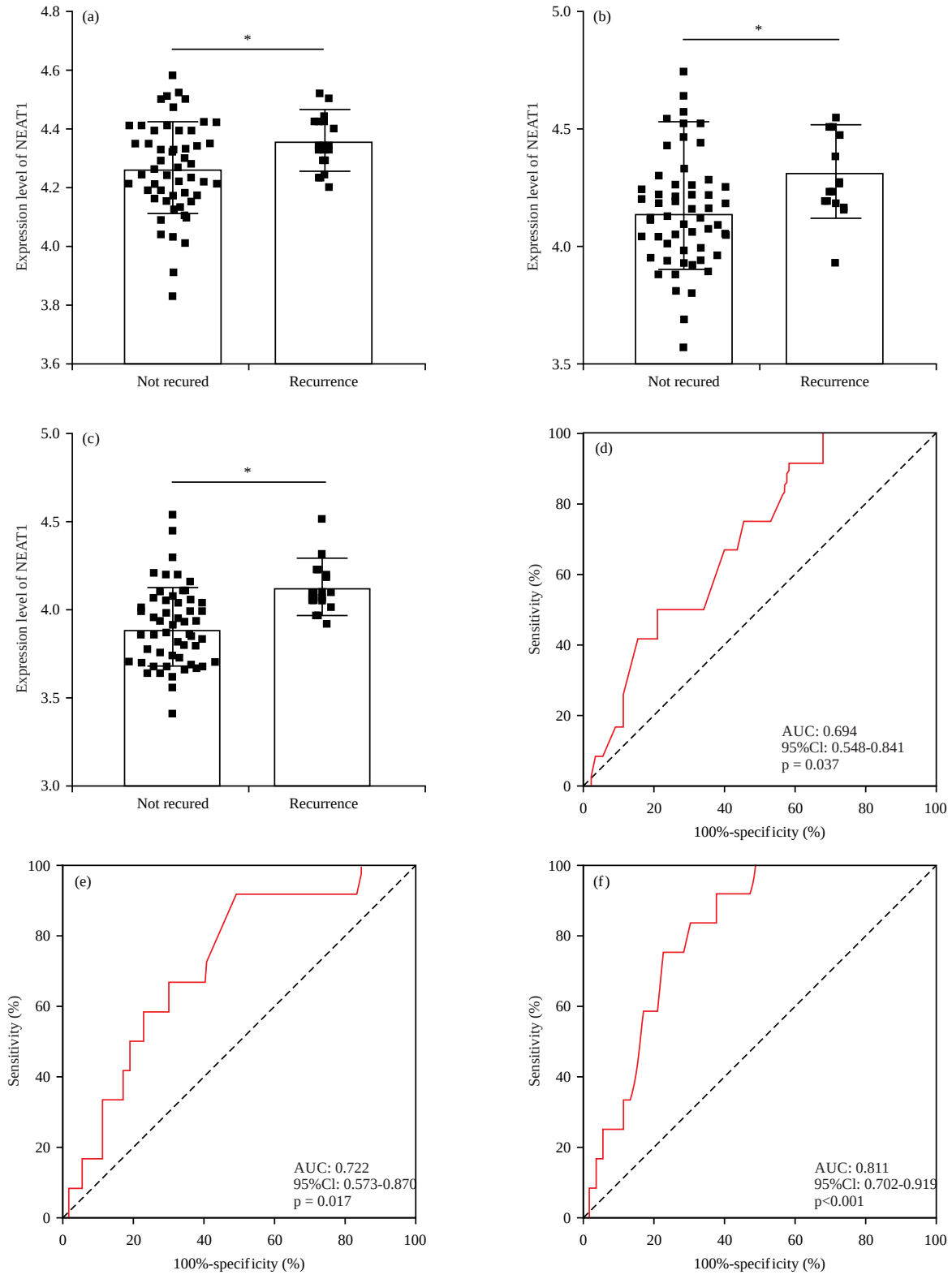

Fig. 3(a-f): Prognostic significance of NEAT1 in NAFLD, (a) Comparison of NEAT1 level at T_0, (b) Comparison of NEAT1 level at T_1, (c) Comparison of NEAT1 level at T_2, (d) ROC curve of NEAT1 at T_0 in predicting recurrence of NAFLD, (e) ROC curve of NEAT1 at T_1 in predicting recurrence of NAFLD and (f) ROC curve of NEAT1 at T_2 in predicting recurrence of NAFLD
*p<0.05

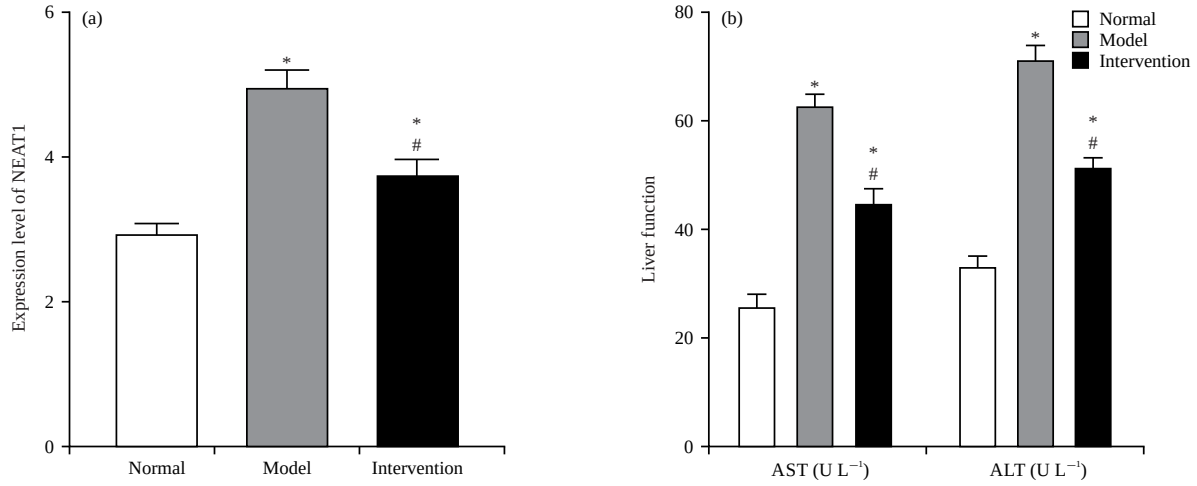

Fig. 4(a-b): NEAT1 expression in NAFLD rats, (a) Comparison of NEAT1 levels among the three groups of rats and (b) Comparison of liver function among the three groups of rats
Compared with the normal group *p<0.05 and model group #p<0.05

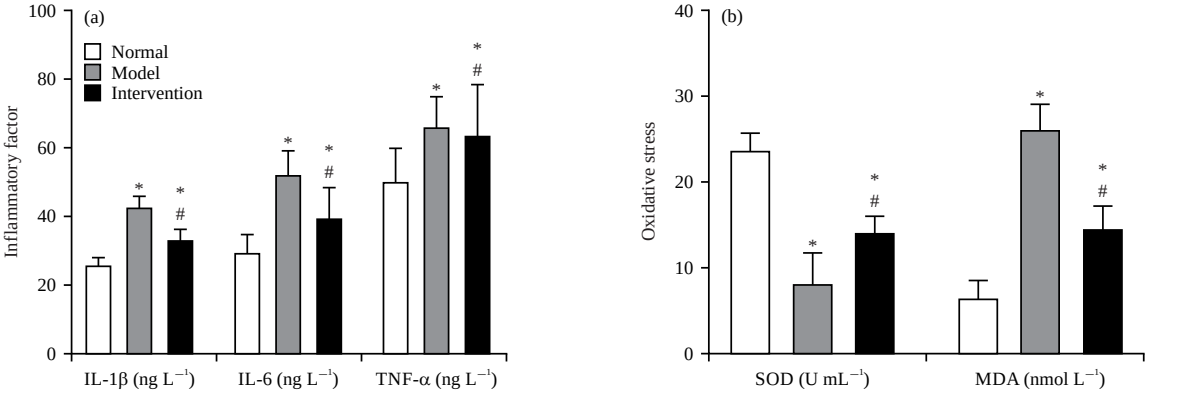

Fig. 5(a-b): Impacts of NEAT1 on ICs and oxidative stress (OS) in NAFLD rats, (a) Comparison of an inflammatory factor among the three groups of rats, (b) Comparison of oxidative stress among the three groups of rats
Compared with the normal group *p<0.05 and model group #p<0.05

Impacts of NEAT1 on GMB in NAFLD rats: Finally, the detection results of GMB showed that the model group rats had the lowest count of *Bifidobacterium* (9.91±1.16 CFU g^{-1}) and Lactobacillus (2.94±1.22 CFU g^{-1}), while the highest count of *Enterobacter* (13.20±3.29 CFU g^{-1}) and *Staphylococcus* (12.25±4.03 CFU g^{-1}) among the three groups (p<0.05, Fig. 6a-b). *Bifidobacterium* and Lactobacillus in the intervention group were (5.18±0.52 CFU g^{-1}) and (4.27±0.31 CFU g^{-1}, respectively, significantly higher than those in the model group while still lower than the control group. *Enterobacter* and *Staphylococcus* were 9.26±0.75 and 9.13±2.39 CFU g^{-1}, respectively, which were significantly lower than those in the model group but higher than the control group (p<0.05, Fig. 6c-d). These results suggest that there is a significant imbalance of GMB in NAFLD, which can be reversed by silencing NEAT1 expression.

DISCUSSION

In this study, it is observed, the relationship between NEAT1 and GMB in NAFLD rats. The results showed that *Enterobacter* and *Staphylococcus* in the model group increased, while *bifidobacterium* and lactobacillus decreased, indicating serious GMB imbalance, which also agrees with the past literature. At present, research on lncRNA involvement in disease onset and development has become a hotspot in clinical practice. As a kind of genetic material in the human body, LncRNAs can not only modulate cell activity and life

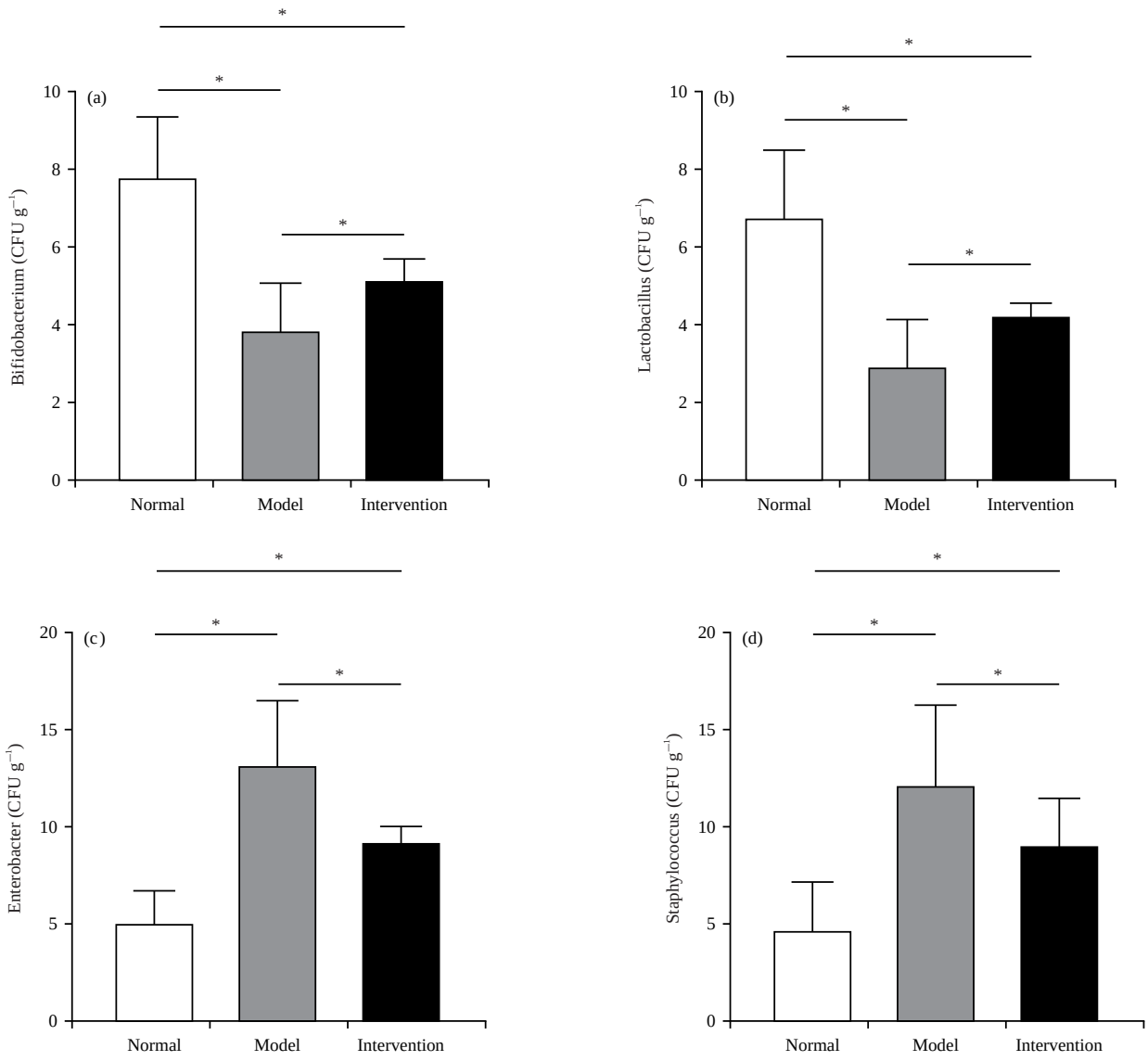

Fig. 6(a-d): Impacts of NEAT1 on GMB in NAFLD rats, (a) Comparison of *Bifidobacterium* among the three groups of rats, (b) Comparison of Lactobacillus among the three groups of rats, (c) Comparison of *Enterobacter* among the three groups of rats and (d) Comparison of *Staphylococcus* among the three groups of rats

*p<0.05

cycle but cause local functional changes in organs and tissues. Moreover, they can be detected in various samples such as human blood, body fluids and tissues, which provides a new diagnosis and treatment direction for future diagnosis and treatment of various diseases[14-16]. Currently, studies on NEAT1 are mostly confined to tumour diseases[17,18], while Chen *et al.*[19] and Ye *et al.*[20] preliminarily revealed the potential significance of NEAT1 in NAFLD. Therefore, analyzing the influence of NEAT1 on NAFLD is of great significance for NAFLD diagnosis and treatment.

Firstly, a highly expressed NEAT1 in NAFLD was found, which was consistent with the results of the previous studies[21]. Through ROC analysis, NEAT1 was also found to have excellent diagnostic implications for NAFLD in diabetic patients, which suggested that NEAT1 can also be an auxiliary diagnostic index for NAFLD in the future to improve the early diagnosis rate of NAFLD. In the course of treatment, NEAT1 showed a trend of gradual decline, which verified the accuracy of the above experimental results and suggested that NEAT1 was closely related to the alterations of NAFLD expression with the

potential to become an evaluation indicator of NAFLD. In the prognostic follow-up, NEAT1 was observed to be markedly higher in relapsed patients with excellent predictive value for prognostic recurrence, which shows that the highly expressed NEAT1 not only interferes with NAFLD development but also predicts the poor prognosis of patients. In the research of Jia, high NEAT1 expression predicted increased prognostic mortality in ovarian cancer patients[22], which can once again prove the potential clinical application value of NEAT1.

To further elucidate the impact of NEAT1 on NAFLD, a NAFLD rat model for *in vitro* experimental analysis was constructed. First of all, the NEAT1 level in NAFLD rats was also found to be increased, which verified the accuracy of the above experimental results. However, AST, ALT, ICs and MDA levels in NAFLD rats decreased while SOD increased after NEAT1-silenced adenovirus intervention, indicating that inhibiting NEAT1 expression can effectively improve the pathological changes of NAFLD and relieve inflammatory reaction and OS reaction in the liver, with potential significance for targeted therapy. This has also been confirmed in previous studies[23,24], indicating that NEAT1 plays an excellent regulatory role in inflammatory response and OS.

The intestinal tract is the main parasitic place of human microorganisms and GMB is crucial to maintaining human health. Therefore, alterations in the body environment can affect the balance of GMB and microorganisms and destroy the integrity of intestinal mucosa, causing pathological changes in the body[25]. The intestinal micro-ecosystem is known to be closely linked to liver function and GMB stability can inhibit the production of massive endotoxin *in vivo*, thus achieving the effect of liver protection. In contrast, unbalanced GMB cannot inhibit the production of intestinal endotoxin, thus accelerating liver damage[26]. More importantly, a large number of gram-negative bacteria breeds in the intestinal tract, resulting in reduced intestinal colonization resistance and damaged intestinal mucosal barrier, which damages a large number of normal liver cells, on the other hand, inhibits the repairability of intestinal mucosa and promotes the release of ICs on the other[27]. Besides, aerobic bacteria decreased notably and anaerobic bacteria increased in the intervention group, suggesting significantly improved GMB at this time, which also preliminarily revealed the mechanism of NEAT1 in NAFLD.

CONCLUSION

The NEAT1 keeps at a high expression in NAFLD, which is closely related to NAFLD progression. Silencing NEAT1 expression can effectively reduce inflammation and OS in NAFLD and regulate the imbalance of GMB, which has important significance for improving NAFLD.

SIGNIFICANCE STATEMENT

This study discovered the LncRNA NEAT1 during the treatment of nonalcoholic fatty liver disease and its relationship with gut microbiota which can be beneficial for disease therapy. These findings suggest that LncRNA NEAT1 may have a significant role in preventing Nonalcoholic Fatty Liver Disease. This study will help the researchers to uncover the critical areas of LncRNA that many researchers were not able to explore in near future.

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