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Overexpression of Long Noncoding RNAs (lncRNA) NF-κβ-Interacting Long Noncoding RNA (NKILA) in Ankylosing Spondylitis is Correlated with Transforming Growth Factor β1 (TGF-β1), Active Disease and Predicts Length of Treatment

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Long noncoding RNAs (lncRNA) NF-κβ-interacting long noncoding RNA (NKILA) is downregulated in various types of cancers, while its involvement in other diseases is unknown. In the present study we found that plasma lncRNA NKILA was expressed at higher levels in active ankylosing spondylitis patients than in healthy controls.

Material/Methods: According to Youden's index, active disease patients were divided into high and low lncRNA NKILA groups.

Results: Patients in the high lncRNA NKILA level group had significantly longer length of treatment and higher re-hospitalization rate at 3 years after discharge. Plasma levels of TGF-β1 were also higher in active ankylosing spondylitis patients than in healthy controls. Levels of plasma lncRNA NKILA and TGF-β1 were significantly and positively correlated in ankylosing spondylitis patients but not in healthy controls.

Conclusions: Overexpression of lncRNA NKILA in ankylosing spondylitis is correlated with active disease and predicts length of treatment. LncRNA NKILA may participate in ankylosing spondylitis through the interaction with TGF-β1, which is a key player in this disease.

MeSH Keywords: Bone Diseases • RNA, Long Noncoding • Spondylitis, Ankylosing

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Background

As a type of medical condition characterized by pathological osteogenesis and systemic inflammation, ankylosing spondylitis is a chronic autoimmune disease that mainly affects ligaments, tendon attachment points, and the axial skeleton [1,2]. The continuous development and progression of ankylosing spondylitis will eventually lead to spinal fusion and ligamentous ossifications, imposing heavy economic and psychological burdens on patients and their families [3]. In spite of the efforts made in the treatment of ankylosing spondylitis, outcomes are still poor in many patients [4,5]. Ankylosing spondylitis patients are also at high risk of other diseases, such as cardiovascular disease [6,7]. Therefore, improving the treatment of this disease is still a focus in this field.

It has been well established that the pathogenesis of inflammation contributes to the progression of ankylosing spondylitis [8]. The involvement of certain cytokines has also been proven [9, 10]. One of these cytokines is transforming growth factor-β (TGF-β), whose signaling plays pivotal roles [8]. TGF-β as a cytokine has dual roles in inflammatory responses by both suppressing inflammatory cytokines and augmenting inflammation [11]. It has been reported that TGF-β1 is upregulated in ankylosing spondylitis [12], and the reduction of TGF-β1 level reflects the recovery from the disease [13]. Long noncoding RNAs (lncRNA) NF-κβ-interacting long noncoding RNA (NKILA) is a well-established tumor-suppression lncRNA in various types of cancers [14,15]. LncRNA NKILA suppresses cancer cell migration and invasion by inhibiting downstream oncogenic pathways [14,15]. In the present study, we found that lncRNA NKILA participates in ankylosing spondylitis, possibly by interacting with TGF-β1, and is correlated with active disease and predicts length of treatment.

Material and Methods

Human specimens and cell lines

Fating blood (5 ml) was extracted from 80 patients with ankylosing spondylitis and 56 healthy controls before treatment. Those participants were admitted by the First People's Hospital of Yunnan Province from January 2012 to January 2014. Inclusion criteria were: 1) patients diagnosed for the first time; 2) patients with complete medical records; and 3) patients willing to participate. Exclusion criteria were: 1) patients with other diseases, and 2) patients who failed to cooperate with researchers. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) was used to measure disease activity. The 170 patients were divided into an active disease group (n=94, BASDAI >4) and an inactive disease group (n=76, BASDAI ≤4). The active disease group include 54 males and 40 females, ages 22–43 years, and mean age 30.6±4.2 years. The inactive disease group include 40 males and 36 females, ages 23–45 years, and mean age 31.4±4.7 years. The control group include 31 males and 25 females, ages 23–45 years, and mean age 29.9±4.5 years (Table 1). The 3 groups showed similar age and sex distributions. This study was approved by the Ethics Committee of the First People's Hospital of Yunnan Province. All participants signed an informed consent form.

Follow-up

All patients received standard treatment and were fully compliant. All patients were followed up for 3 years by outpatient visit after discharge to record re-hospitalization conditions.

Real-time quantitative PCR (RT-qPCR)

The Total RNA Purification Kit (Cat. no. 17200, Norgen Biotek) was used to extract total RNA from plasma of the 3 groups of participants. RNA quality was checked by urea PAGE gel electrophoresis. Synthesis of cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

Table 1. Basic information of 3 groups of patients.

	Ankylosing spondylitis patients (active)	Ankylosing spondylitis patients (inactive)	Healthy controls
Cases (n)	94	76	56
Sex			
Male (cases)	54	40	31
Female (cases)	40	36	25
Age range (years)	22–43	23–45	23–45
Mean age (years)	30.6±4.2	31.4±4.7	29.9±4.5

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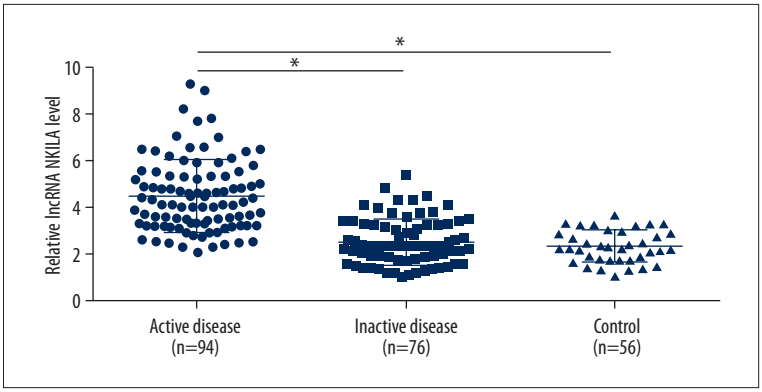

Figure 1. Plasma lncRNA NKILA was overexpressed in active ankylosing spondylitis patients than in healthy controls. Plasma levels of lncRNA NKILA were measured by RT-qPCR. Data are presented by 5 lines, from lower to upper: minimum, lower 25%, median, upper 25%, and maximum. Compared with the inactive disease group and control group, plasma levels of lncRNA NKILA measured by RT-qPCR were significantly higher in the active disease group. In addition, the control group and inactive disease group showed very close plasma levels of lncRNA NKILA (* p<0.05).

We used the Invitrogen SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit to prepare all PCR reaction systems. Primers of lncRNA NKILA and endogenous control GAPDH were designed and synthesized by Sangon (Shanghai, China). Primer sequences were: 5'-TGGATTGTTGGGTATATTTTGGA-3' (forward) and 5'-TGTATGAAGAGGATGCTGAAGGC-3' (reverse) for lncRNA NKILA; 5'-CCTCGTCTCATAGACAAGATGGT-3' (forward) and 5'-GGGTAGAGTCATACTGGAACATG-3' (reverse) for GAPDH. PCR reactions were performed under the following conditions: 95°C for 1 min, and then of 95°C for 15 s and 56.5°C for 28 s for 40 cycles. Expression of lncRNA NKILA was normalized to GAPDH by $2^{-\Delta\Delta CT}$ method. The sample with the lowest ΔCT value was set to "1", and all other samples were normalzied to this sample.

Enzyme-linked immunosorbent assay (ELISA)

We used the LEGEND MAX Free Active TGF-beta1 ELISA Kit (Biolegend, San Diego, CA) to measure plasma levels of TGF-β1. All operations were performed in strict accordance with manufacturer's instructions. Plasma levels of TGF-β1 were normalized to ng/ml.

Statistical analysis

Sample sizes met the requirement of statistical power of the experiments. All experiments were repeated 3 times and data are expressed as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 6 software. Pearson's correlation coefficient analysis was performed to analyze the correlations between plasma levels of lncRNA NKILA and TGF-β1. The unpaired *t* test was used for comparisons between 2 groups. Comparison of re-hospitalization rate was performed by chi-square test. One-way ANOVA followed by Tukey test was used for comparisons among multiple groups. Differences were statistically significant at $p<0.05$.

Results

Plasma lncRNA NKILA was expressed at higher levels in active ankylosing spondylitis patients than in healthy controls

Results of RT-qPCR showed that, compared with the control group and inactive disease group, plasma levels of lncRNA NKILA were significantly higher in the active disease group (p<0.05) (Figure 1). Although slightly higher plasma levels of lncRNA NKILA were observed in the inactive disease group than in the healthy control group, the difference was not significant (p<0.05) (Figure 1).

High plasma lncRNA NKILA level predict long treatment course and high re-hospitalization rate

According to Youden's index (an optimal cut-point determination method), active disease patients were divided into high (n=48) and low (n=46) lncRNA NKILA level groups (cutoff value was 4.53). Compared with the low lncRNA NKILA level group, patients in the high lncRNA NKILA level group showed significantly longer treatment course (Figure 2A, p<0.05). The 3-year follow-up data showed that a total of 39 patients were re-hospitalized during this time period. There were 29 cases in the high lncRNA NKILA level group, accounting for 60.4%, and 10 cases in the low lncRNA NKILA level group, accounting for 21.7%. Therefore, the re-hospitalization rate was significantly higher in the high lncRNA NKILA group than in the low lncRNA NKILA group (Figure 2B, p<0.05).

Plasma levels of active TGF-β1 were higher in the active disease group than in the inactive disease group and healthy controls

Results of ELISA showed that, compared with the control group and inactive disease group, plasma levels of active TGF-β1 were significantly higher in the active disease group (p<0.05) (Figure 3).

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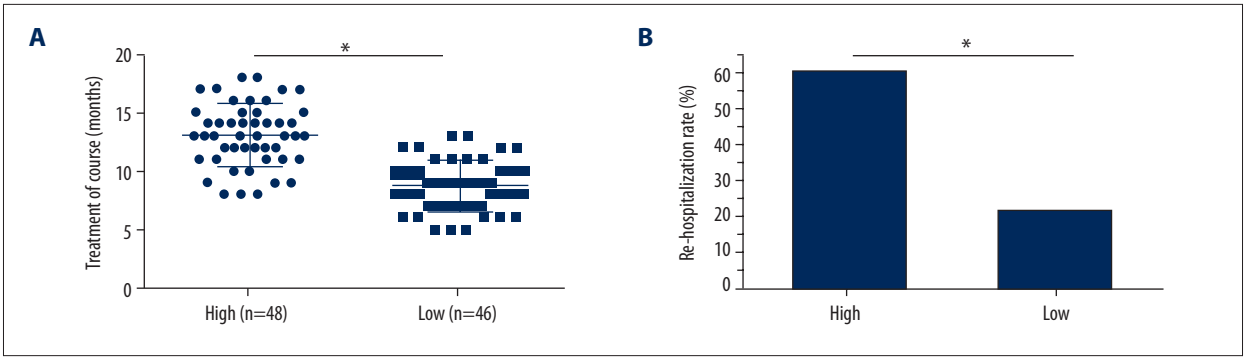

Figure 2. High plasma lncRNA NKILA level predicts long treatment course and high re-hospitalization rate. Treatment course (months) data are presented by 5 lines, from lower to upper: minimum, lower 25%, median, upper 25%, and maximum. The treatment course of patients in the high plasma lncRNA NKILA level group was significantly longer than that of the low lncRNA NKILA level group (**A**). Comparisons of hospitalization rate between 2 groups were performed by chi-square test. A higher re-hospitalization rate was observed in the high plasma lncRNA NKILA level group than in the low plasma lncRNA NKILA level group (**B**) (* p<0.05).

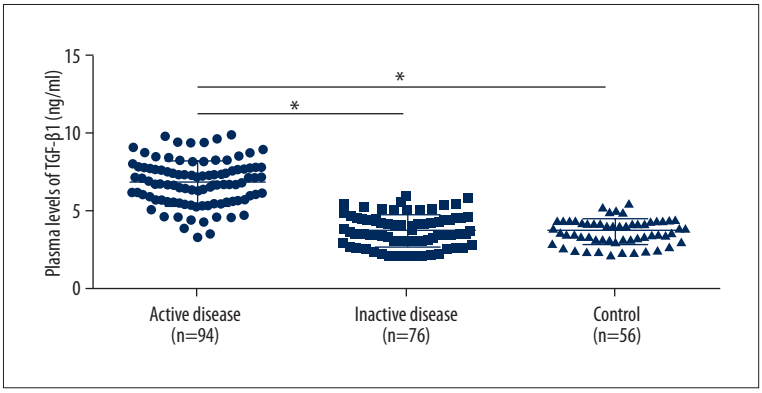

Figure 3. Plasma levels of active TGF-β1 were higher in active ankylosing spondylitis patients than in healthy controls. Plasma levels of active TGF-β1 were measured by ELISA and data are expressed by 5 lines, from lower to upper: minimum, lower 25%, median, upper 25%, and maximum. Compared with the control group and inactive disease group, plasma levels of active TGF-β1 were significantly higher in the active disease group. In addition, the control group and inactive disease group showed very close plasma levels of active TGF-β1 (* p<0.05).

Although slightly higher plasma levels of active TGF-β1 were observed in the inactive disease group than in the healthy control group, the difference was not significant (p<0.05) (Figure 3).

Levels of plasma lncRNA NKILA and TGF-β1 were significantly and positively correlated in active ankylosing spondylitis patients

Pearson's correlation coefficient analysis was performed to analyze the correlations between plasma levels of lncRNA NKILA and TGF-β1. As shown in Figure 4A, levels of plasma lncRNA NKILA and TGF-β1 were significantly and positively correlated in the active disease group (Fig.4A). In contrast, the correlation between levels of plasma lncRNA NKILA and TGF-β1 was not significant in the inactive disease group (Figure 4B) and control group (Figure 4C).

Discussion

LncRNA NKILA has been characterized as a tumor-suppression lncRNA in various types of cancers [14,15]. The key finding of the present study is that lncRNA NKILA is overexpressed in active ankylosing spondylitis and is correlated with active disease, and it predicts length of treatment. Expression of lncRNA NKILA is positively correlated with TGF-β1 in active ankylosing spondylitis.

The development and progression of ankylosing spondylitis is accompanied by changes in expression patterns of a large set of lncRNAs [16], indicating the involvement of lncRNAs in this disease. Our preliminary microarray data showed that lncRNA NKILA, which was usually downregulated in cancer patients, is upregulated in plasma specimens of patients with ankylosing spondylitis (data not shown). In the present study, we found that lncRNA NKILA was upregulated in patients with active ankylosing spondylitis but not in patients with inactive ankylosing spondylitis. Therefore, the upregulation of lncRNA NKILA may be used as an indicator of the activity of ankylosing spondylitis.

Treatment course of ankylosing spondylitis is generally long [17,18]. Shortening the treatment procedure is critical to

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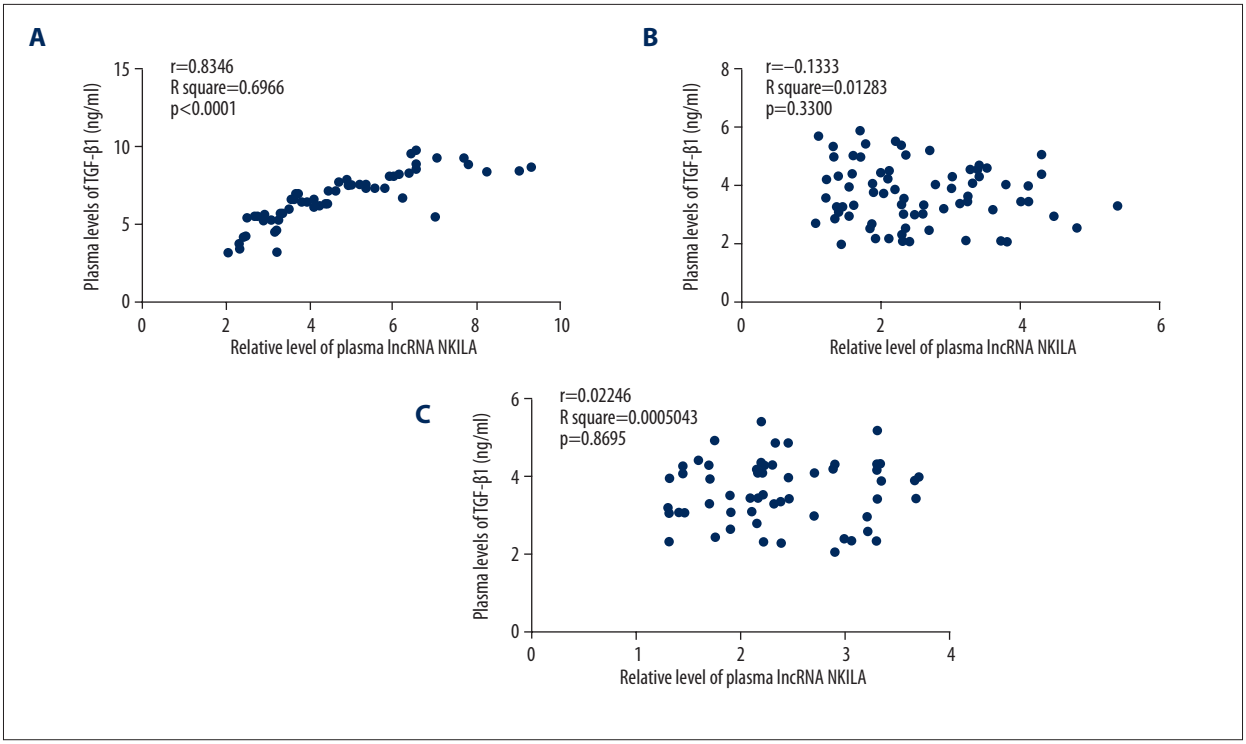

Figure 4. Levels of plasma lncRNA NKILA and TGF-β1 were significantly and positively correlated in active ankylosing spondylitis patients. Correlation analysis was performed by Pearson' correlation coefficient. A significant and positive correlation between levels of plasma lncRNA NKILA and TGF-β1 was observed in the active disease group (**A**) but not in the inactive disease group (**B**) and control group (**C**).

reduce the economic and psychological burdens on patients and their families. Our study showed that the high plasma levels of lncRNA NKILA were significantly correlated with prolonged treatment course. Therefore, lncRNA NKILA may serve as an indicator of the length of treatment course or even as a treatment target. Re-hospitalization is common in many patients with ankylosing spondylitis [19]. In a recently study, a lncRNA named TUG1 was proven to be significantly correlated with re-hospitalization rate of patients with ankylosing spondylitis. In the present study, we showed that the higher lncRNA NKILA levels were also correlated with the increased re-hospitalization of ankylosing spondylitis after discharge.

TGF-β is a cytokine that plays pivotal roles in the development and progression of ankylosing spondylitis [12]. Expression levels of TGF-β1 are positively correlated with the activity of disease [12]. Consistent with previous studies, plasma levels of TGF-β1 in the present study were also found to be significantly higher in active ankylosing spondylitis patients, but not in inactive ankylosing spondylitis patients, than in healthy controls. It is known that TGF-β1 can interact with lncRNAs to achieve its biological roles [20]. In the present study, we showed that levels of plasma lncRNA NKILA and TGF-β1 were significantly and positively correlated only in ankylosing spondylitis patients. Further studies using animal models are needed to assess the potential interactions between lncRNA NKILA and TGF-β1.

Conclusions

lncRNA NKILA overexpression is correlated with active disease and predicts length of treatment of ankylosing spondylitis. lncRNA NKILA can interact with TGF-β1 in ankylosing spondylitis patients. Our study suggests that lncRNA NKILA is a potential biomarker for the treatment of ankylosing spondylitis.

Conflict of interests

None.

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