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Cloning identification and functional analysis of human IL-17A promoter

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ABSTRACT

Objective: To conduct the cloning identification and characterization of the sequence of human IL-17A promoter so as to analyze the regulatory mechanism of the gene expression of IL-17.

Methods: First of all, the potential promoter region of IL-17A was found by means of the bioinformatics methods. Then, it was cloned into the reporter vector with PCR technique. Finally, the activity of the test promoter was determined by dual luciferase reporter system.

Results: Two transcriptional start points of the upper region, 600 bp and 1000 bp, of IL-17A were obtained by PCR clone and proved to have certain activities by dual luciferase reporter system. Also, they could be activated by IL-17A activator STAT3, which could start the expression of the reported gene.

Conclusions: Clone established the regulatory region of human IL-17A promoter, which provided bases to the subsequent function research.

1. Introduction

IL-17 is a kind of proinflammatory cytokine derived from TH17 cells which is quite different to Th1 and Th2 cell subsets. IL-17 axis is a hot point in basic and clinical studies at present, since it play an important role in host defense, autoimmune diseases and the course of tumors [1].

The discovery of IL-17 gene and IL-17 protein goes back to the CTLA-8 cloned by activated rodent T hybridoma, and they are found to have a high homology with the 13th ORF of Herpes virus saimiri. Later, researchers found that CTLA-8 protein could be secreted to the outside of the cell and induce the expression of IL-6 and IL-8 by activating the NF-kB pathway. Meanwhile, it can activate the proliferation of T cells so that it is named IL-17 [2,3].

The biological function of IL-17 depends on the forms of the homodimer or heterodimer (built by intramolecular disulfide bond). At present, it is found that IL-17A and IL-17F are the main members of the IL-17 family. The others are IL-17B, IL-17C, IL-17D and IL-17E [4]. Among them, the function of IL-17B, IL-17C and IL-17D remains undefined, but the expressions of proinflammatory factors such as TNFa, IL-6 and IL-1b can be induced by the proinflammatory effect of IL-17E, IL-17A and IL-17F [5,6].

IL-17 plays a key role in the host defense against infections. For instance, it works through mucosal immunity in bacterial and fungal infection [7]. IL-6 induced by IL-17 has a quite strong positive feedback promotion effect on the differentiation of TH17 cells and the activation of acute phase response and complement [8]. IL-17 can also adjust neutrophilic granulocyte by inducing and promoting G-CSF and CXCL. On the other hand, IL-17 can gather monocytes (by inducing CCL2 and CCL7) by induced proteins with chemotactic activity such as dendritic cells to target them to the mucosal surface. It-17 can also raise the expression of antimicrobial peptide, the latter can kill the invaded pathogens directly and strengthen the immune function of the host [9]. The above explains the effects of pathogenic TH17 and IL-17 on autoimmunity diseases.

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Now, the existed clinical and fundamental researches have discovered that serum IL-17A increases in patients with influenza virus infection, which also reflects in patients infected with macrophage and helicobacter pylori infection after infecting by mycobacterium tuberculosis [10–12]. Therefore, the regulatory analysis of IL-17A in researches of common infectious diseases is very important. There are many different molecular mechanisms which are found to control the activation and expression of IL-17, such as STAT3, Oct1, CNS2, *etc*. [13–15].

Other researchers and we have found in the early researchers that the expressions of many immune response-related inflammatory factors were adjusted and controlled by DNA methylated modification after bacterial or viral infection [16–18]. In order to further study whether the expression of IL-17A is regulated and controlled by that mechanism, it is necessary to explore the DNA methylated modification and the combination of transcription factors in the promoter region. So, in this study, we cloned the regulatory region of IL-17A promoter firstly and then analyzed and identified its biological function to provided basis for the follow-up researches.

2. Materials and methods

2.1. Cells

Human embryonic kidney-derived HEK293T cell strain medium consisted of DMEM containing 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/L streptomycin. The required temperature: 37 °C, incubator: 5% CO_2. The synthesis of primers and DNA sequencing were completed by Invitrogen. *Escherichia coli* DH5 strains used for clone construction were kept in our lab.

2.2. Reagents

Instrumental enzymes used for clone were bought from Takara; plasmid extraction, gel extraction, product purification and non-endotoxin hyperpure plasmid midi kit were purchased from Axygen; luciferase detection reagent Dual-Luciferase Reporter Assay Systemp, GL3 and pRL plasma were bought from Promega; cell culture media, Lipofeatmin2000 were provided by Invitrogen; and other analytical reagents were purchased from Shanghai Pharmaceutical Group.

2.3. Bioinformatics analysis

The gene sequence of human IL-17A was obtained from National Center for Biotechnology Information (NCBI). The promoter was predicted and the transcription factor was analyzed by using online database TFSEARCH and Promoter 2.0 Prediction Server.

2.4. Primers design and PCR amplification

According to the predicted sequence, two primer segments compounded by Invitrogen, 600 bp and 1 000 bp, were designed (Table 1). The template was given PCR amplification with the human-derived genome. The reaction conditions were 95 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s and 72 °C for 70 s, which would repeat 30 times. PCR instrument of Eppendorf was used. The products of PCR were detected by 1% sepharose gel.

2.5. The construction of luciferase vector

The products of PCR, 600 bp and 1 000 bp purified in electrophoresis with restriction enzyme Sac I and Xho I double digested gel, and pGL3-Basic plasma were used to purify, connect and transform competence *Escherichia coli* DH5α cells. Bacteria liquid polymerase chain reaction and positive clones were detected by screening, double enzyme digestion and sequencing. Luciferase reporter vector containing IL-17A promoter region was acquired and named p17A-600 and 1000.

2.6. Cell transfection

HEK293T cells were planked on 24-well plates (4×10^5) and cultured to a certain concentration (70–80%). Then, p17A-600, p17A-1000 and pRL were applied for co-transfection. The mass ratio was 20:1. The co-transfection volumes of pCMV-STAT3 were 3 μg and 5 μg. Lipofectamine 2000 (Invitrogen) was employed for the transfection. The application amount of plasma was in accordance with the instruction book. OPTI-MEM medium was used for the transfection. Four hours later, normal serum medium was applied.

2.7. The detection of the activity of promoter

After transfected for 48 h, the activity of luciferase was tested by chemiluminescence apparatus. Every step was conducted in accordance with the instruction of Dual-Luciferase Reporter Assay System strictly. Every sample received 3 parallel hole experiments. The relative intensity of fluorescence (RLA, refers to the ratio of fluorescence signal strength, i.e. catalytic reaction substrates of luciferase) was used to start the promoter activity. SPSS16 software was used for statistical analysis. Mean ± standard deviation was used to express measurement data. The comparison was tested by *t*-test. Differences were statistically significant ($P < 0.05$).

Table 1
The activation analysis of IL-17A promoter.

Promoter	pSTAT3			*P* (3 *vs.* 0)	*P* (5 *vs.* 0)
	0 μg	3 μg	5 μg		
pGL3	10.52 ± 2.48	12.38 ± 1.67	13.01 ± 2.02	0.339	0.259
p17A-600	24.37 ± 2.33	103.58 ± 15.27	84.93 ± 14.32	0.009	0.014
p17A-1000	37.12 ± 1.98	104.65 ± 12.52	123.18 ± 17.17	0.008	0.009

The activation of IL-17A promoter analyzed the RLA intensity of luciferase after p17A-600, p17A-1000 and pGL3 empty vectors and expression vector STAS3 co-transfected 293T and compared with the 0 of the same group.

3. Results

3.1. Bioinformatics analysis

The gene sequence of IL-17A was obtained from NCBI. The region between the 30 bp after the transcription start site which was set to be the starting point and the 5000 bp in the upper region which was designed to be the section was cut out and analyzed. After analyzed by Promoter 2.0, it was found that the highest score appeared in the previous 600 bp and 1000 bp regions and following the TFSEARCH Search analysis indicated that a large amount of transcription activator binding sites such as OCT1, STAT3, ROR and AP1 also existed in the previous 600 bp and 1000 bp regions. So, we cloned these two regions at the same time, respectively.

3.2. The construction of the reporter vector of IL-17A promoter

Human peripheral blood-originated genome was used as a template and two pairs of primers were used respectively for PCR amplification. Promoter segments of IL-17A with expected size of about 600 bp and 1000 bp were found to be amplified successfully after the products had been tested with electrophoresis by 1% agarose gel. When targeted segments were connected with pGL3 vector and transformed and identified by colony polymerase chain reaction, positive clones appeared, and its sequencing result was equivalent to the reference sequence of NCBI, which further implied the success of the construction of the two luciferase reporter vectors (containing promoter region of IL-17).

3.3. Analysis of the promoter activity of Il-17A

P17A-600, p17A-1000 or pGL3 empty vectors and internal control pRL were used to transfect HEK293T cells, which aimed to verify the activity of the constructed pIL-17A vector. After 48 h, the relative intensity of fluorescence was used for the detection. The results showed that pGL3 vector had no activity, while p17A-600 and p17A-1000 can promote the activity of luciferase significantly, which indicated the success of the construction of pIL-17A promoter vector.

3.4. The activation analysis of IL-17A promoter

In order to further detect the response of constructed p17A-600 and p17A-1000 towards IL-17A activating agent, STAT3, p17A-600 and p17A-1000 were co-transfected to observe the activity of IL-17A. The result showed that after the activation analysis of IL-17A promoter, the RLA intensity of luciferase after p17A-600, p17A-1000 and pGL3 empty vectors and expression vector STAS3 co-transfected 293T were all compared with the 0 of the same group, $^{**}P < 0.01$ (Table 1). Without STAT3, the values of p17A-600 and p17A-1000 agreed with the about above validation. However, after adding 3 μg and 5 μg pCMV-STAT3 expression plasma for co-transfection, the activities of p17A-600 and p17A-1000 increased 3–5 times, which indicated that the cloned IL-17 possessing binding points of STAT3 had effects of promotion and regulation and further proved that the cloned amplified regions also contained biological function which could be used in the subsequent mechanism analysis and researches.

4. Discussion

In many physiological processes of infection and immunity, IL-17 is an very important inflammatory factor, so that it is very important to study the signal transduction mechanism worked by IL-17. In this study, we carried out a cloning identification of IL-17A promotion regions, which was the most important promoter region of the IL-17 family. Firstly, we obtained the possible region, 600 bp and 1000 bp of the upper region, by bioinformatics prediction. Next, they were cloned to luciferase reporter vector pGL3. Finally, luciferase report system was used to prove that the segment contained promoter activity and was sensitive to IL-17 activating reagent STAT3. All these results demonstrated that we have cloned IL-17A containing promoter activity regions, which provided basis for the follow-up studies.

In recent years, 6 numbers of the IL-17 family and 5 numbers of the IL-17 receptors (IL-17RA/B/C/D/E) family have been discovered, but only the proinflammatory cytokines of IL-17A, IL-17E and IL-17F are confirmed, and IL-17A is the most important one [19]. Hence, the regulation and control of the expression of IL-17 remains very important. In studies of immune cells, the characteristic transcription factor RORγt of IL-17 was found. It was found by knocking out RORγt of gene in mice that IL-17 cells could not be induced by TGFb and IL-6, and IL-17 could be induced specifically (over-expressed RORγt) without affecting IFNγ, which indicated that the key transcription factor of IL-17 controlling differentiation was RORγt [20]. A research also found that without IL-6 and TGF-β IkBf could adjust and control the differentiation of TH17 and the expression of IL-17 by co-working with ROR nuclear receptor [21].

With the intensive study of TH17 cells, other factors have been found successively, positive regulation factors such as STAT3, IRF4 and NLRP3 and negative regulation factors such as Ets-1, STAT5 and Foxp3. The activating agent STAT3 used in this study was a positive regulation factor of IL-17A, too. It could strengthen the activity of IL-17 promoter at the cellular level. Besides, STAT3 was also the downstream regulation pathway of IL-17. New researches have revealed that IL-17A would facilitate the transformation from chronic pancreatitis to pancreatic cancer by REG3β-JAK2-STAT3 inflammatory corpuscle pathways [22].

In addition, epigenetic is a branch of genetics referring to the inheritable changes of genetic expression (under the condition of the unchanged gene nucleotide sequences), which has become the hot point of in the field of biomedicine. The epigenetic mechanism mainly includes DNA methylation, histone acetylation, microRNA, genomic imprinting, etc. Epigenetic is also found in the research area of IL-17 and TH17. Multiple researches have found that methylation participates in adjusting and controlling the expression of IL-17 [23]. When IL-6 and TGF-β facilitate the differentiation of Naive CD4(+) T cells towards TH17, conserved intergenic elements and DNA methylation work together to regulate the expression of IL-17 gene blocks. Methylation in the IL-17 promoter region would lead to the decrease of the combination of STAT3 transcription factors, which would further affect the expression of IL-27 [24]. Similarly, histone acetylation was also found to work in this process, which indicated the phenomenon of H3K27 demethylation and H3K4 methylation [24]. Extended researches have revealed that histone H3 lysine-27 demethylase Jmjd3 plays a key role in the differentiation process of TH17. Jmjd3

combined H3K27 directly in the key locus of Rorγt transcription factors and declined the level of methylation [25]. In patients with acute asthma, Estrogen and progesterone enhanced the expression and secretion of IL-23/IL-23 receptor signaling and IL-17A by decreasing the expression of let-7f microRNA [26].

Due to the strong effect expressing by IL-17 in induced inflammations, especially the effects of fending off pathogens and inducement and maintenance of chronic inflammations which have attached more and more attentions. In this study, we focused on the epigenetic regulation after infection, so we preliminary obtained the potential promoter clone with biological activity by cloning and functionally identified the promoter regulatory region of IL-17A, which provided working foundations to the subsequent molecular mechanism researches.

Conflict of interest statement

We declare that we have no conflict of interest.

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