Detection of gene expression and sequence analysis of chicken class II trans activator (*CIITA*)

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Abstract:

BACKGROUND: Class II transactivator (*CIITA*) is a dominant transcriptional element, controlling numerous genes in the immune system. *CIITA* is expressed in a constitutive pattern in antigen presenting cells although its expression can occur in other cell types. Since the revelation of *CIITA*, there have been considerable advances toward understanding its role as an activator of MHC II genes in humans and mice; nonetheless, there is a lack of published data for this gene in other animals such as chickens. **OBJECTIVES:** The goals of this study were to determine the expression of class II transactivator (*CIITA*) in chicken and analysis of the *CIITA* gene sequence between four Iranian indigenous chicken ecotypes. **METHODS:** After securing the research accuracy and optimization of reaction conditions, cDNA and DNA samples of gene were obtained from four Iranian indigenous chicken ecotypes. The PCR and RT-PCR products were sequenced and the data were analyzed by bioinformatics software. **RESULTS:** Comparison of the sequencing results with the reference sequence of the red jungle fowl revealed that these sequences belonged to the predicted *CIITA* gene. There was a high conservation rate in the sequence of *CIITA*. **CONCLUSIONS:** Our results indicated that like other species, *CIITA* is transcripted in chickens' immune system cells. Further studies on chickens must be done to reveal *CIITA* roles in immune responses of chickens.

Introduction

Class II transactivator (*CIITA*) is an effective transcriptional factor regulating various genes in the immune system (Harton and Jenny, 2000). *CIITA* is a member of a famous family of cytosolic proteins under numerous names, including NOD and NACHT proteins containing caspase activation and recruitment domain (CARD). The Nod like receptors (NLR) family is an evolutionary important immune gene as it consists of three distinct conserved motifs. Main features in the principal amino acid sequence of *CIITA* include an N-terminal section with acidic amino acids, three segments rich in proline, serine and threonine, a centrally placed GTP binding domain, and a C-terminal area consisting of leucine rich repeats (LRR) (Ting and Davis, 2005). *CIITA* is the only component of NLR family that has an N-terminal transcription activation domain with recognized function as a transcription activator (Krawczyk and Reith, 2006).

CIITA is expressed in a constitutive manner on macrophages, B lymphocytes, and dendritic cells (Muhlethaler et al., 1997) although its expression can be induced on various tissues by IFN-γ (Steimle et al., 1994). *CIITA* is expressed in activated human T cells, which is related to class II MHC, but not in mouse T cells (Harton and Jenny, 2000). Microarray experiments recognized over 40 genes that were anticipated to be regulated by *CIITA* (Nagarajan et al., 2002). Genes proposed to be withdrawn by *CIITA* consist of those encoding interleukine-4 (IL4), Fas ligand (Fas

L), cathepsin E, IL10, collagen type I a2 (COL1a2), tymidine kinase, cycline D1, and 16 other proteins with various functions (Sisk et al., 2000 and Nagarajan et al., 2002). Recently, it has been revealed that *CIITA* plays an important role in fighting against infectious diseases, cancer, and autoimmunity (Rasmussen et al., 2001). *CIITA* is necessary for the regulation and transcription of major histo-compatibility complex (MHC) class II genes in the normal immune reaction; therefore, it is a regulator for antigen presentation to CD4 T cells and often referred as the master regulator of MHC class II expression (Harton and Jenny, 2000). It is a non DNA-binding co-activator that is recruited to MHC promoters via many proteins bound to DNA (Zhu et al., 2000). The relationship between the expression of MHC class II molecules and the expression of *CIITA* was well defined (Day et al., 2003).

Since the detection of *CIITA* in 1993, there have been considerable progresses toward understanding its role as an activator of *MHC II* genes in humans and mice (Cheong et al., 2002); however, there are a few published data for this gene in other animals such as chickens. The results of in vitro experiments on *CIITA* gene have indications for practical applications. Transitory stimulation or inhibition of class II MHC expression, can hang the immune response during important situations (reviewed in Harton and Jenny, 2000, Waldburger et al., 2000, Kuipers and Elsen, 2005). By understanding the structure and function of *CIITA*, we could be able to use it in vaccination, prevention, and treatment of chicken diseases. Genetic variation in chicken populations would reveal the effects of polymorphism on the *CIITA* functions and occupation of different parts of *CIITA* in gene regulation. Finally, chickens could be as a laboratory animal for research purposes.

The goals of th present study were to determine the expression and phylogenetically analysis of chickens' class II transactivator gene. Sequence comparisons of the *CIITA* gene segment from four Iranian indigenous chicken ecotypes with related sequences from red jungle fowl is another purpose which discloses the genetic variation of this gene.

Materials and Methods

Sampling and isolation of genomic DNA: Four Iranian indigenous chicken ecotypes, consisting of local chickens of Mazandran, Isfahan, Urmia, Khorasan, along with Ross 308 chickens, were examined. Blood samples with EDTA were collected and preserved at -20^{o}C. Genomic DNA was isolated from whole blood using the extraction kit (AccuPrep Genomic DNA Extraction Kit Cat. No. K-3032, Bioneer corporation, Korea) as recommended by the manufacturer, and it was dissolved in deionized water for polymerase chain reaction (PCR) analysis.

PCR condition and procedures: The specific primer sequences used for the amplification of *CIITA* gene in chicken are shown in Table 1 and Figure 1. These primers were designed according to the sequence of inbred red jungle fowl (Gallus gallus), retrieved from the Ensemble database (ENSGALG-00000007171, Chromosome 14: 9, 290, 165-9, 298, 267). The expected size of the PCR products was around 388 and 259 bp.

The PCR mixture (total volume 25 µL) contained genomic DNA (0.5-1 µL), 2.5 µL of 10 mmol/L PCR buffer, 0.75 µL of 50 mmol/L MgCl2, 4 µL of 1.25 mmol/L dNTPs, 14 µL of distilled water, and 1 unit of Taq DNA polymerase (Cinnagen Co., Tehran, Iran). In addition, primer sets 1 and 2 were added at 1 µL into the reaction at two separate mixes. The PCR mixture was pre-denatured at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 62°C for 30s, and an extension at 72°C for 40s, and the final extension at 72°C for 5min. The amplified fragments were run on a 1.7% agarose gel and visualized in gel document system after staining with ethidium bromide.

RNA isolation and reverse transcription PCR: RNA was isolated from spleen and Buffy coat of Ross 308 chicken using TRIzol/TriPure (Roche, Germany) and then the RNA was digested with RNase-free DNase (Fermentas, Germany) according to manufacturer's instructions. To synthesize the first-strand cDNA, the RNA reverse-transcribed in a reaction with total volume of 20 µL by using random hexamer hexanucleotide primer and M-MLV reverse transcriptase (Invitrogen), according to manufacturer's instructions. The prepared cDNA was amplified using Taq DNA polymerase (Cinnagen Co., Tehran, Iran) and the specific primers for *CIITA*. PCR conditions and procedures were the same as the conditions and procedures described above.

Table1. Primer sequences for the PCR amplification of chicken *CIITA* gene.

Primer name	Sequence (5'->3')	Nucleotide position	Product length
F1	ACTTTGAAGGATTACCCTCGTGCG	9069059- 9069082	388
R1	TGGGGAATGCAAATGGCAGGAA	9068699- 9068720	
F2	TGGGAGATCAAGGCCTTCCTTCAA	9068930- 9068953	259
R2	TGTTTGGGGAATGCAAATGGCAGG	9068695- 90686718	

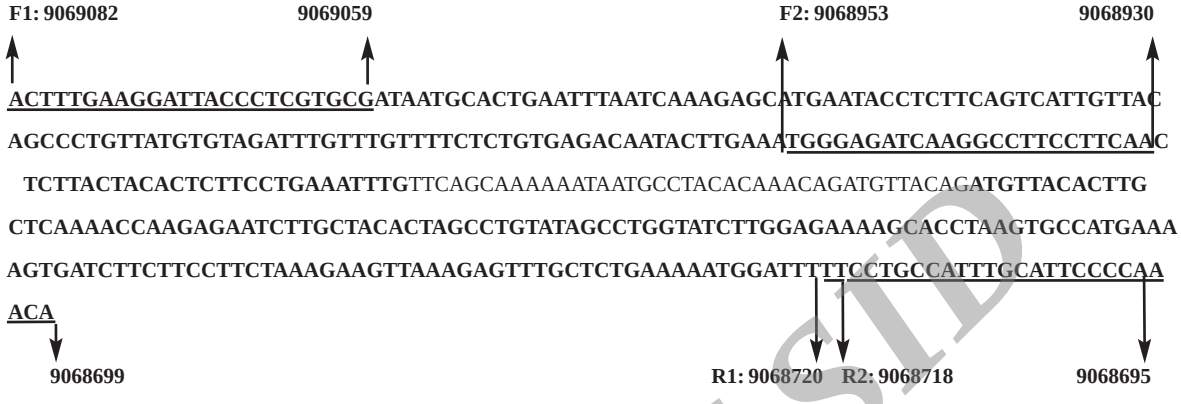

Figure 1. Primer sequences and locations for the amplification of chicken *CIITA* gene. The exonic regions are bolded.

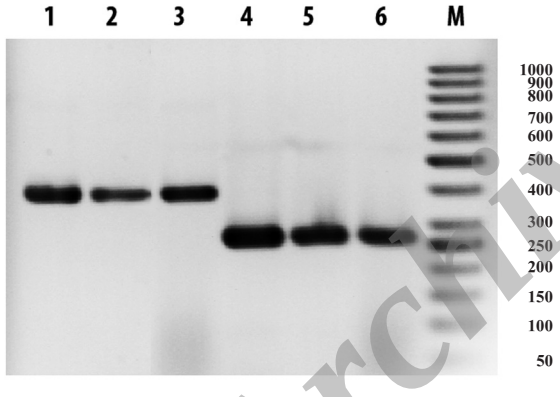

Figure 2. Electrophoresis of PCR products by using primer set 1 (lanes 1- 3) and primer set 2 (lanes 4- 6); Lanes 1 and 4: extracted DNA samples,; 2 and 5: cDNA samples from WBC, 3 and 6: cDNA Samples from the spleen and M: Marker (50 bp ladder) (Fermentas, Germany).

Direct sequencing and data analysis: The PCR products were purified using the purification kit (Bioneer, Cat. No. K-3034) and were also bidirectionally sequenced by using forward and reverse (F1 and R1) primers by an automatic DNA sequencer (ABI 3730 XL, Bioneer, South Korea). Sequences were analyzed by BLAST through the national center for biotechnology information website. The sequence alignment was made using clustal software, and the amino acids prediction was done using the Bioedit version 7.0.5.3 software package.

Phylogenetic analysis: The *CIITA* sequences from other organisms with the following accession numbers: *Homo sapiens* NM-000246, *Macaca mulatta* NC-007877, *Mus musculus* AK-080723, *Taeniopygia guttata* XM-002195026, *Meleagris gallopavo* MX-003210727, *Rattus norvegicus* NM-053529, *Cricetulus griseus* NW-00361363, and *Heterocephalus glaber* EHB13615 were obtained from the NCBI Genome database and were compared by phylogenetic analysis, using MEGA4 software.

Results

PCR produced 388 and 259 base pair amplification products from *CIITA* gene (DNA and cDNA) of chickens, regardless of their ecotypes (Figure 2).

The amplified products of cDNA show the same molecular weight as PCR products (Figure 2).

Comparison of Our sequences with the sequence of the red jungle fowl revealed that these sequences belonged to the predicted *CIITA* gene. Sequence analysis showed no nucleotide mutation in the sequences (Figure 3). In contrast with the Ensemble data, this area is free of introns.

ACT TTG AAG GAT TAC CCT CGT GCG ATA ATG CAC TGA ATT TAA TCA AAG AGC ATG AAT ACC TCT TCA GTC ATT GTT ACA {78}

GCC CTG TTA TGT GTA GAT TTG TTT GTT TTC TCT GTG AGA CAA TAC TTG AAA TGG GAG ATC AAG GCC TTC CTT CAA CTC {156}

TTA CTA CAC TCT TCC TGA AAT TTG TTC AGC AAA AAA TAA TGC CTA CAC AAA CAG ATG TTA CAG ATG TTA CAC TTG CTC {234}

AAA ACC AAG AGA ATC TTG CTA CAC TAG CCT GTA TAG CCT GGT ATC TTG GAG AAA AGC ACC TAA GTG CCA TGA AAA GTG {312}

ATC TTC TTC CTT CTA AAG AAG TTA AAG AGT TTG CTC TGA AAA ATG GAT TTT TCC TGC CAT TTG CAT TCC CCA AAC A {388}

Figure 3. Nucleotide sequences of four Iranian ecotypes and Ross 308 chickens DNA and cDNA.

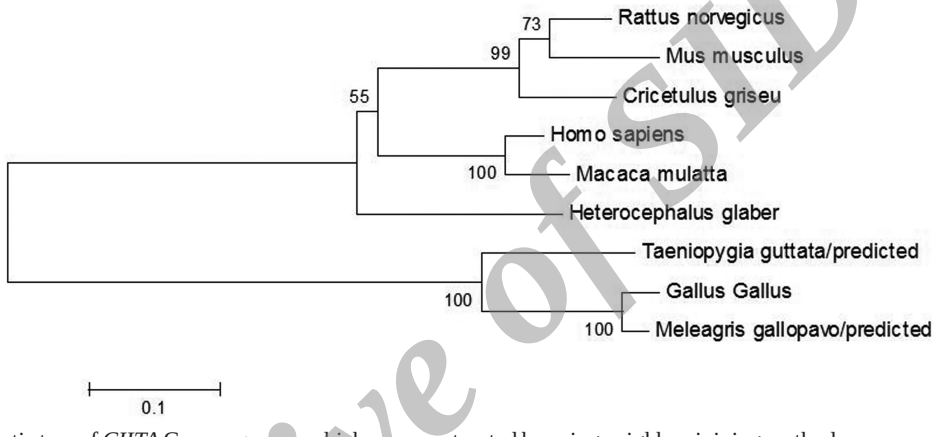

Figure 4. Phylogenetic tree of *CIITA* Gene sequence which was constructed by using neighbor-joining method.

The results of phylogenetics analysis are shown in figures 4. Phylogenetic tree consisted of two main clusters namely, mammals and birds. *Gallus gallus* was much closer to *Meleagris gallopavo* than *Taeniopygia guttata*.

Discussion

Gene's data from any completely sequenced genome give numerous levels of information. The creation of a high quality draft sequence of the chicken genome is a main progress in the field of animal genetics (International Chicken Polymorphism Map Consortium, 2004). Accessible sequence from the chicken included apparent orthologs of *CIITA*. The apparent orthologs of *CIITA* supports the suggestion that this protein originated as a result of gene duplications before the bird-mammal deviation; anticipated to have occurred 310 million years ago (Hughes A, 2006). Comparative investigation of genomes can be used to find conserved sequence elements, which may include formerly unknown genes. Comparison of two closely allied species like humans and mice will help us to find many conserved regions within coding and non-coding DNA. In contrast, a comparison between distantly related groups, such as fish and humans may detect only well-conserved exonic sequences; chicken represents an intermediate level of evolutionary distance, which makes them very useful for determining the essential features of vertebrate genomes (Furlong, 2005).

Our results indicated that the mRNA of *CIITA* is present in the chicken blood leukocytes and spleen cells. According to NCBI data, the sequence of this gene in humans is defined by synthesizing cDNA from different sources including lymph, germinal center B cells, lymphoma, cell lines, lung and uterus tumors, bone marrow, and some other tissues. In mice, the sequence of this gene is obtained from cDNA of tissues such as 4-cell stage embryo, aorta,

vein, spleen, thymus, kidney, germinal center B cells and mammary.

Sequencing analysis of different Iranian chicken ecotypes indicated that the studied fragments are highly conserved. According to the database (Beijing Genomics Institute, NCBI-dbSNP: International Chicken Polymorphism Map Consortium, 2004), at the position of 9297624_ the single nucleotide polymorphism (C-T) exists in different genotype; however, our results did not show this variation.

Certain duplications of the genes encoding Nod like receptors (NLR) preceded the divergence of tetrapods and bony fishes, occurring about 450 million years ago (Kumar and Hedges 1998). The function of *CIITA* in the regulation expression of the class II MHC molecules, supported by the presence of the *CIITA* gene in bony fishes, as well as birds and mammals, are known to be present in all jawed vertebrates (Kelley et al., 2005). Functional description of NLRs, particularly in lower vertebrates, would elucidate the *CIITA* evolution (Liu et al., 2012). Our phylogenetic results for mRNA sequences corroborates the findings of Hughes (2006) and Liu (2012) who reported that the phylogenetic tree with the same arrangement was mainly divided into two clusters.

This study evaluated DNA sequences and gene expression of chicken *CIITA*. To the best of our knowledge, this was the first report on *CIITA* expression in chickens. The primers used in this study were designed according to the related sequences in the Ensemble database. As expected, based on human and mouse information, the *CIITA* sequence is also highly conserved among different chicken ecotypes. In addition, the gene expression in leukocytes and spleen may indicate that *CIITA* protein is important in the immune responses of chickens as well as humans and other animals.

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بررسی بیان و آنالیز توالی ژن *CIITA* در ماکیان

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چکیده

زمینه مطالعه: فعال کننده ترانس کلاس دو (*CIITA*) از عوامل تنظیم کننده رونوشت برداری است که بیان چندین ژن در سیستم ایمنی را کنترل می نماید. بیان *CIITA* می تواند به صورت دائمی در سلول های عرضه کننده پادگن و یا به صورت القایی در دیگر سلول های بدن باشد. در سال های اخیر پیشرفت هایی در زمینه درک نقش این مولکول در انسان و موش حاصل شده است ولی در زمینه این ژن در سایر حیوانات از قبیل ماکیان اطلاعات منتشر شده‌ای وجود ندارد. **هدف:** هدف این مطالعه بررسی بیان ژن *CIITA* و مطالعه فیلوژنتیکی توالی آن است. مقایسه توالی ژن *CIITA* در طیور بومی ایران با توالی red jungle fowl به منظور مشخص نمودن تغییرات از دیگر اهداف است. **روش کار:** پس از اطمینان از صحت آزمایش و بهینه سازی واکنش، DNA و cDNA مربوط به ژن مورد نظر از چهار سویه ماکیان بومی ایران به دست آمد. نمونه های حاصل از PCR و RT-PCR پس از تعیین توالی توسط نرم افزارهای بیوانفورماتیک مورد بررسی قرار گرفتند. **نتایج:** نتایج این تحقیق نشان دهنده بیان ژن *CIITA* در گلبول های سفید خون و طحال ماکیان است. برخلاف اطلاعات موجود در پایگاه های اطلاعاتی ناحیه مورد بررسی فاقد اینترون می باشد. **نتیجه‌گیری نهایی:** قطعه بررسی شده در ماکیان همانند انسان و سایر حیوانات بسیار حفاظت شده است. بیان ژن *CIITA* در ماکیان بیانگر آن است که احتمالاً این پروتئین در پاسخ ایمنی ماکیان نیز همانند انسان و سایر حیوانات نقش دارد که لازم است در مطالعات آینده روشن شود.

واژه های کلیدی: ماکیان، بیان ژن *CIITA*، ایمنی

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