Cloning and Genetic Evolution Analysis of chIL-2 Gene of Chinese Local Breeds*

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Abstract Chicken counterpart of interleukin-2 (chIL-2) is discovered recently which shows great potential in immunotherapy and immunoadjuvant applications. In view of its immunomodulating effects and involvement in disease resistant traits, this study compared the sequence relatedness and the genetic evolution of chIL-2 genes of local breeds with other geographical isolates. The chIL-2 genes from two Chinese local breeds (Xianju and Silky) and a commercial broiler breed (Avian) were amplified and cloned from Con A-stimulated chicken spleen lymphocytes. Sequence comparison revealed a similar genetic organization among the breeds with a coding region of 429 bases, a 5′-terminal untranslated region (UTR) with 17 nucleotides in length and an UTR with 285 nucleotides with five repetitive sequence of ATTTA at 3′-terminal. The deduced amino acid sequences were compared with strains Kestrel, Obese and SC Leghorn chickens from GenBank. Results showed that Xianju, Silky and Kestrel breeds shared an identical chIL-2 gene while the chIL-2 from Avian commercial broiler, Obese and SC Leghorn chickens was identical. In addition, a hypermutation region between residue 28 to 32 was identified. Phylogenetic analysis grouped Xianju, Silky and Kestrel, and Avian, Obese and SC into a separate group. Furthermore, a mutation from glutamic acid (E) to glycine (G) was found at the conserved residue 133 in Chinese official chicken breed-Silky chicken.

Key words chicken interleukin-2, cloning, genetic evolution

Interleukin-2 (IL-2) is essential for regulating several crucial host immune processes. This cytokine serves as a growth factor for variety of cell types and plays key roles in T cell growth and differentiation, functional B cell development and activation of NK cells following stimulation with antigens or mitogens[1]. It is a potent immune system modulator affecting nearly every facet of host immune response. IL-2 has also been used as an immunotherapeautic agent, both in the treatment of certain malignancies and as an enhancing agent in vaccines. Chicken IL-2 (chIL-2) also presents similar bioactivity both *in vivo* and *in vitro*[2,3].

Currently, IL-2 genes from more than thirty species have been cloned since the human IL-2 (hIL-2) gene was first cloned in 1983[4]. Monoclonal antibodies to hIL-2 have been produced[5~7]. However, the research of IL-2 in lower animals is lagging behind. Until recently, a cDNA clone encoding chIL-2 which showed homology to mammalian IL-2 and IL-15 has been described[8]. The gene structure of chIL-2 closely resembles that of human and mouse IL-2 rather than IL-15, which comprises of a promoter, four exons and three introns. The chIL-2 gene has been mapped to chicken chromosome 4 and is linked to a gene encoding annexin V[9]. A functionally bioactive recombinant chicken IL-2 (rchIL-2) has been successfully expressed in prokaryotic system like *E.coli* and in eukaryotic cells like COS-7, CHO-K1, while monoclonal antibodies to chIL-2 has been produced[10~13].

Recently, many new vaccines are being developed using recombinant viral proteins expressed in viral vectors. Vaccination studies in mammals and birds using exogenous IL-2 showed an enhancement of viral-based vaccines efficacy in young animals[14,15]. Therefore, it is possible that the delivery of chIL-2 gene or rchIL-2 proteins can significantly enhance the efficacy of avian vaccines administered *in ovo* and at hatching. In order to understand the genetic conservation of chIL-2 gene in both local and foreign breeds, the local breeds Xianju, Silky and foreign breed Avian commercial broiler were used in this study. Xianju layer chicken is a local layer breed originated from Xianju county of Zhejiang province while silky broiler chicken is a local official broiler breed originated from Taihe county of Jiangxi province, which is used mainly for the production of drug in Traditional Chinese Medicine. Avian commercial broiler is a foreign broiler breed[16]. Our objectives were to clone, sequence and analyze the chIL-2 gene from three different breeds. The information provided an insight to understand better the evolutional stabilization of chIL-2 among different breeds and to establish a foundation for application of avian cytokines as immunotherapeutics and vaccine adjuvant.

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1 Materials and methods

1.1 Materials

1.1.1 Chickens: 1-day old Xianju chickens were purchased from Chunan Zhenxing Poultry Co. Ltd. 1-day old avian commercial broiler chickens were purchased from Yuyao Shennong Livestock Co. Ltd. 1-day old Silky officinal broiler chickens were purchased from Jangshan Preservation Center of Silky broiler breed. The chickens were reared in individual wire cages with unlimited access to feed and water.

1.1.2 Enzymes and reagents: Complete RPMI 1640 growth medium [GIBCOBRL, Grand island, New York, supplemented with 10% fetal calf serum (FCS), 25 mmol/L HEPES, 20 mmol/L sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 mg/L)]. FCS was purchased from Hangzhou SIJIQING Bioengineering Material Co. Ltd. (Hangzhou, China). Con A (Sigma, 10 mg/L) was diluted in RPMI 1640. Total RNA Isolation System Kit were purchased from Promega (Madison, WI). Lymphoprep was obtained from Shanghai Hengxin Chemicals Co. Ltd. UNIQ-10 column plasmid minipreps kit, UNIQ-5 DNA gel extraction kit, Taqplus DNA polymerase, T4 DNA ligase and restriction endonuclease *Eco*R I and *Bam*H I were purchased from Sangon (Shanghai, China).

1.1.3 Plasmid and bacteria stock: pGEM-T Easy Vector System was used for cloning (Promega, Madison, WI) and recombinant plasmids were maintained in *E.coli* DH 5α.

1.2 Methods

1.2.1 Isolation of splenic mononuclear cells (SMC): 30-day old chickens were sacrificed by intravenous inoculation of barbiturate. Spleen collected under aseptic conditions was washed with Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS, 717 mmol/L K_2HPO_4, 283 mmol/L KH_2PO_4, pH 7.2), was minced with a pair of scissors and then passed through a stainless steel screen to obtain a homogeneous cell suspension. The cells were collected after spinning at 300 *g* for 10 min at 4℃ and resuspended in HBSS (Hanks' Balanced Salt Solution). Cell suspension was carefully overlayed on equal volume of Lymphoprep. An interface rich in mononuclear cells was recovered after spinning at 500 *g* for 30 min at 4℃. Cells were washed twice in serum free RPMI 1640 and then adjusted to three different cell concentration of 2×10^6/ml, 3×10^6/ml and 1×10^7/ml in complete RPMI 1640 medium. Cell viability (> 99%) was detected by 0.1% Trypan blue dye staining. SMC (splenic mononuclear cell) of each concentration was stimulated with Con A at a final concentration of 5, 10 and 20 mg/L at 40℃ in 5% CO_2 for 18, 19 and 20 h.

1.2.2 RNA isolation and RT-PCR: Cells with different densities stimulated for 18, 19 and 20 h were harvested by centrifugation at 500 *g* for 3 min at 4℃ and followed by washing three times with PBS. Total cellular mRNA was extracted using RNAgents Total RNA Isolation System. Oligo-dT was used in first strand cDNA synthesis and two chIL-2 specific PCR primers were designed based on the published sequence[8]. The upstream primer (5′-GCGAATTATAACTGGGACACTGCCATG-3′) and the downstream primer (5′-GCGGATCCAAATG-TCATCTAGAAGTGACT-3′) were designed to carry out an *Eco*R I or *Bam*H I restriction site, respectively, to facilitate cloning. PCR was performed for 30 cycles at 95℃ for 1 min, 56℃ for 45 s and 72℃ for 2 min, and a final extension at 72℃ for 10 min. PCR product was detected in 1% agrose gel.

1.2.3 cDNA sequencing: RT-PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and then cloned into pGEM-T vector by T-A ligation method and transformed into competent *E.coli* DH5α as described[17]. The recombinant plasmid were confirmed by PCR screening and the restriction enzymes digestion using *Eco*R I / *Bam*H I. At least five positive chIL-2 clones from each breed were sequenced with an automated DNA sequencer (ABI PRISM 377).

1.2.4 Nucleotide analysis, secondary structure prediction and phylogenetic study: The sequences and deduced amino acid sequence were analyzed using Lasergene software package (DNASTAR, Madison, Wisc., USA) and Antheprot software (Version 4.5) (Institute of Biology and Chemistry of Proteins, France) was used for secondary structure analysis. The phylogenetic analysis of chIL-2 from local breeds was done by CLUSTAL using additional IL-2 protein sequences from various species found in GenBank namely Red-crowned mangabey (U19846), Rhesus monkey (U19847), Pig-tailed macaca (U19852), Cynomolgus monkey (D63352), Human (U25676), Gibbon(K03174), Baboon (U88365), House mouse (NM-008366), Mouse (K02292), Rat (M22899), Mongolian gerbil (X68779), Guinea pig (AB010093), Rabbit (AF068057), Canis (D30710), Gray seal (AF072871), Northern elephant seal (U79187), Cat (L19402), Horse (X69393), Pig (X58428), Beluga whale(AF072870), Killer whale (AF009570), Bovine (M13204), Red deer (U14682), Sheep (X53934), SC chicken (AF017645), Obese chicken (AF000631), Kestrel leghorn Chicken (AF033563), Silky chicken (AF502412), Xianju chicken (AF483600), Avian commercial broiler chicken (AF483599).

2 Results and analysis

2.1 *In vitro* induction of chicken IL-2 mRNA and RT-PCR detection

In order to determine the optimal inducing condition for chIL-2 trancription *in vitro*, a comparison study using different cell densities versus mitogen concentrations was established. SMC obtained from chicken vaccinated with Newcastle disease vaccine was cultured at three densities and each preparation was stimulated with three different concentration of Con A for three durations (18 h, 19 h, 20 h). Optimal secreting condition of chIL-2 was obtained when cell with a density of 2×10^6/ml was stimulated with 10 mg/L Con A for 20 h at 40 ℃. A DNA fragment with size greater than 700 bp was specifically amplified by RT-PCR from total cellular mRNA obtained from SMC incubated for 20 h (Figure 1). However, no similar DNA product could be detected from the SMC treated with other conditions (data not shown).

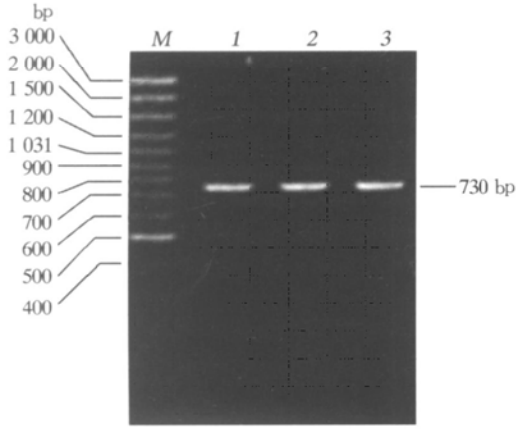

Fig. 1 RT-PCR products of interleukin 2 gene for the different chicken breeds

M: marker(3 000~ 100 bp); *1*: Silky chicken; *2*: Xianju chicken; *3*: Avian chicken.

2.2 Nucleotide sequence of chIL-2 gene and structural analysis

PCR product amplified from each breed was cloned and sequenced. Sequence analysis revealed the chIL-2 gene obtained from the three chicken breeds (Xianju, Silky and Avian) were consistently 734 bp in length, which encode a precursor polypeptide of 143 amino acid residues (GenBank accession number: AF483600, AF502412, AF483599). An UTR of 17 nucleotides was found at the 5′-terminal of all chIL-2 genes. On the other hand, an UTR of 285 nucleotides with 4 repetitive sequence of "ATTTA" was found in 3′-terminal (482~ 490, 494~ 498, 509 ~ 513, 731~ 735). The identity of chIL-2 genes of the different chicken strains is shown in Table 1. Amino acid sequence variation between China local breeds with chicken from different geographical locations was unclear. The significant of variation at amino acid level was further characterized with the deduced amino acid of Kestrel, Obese and SC leghorn chicken from GenBank. Alignment results displayed in Figure 2 showed nearly identical chIL-2 sequence among different geographical isolates except at residues 15, 28, 30, 32 and 133. Mutations found at these residues suggested that the chicken breeds used in this study could be grouped into two groups. Xianju, Silky and Kestrel were possibly derived from a common source whereas Avian, Obese and SC were placed under another group. Intriguingly, a mutation was found at the conserved residue 133 of Chinese official chicken breed-Silky chicken. A conserved glutamic acid (E) found in other chicken breeds was substituted by a glycine (G) in Silky chicken.

Table 1 Identity levels of nucleic and deduced amino acid sequences of chicken IL-2 cDNA sequences of several chicken strains*

%

	Kestrel	Obese	SC	Avian	Silky	Xianju	
Kestrel		98.8	99.1	99.1	99.8	100.0	Kestrel
Obese	96.5		99.8	99.8	98.6	98.8	Obese
SC	97.2	98.6		100.0	98.8	99.1	SC
Avian	97.2	98.6	99.3		98.8	99.1	Avian
Silky	98.6	95.8	96.5	96.5		99.8	Silky
Xianju	99.3	96.5	97.2	97.2	98.6		Xianju
	Kestrel	Obese	SC	Avian	Silky	Xianju	

*Upper section: percentage of nucleic acid sequence identity; Lower section: percentage of deduced amino acid sequence identity.

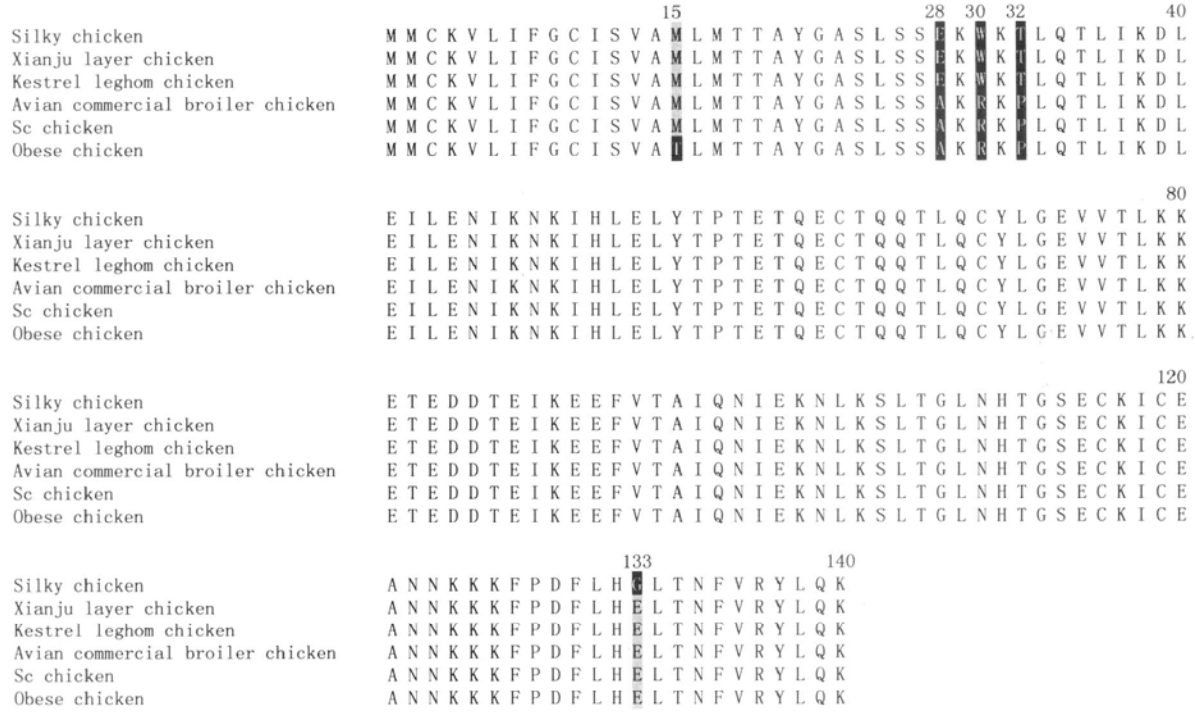

Fig. 2 Comparison of identity of the deduced amino acid sequence of chIL-2 gene

2.3 Prediction of secondary structure

Chicken IL-2 has a predicted signal peptide of 22 amino acids, similar to the signal sequence of mammalian IL-2s in size and distinctly shorter than the signal sequence of chicken IL-15 (66aa) and mammalian IL-15 (45aa)[18]. Amino acid sequence analysis revealed that the mature chIL-2 has four conserved cysteines located at residues 42, 49, 95 and 98, allowing the formation of two intrachain disulfide bonds, which a similar structure that could also be found in mammalian IL-15. However, mammalian IL-2 mature protein contains only 3 conserved cysteines at the site of 58, 105 and 125, and formed one intrachain disulfide bond between 58 and 105[19]. Further analysis indicated that chicken IL-2 contained four regions of heptad repeat (LQTLIKDLEIL, LQCYLGEVVTL, IQNIEKNLKSL and LHELTNFVRYL), and the predicted secondary structure of the mature chIL-2 formed 4α helices at regions covered by residues 10~ 30, 39~ 48, 64~ 85 and 108~ 117(data not shown).

2.4 Phylogenetic analysis

chIL-2 amino acid sequence from six different breeds were aligned with IL-2 from 23 mammalian species from GenBank. We computed the genetic distance amongst 23 mammalian IL-2 proteins and 6 chIL-2 protein. These distances and the dendogram constructed (Figure 3) indicated that the chIL-2s and the mammalian IL-2s were grouped into two families: mammalian IL-2 family and chicken IL-2 family. The establishment of the phylogenetic tree showed that the molecular evolution was similar to the evolution tree of IL-2 family and IL-15 family. In avian IL-2, the Xianju chicken IL-2 and Silky chicken IL-2 had close relationship with Kestrel leghorn chicken IL-2; Avian commercial broiler chicken IL-2 had close relationship with Obese chicken IL-2.

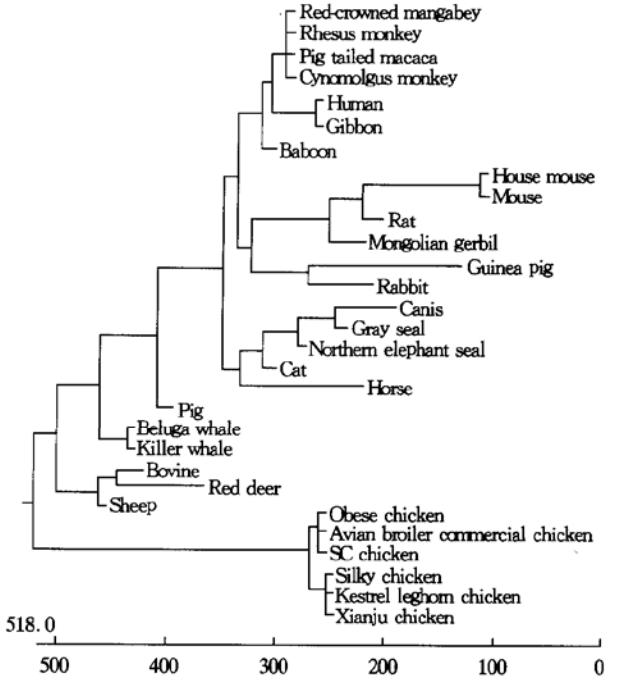

Fig. 3 Phylogenetic relationship based on deduced amino acid sequences of seveal IL-2 mammalian and bird species

3 Discussion

Chicken IL-2 is a cytokine that play an important role as an immunoregulatory molecule identical to mammalian IL-2s[20]. It has been shown to stimulate T lymphoblast cells, enhance not only NK-cell activity, but also *in vivo* disease resistance against various infections[2, 21~24]. Pharmacologic doses of rIL-2 have proven valuable for the treatment of cancer and as an adjuvant for vaccines[25, 26]. chIL-2 exhibits similar bioactivity to mammalian IL-2[2]. Chicken IL-2 gene was first obtained by constructing cDNA library[8] and was further confirmed with RACE technology[9]. In this study, chicken immune system was first stimulated with live Newcastle disease virus and chIL-2 gene was then amplified by RT-PCR from chicken spleen lymphocytes which had been co-cultured with ConA for 20 h at 40 ℃ *in vitro*. Consistent with the report of Thiagarajan *et al*.[27], it was noticed that the concentration of Con A, lymphocyte or FBS could significantly affect the production of chIL-2 by SMC. As to the most appropriate time to detect the mRNA of chicken IL-2 *in vitro*, no consensus point of view has been agreed to so far. Sundick *et al*.[8] detected the mRNA 5 h after stimulating with Con A. Choi *et al*.[10] reported that mRNA of chicken IL-2 can be detected at 2 h, 4 h and 16 h by RT-PCR and Northern hybridization after stimulation with Con A. Liu *et al*.[28] detected the mRNA of chicken IL-2 at 2 h, 3 h, 4 h, 8 h, 12 h, 24 h and 48 h after stimulation with Con A. In this study, total cellular mRNA was extracted at the time of 18, 19 and 20 h for RT-PCR, chIL-2 mRNA could only be detected at 20 h. These results were different from previous studies. We considered the discrepency was largely due to the route and type of antigens used for stimulation. In the present study, the chickens were stimulated by injecting ND vaccines.

Point mutations were found at nucleotides 44, 83, 88, 94, 144 and 398 of chIL-2 gene of Xianju layer, silky and Avian commercial broiler. Among these mutations, mutations in position 44, 144 and 398 were non-sense mutations. Nevertheless, mutations at nucleotides 83, 88 and 94 resulted in the change of amino acid at residues 28, 30 and 32 to E, W and T in Xianju, Silky and Kestrel when compared to the Avian commercial broiler chicken IL-2 which has amino acid residues A, R and P in the corresponding positions. Analysis of the molecular evolution of chIL-2 showed that Silky and Xianju chicken were in the same subfamily with Kestrel chicken while avian commercial broiler chicken belonged to the same subfamily of SC and Obese chicken. These findings showed that Silky and Xianju chicken have close ancestor with Kestrel chicken and Avian commercial broiler chicken shares a close ancestor with SC and Obese chicken. However, mutations found in region covered by amino acid residues 28~ 32 resulted in variations of the biological functions of the different breed chicken IL-2 remained unanswered. Moreover, it is worth noting that a mutation was found at the very conserved residue of 133 in Chinese official chicken breed-Silky chicken. Further study is required to determine if this single mutation would lead to disease resistance associated with this breed of chicken.

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中国地方品种鸡白细胞介素 2 基因的克隆及遗传进化分析*

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摘要 鸡白细胞介素 2 (chIL-2) 是近年来新发现的一类细胞因子. 根据 Sundick 等发表的鸡 IL-2 基因序列设计一对特异性引物, 从 ConA 体外激活的脾淋巴细胞中提取 mRNA, 通过 RT-PCR 方法分别扩增和克隆了我国仙居鸡、丝羽乌骨鸡两个地方品种和艾维茵商品肉鸡 IL-2 cDNA. 仙居鸡、丝羽乌骨鸡和艾维茵商品肉鸡 IL-2 基因的编码区均由 429 nt 组成, 编码一个由 143 个氨基酸组成的前体蛋白. 基因 5′端含有 17 个核苷酸, 3′端含有 285 个核苷酸组成的非编码区, 3′-UTR 中含有 5 个重复的 "ATTTA" 序列. 编码蛋白氨基酸与来自 GenBank 的 Kestrel、Obese 和 SC 品系的来杭鸡比较, 氨基酸的突变主要发生在 28~ 32 位. 而这一区域仙居鸡和丝羽乌骨鸡的编码氨基酸序列与 Kestrel 来杭鸡相同, 艾维茵商品肉鸡与 Obese 和 SC 来杭鸡相同. 基因系统进化树分析表明, 仙居鸡、丝羽乌骨鸡和 Kestrel 来杭鸡具有很近的亲缘关系, 艾维茵商品肉鸡与 Obese 和 SC 来杭鸡具有很近的亲缘关系. 中国的药用鸡品种——丝羽乌骨鸡在非常保守的 133 位发生了氨基酸突变.

关键词 鸡白细胞介素 2, 克隆, 系统进化

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