

Identification of Differentially Expressed Genes between Neonatal and Peripubertal Rat Thymi Using GeneFishing™ Polymerase Chain Reaction

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ABSTRACT

Aging causes thymus involution, and genes in thymus play an important role in the development of the immune system. In this study, we compared genes expressed in thymus of neonatal and peripubertal rats using annealing control primers (ACPs)-based GeneFishing polymerase chain reaction (PCR) and semiquantitative reverse transcription (RT)-PCR. We identified 10 differentially expressed genes (DEGs) with 20 ACPs. Of 10 DEGs, *bystin*-like, collagen type V alpha 1 (COL5A1), and T-cell receptor beta-chain segment 2 (TCRB2) that are related to immune-function were detected in rat thymus. *Bystin*-like and TCRB2 were up-regulated, while COL5A1 was down-regulated in peripubertal thymus. Semiquantitative RT-PCR confirmed postnatal changes in expression of *bystin*-like, COL5A1, and TCRB2. These results suggest that *bystin*-like, COL5A1, and TCRB2 could regulate immune function controlled in thymus as age increases.

(Key words : Aging, Gene expression, Polymerase chain reaction, Rats, Thymus gland)

INTRODUCTION

Thymus plays an important role in the development of the immune system in mammals, and lymphocytes mature into T cells in its lobules (Miller, 2002). Thymus acts like a thermostat to provide the right balance of immunity. Therefore, it adjusts the body tolerance to fight against infection or tumor, and to prevent autoimmune disease. However, thymus undergoes physiological changes associated with aging that is termed thymic involution (Stutman and Good, 1974; Thoman, 1995).

Aging is a complex, continuous, and slow process that involves most organs of the organism, causing their abnormal function of both qualitative and quantitative terms, as well as morphological or structural changes. Thymus atrophy commences with the onset of puberty in many species, and sex hormones partially mediate this atrophic process (Nunn *et al.*, 1999). Therefore, the identification of genes expressed in thymus is critical for understanding the mechanism of thymic atrophy or involution with age. In this study, we identified differentially expressing genes between neonatal and peripubertal rat thymus using annealing control primers (ACPs)-based GeneFishing PCR. Our results

show that *bystin*-like and T-cell receptor beta-chain segment 2 (TCRB2) are up-regulated while collagen type V alpha-1 (COL5A1) is down-regulated in peripubertal rat thymus. These results suggest that *bystin*-like, TCRB2 and COL5A1 might regulate various immune functions including atrophy and involution of thymus.

MATERIALS AND METHODS

Animals

The neonatal (6.3 g, postnatal 1 day; P1) and peripubertal (200 g, postnatal 60 days; P60) female rats of Sprague-Dawley were used (n=12). The rats were purchased from Samtako Co. (Animal Breeding Center, Korea). Animals were maintained under controlled temperature (25°C) and lighting (14/10, light/dark cycle), and were allowed to have free access to food and water. The rats were used in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health, Education and Welfare publication no. NIH85-23).

First-strand cDNA Synthesis

Total RNAs extracted from rat thymus were used for

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the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 hr at 42°C in a final reaction volume of 20 μ l containing 3 μ g of the purified total RNA, 4 μ l of 5 reaction buffer (Promega, Madison, WI, USA), 5 μ l of dNTPs (each 2 mM), 2 μ l of 10 μ M dT-ACP1 (5'-CGTGAAT-GCTGCGACTACGATTTTT(18)-3'), 0.5 μ l of RNasin[®] RNase Inhibitor (40 U/ μ l; Promega), and 1 μ l of Moloney murine leukemia virus reverse transcriptase (200 U/ μ l; Promega). First-strand cDNAs were diluted by the addition of 80 μ l of ultra-purified water for the GeneFishing[™] PCR, and stored at -20°C until use.

ACP-based GeneFishing[™] PCR

Differentially expressed genes (DEG) were screened by annealing control primers (ACP)-based PCR method (Kim *et al.*, 2004) using the GeneFishing[™] DEG kits (Seegene, Seoul, Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 μ l containing 3-5 μ l (about 50 ng) of diluted first-strand cDNA, 1 μ l of dT-ACP2 (10 μ M), 1 μ l of 10 μ M arbitrary ACP, and 10 μ l of 2 \times Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 sec, followed by 65°C for 40 sec, 72°C for 40 sec, followed by a 5 min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

Cloning and Sequencing

The differentially expressed bands were extracted from the gel by using the GENCLEAN[®] II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cloned plasmids were sequenced with ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Confirmation

The differential expression of DEG was confirmed by RT-PCR using each gene specific primer pair. Specific primers [5'-CCCAGCTGGTGTAGTTAAAG-3' (sense) and 5'-GAAACCCAGTGAGCTTACAC-3' (antisense)] for bystin, [5'-TACCAAGAAAGGCTACCAGA-3' (sense) and 5'-GCTCTTCCCTTTGTTCTCT-3' (antisense)] for COL5-A1 and [5'-AAGGTAAGCTTAGGCCTGTT-3' (sense) and 5'-TGAATTGTGCATAAGTGAGC-3' (antisense)] for TC-RB2 were used in PCRs with *Taq* polymerase (Takara, Japan). The first-strand cDNA was normalized by the

rat glyceraldehyde-3-phosphate dehydrogenase (GAP-DH). The normalized cDNA was used as a template. PCR conditions were initial denaturation at 94°C for 5 min, then 28 or 30 cycles at 94°C for 20 sec, 55°C for 20 sec, 72°C for 20 sec, and a final extension step at 72°C for 10 min. The amplified PCR products were separated in 1.5% agarose gel stained with ethidium bromide. The differentially expressed bands obtained by RT-PCR using P1 and P60 rat thymus were directly sequenced with ABI PRISM3100-Avant Genetic Analyzer (Applied Biosystems).

RESULTS

Differentially Expressed Genes in Rat Thymus

Thymus is responsible for changes in the immune system with age. To identify DEGs in thymus with age, we compared the mRNA expression profiles in rat thymus between neonate (P1) and peripuberty (P60). The mRNAs isolated from each thymus of the rat were subjected to the ACP system. The structure of ACP is shown in Fig. 1. ACP technology based on the unique tripartite structure of a specific oligonucleotide primer, which has 3'- and 5'- end distinct portions separated by a regulator, specifically targets sequence hybridization to the template via a polydeoxyinosine linker. Using a combination of 20 arbitrary primers of two anchored oligo (dT) primers of ACP-based GeneFishing PCR kit, we identified 10 DEGs in rat thymus between P1 and P60. Among the 10 DEGs analyzed, 7 genes were up-regulated in P60 thymus, compared to those in P1. The sequence similarities and characterization of differentially expressed transcripts are summarized in Table 1 and 2. BLASTN and BLASTX searches in NCBI GeneBank revealed that DEGs identified in this study displayed marked similarities with known genes or ESTs

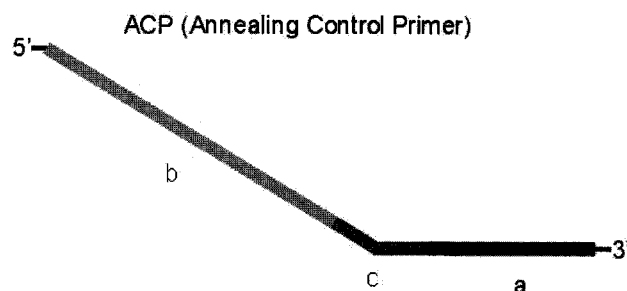


Fig. 1. The structure of annealing control primer (ACP). The ACP is composed with three sequence regions: a, core sequence (3'-end targeting portion; 10 nts) having a hybridizing sequence substantially complementary to a site on a template nucleic acid to hybridize; b, universal sequence (5'-end portion; 22 nts); c, regulator sequence (linker portion separated 3'-end portion and 5'-end portions; 5 nts).

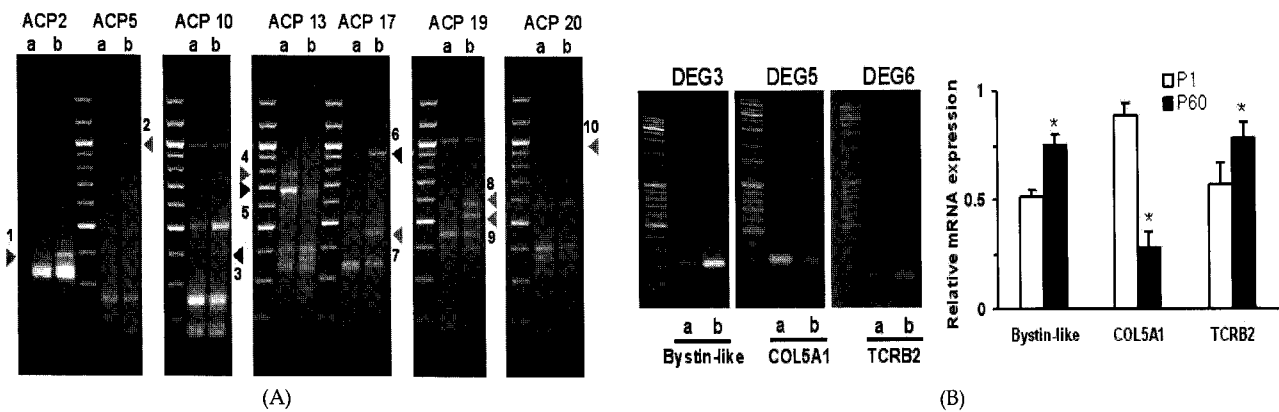


Fig. 2. Differentially expressed genes (DEGs) between neonatal and peripubertal rat thymus. (A) Gel is photographed to show DEGs obtained from thymus of different age. Using 20 ACPs, 10 DEGs were identified in P1 and P60 thymuses. Arrows indicate DEG bands (1~10). These DEG bands were excised from the gel for further cloning and sequencing. Three black triangles represent bystin-like, COL5A1, and TCRB2. ACP, annealing control primer; a, post neonatal 1 day thymus; b, post neonatal 60 days thymus. (B) Expression level of DEGs derived from P1 and P60 rat thymus using semi-quantitative RT-PCR. Data of bar graph are shown as mean±SD of triplicate determinations. COL5A1, Collagen type V alpha 1; TCRB2, T-cell receptor beta-chain segment 2.

Table 1. Up-regulated genes in peripubertal rat thymus

DEG no.	Genebank accession no.	Identity	Homology
DEG1	AK141017.1	Mus musculus 0 day neonate cerebellum cDNA, product : nuclear receptor	169/179 (94%)
	AF287263	Mus musculus ATP-binding cassette 1, sub-family A, member 1 (Abca1) gene	166/175 (94%)
	MMU58105 DN933925 (EST)	Mus musculus Btk, alpha-D-galactosidase A (Ags), Ribosomal protein (L44L) AGENCOURT_50149273 NCI_CGAP_Pr50 Rattus norvegicus cDNA clone IMAGE: 7934477	166/175 (94%)
DEG2	AK134048.1	Mus musculus adult male thymus cDNA, product: hypothetical protein	201/228 (88%)
DEG3	BC079030.1	Rattus norvegicus bystin-like, mRNA complete cds	324/326 (99%)
DEG6	M63794.1	Rat T-cell receptor beta-chain, segment 2 (TCRB2)	286/289 (98%)
DEG7	BQ210711	UI-R-DY1-com-c-12-0-UI.sl NCI_CGAP_DY1 Rattus norvegicus cDNA clone	73/76 (96%)
	NM_021745.1	Rattus norgicus nuclear receptor subfamily 1, group H, Member 4 (Nr1 h4)	
	RNU18374	Rattus norgicus famesoid X activated receptor mRNA, complete cds	73/76 (96%)
	AB180912.1	Rattus norgicus mRNA for novel serine protease like protein, complete cds	72/75 (96%)
DEG8	CX571118 (EST)	RV L2366 Wackym-Soares normalized rat vestibular cDNA library Rattus norgicus cDNA	38/40 (95%)
DEG9	AL732502.32	Mouse DNA sequence from clone RP23-292G12 on chromosome 4	
DEG10	BQ191723 (EST)	UI-R-DY0-cmh-n-21-0-UI.sl NCI_CGAP_DY0 Rattus norvegicus cDNA clone IMAGE: 7328951	

Table 2. Up-regulated genes in neonatal rat thymus

DEG no.	Genebank accession no.	Identity	Homology
DEG4	CB325917 (EST)	UI-R-DZ0-cro-o-08-0-UI.sl NCI_CGAP_DZ0 Rattus norvegicus cDNA clone IMAGE: 7342786	
	AK146823.1	Mus musculus 16 days embryo kidney cDNA, thyroid hormone receptor interactor 6	461/493 (93%)
	AF097511	Mus musculus zyxin related protein-1 (Zrpl) mRNA, complete cds	460/493 (93%)
DEG5	AJ005394.1	Rattus norgicus mRNA for collagen alpha 1 type V (COL5A1)	550/556 (98%)

(Table 1 and 2). As shown in Fig. 2, Table 1 and 2, bystin-like and TCRB2 were up-regulated but COL5A1 was down-regulated in P60 rat thymus, suggesting that these genes might involve in the mechanism of thymic involution.

The results of the ACP analysis were confirmed by semi-quantitative RT-PCR. Total RNAs isolated from P1 and P60 thymus were reverse transcribed and subjected to semi-quantitative PCR using specific primers. All PCR reactions were conducted in triplicate and normalized relative to GAPDH expression. In consistent with results using ACP, RT-PCR data showed that bystin-like and TCRB2 were up-regulated while COL5A1 was down-regulated in P60 rat thymus (Fig. 2B)

DISCUSSION

This study reports for the first time that mRNA expression levels of bystin-like, TCRB2 and COL5A1 are changed in developing rat thymus. Our results show that bystin-like and TCRB2 are up-regulated while COL5A1 is down-regulated in peripuberty rat thymus as judged by ACP analysis and RT-PCR data.

Thymus with Age

The rat is a central experimental animal in several fields of biomedical research, such as aging, cardiovascular diseases, infectious diseases, autoimmunity, cancer models, transplantation biology, inflammation, cancer risk assessment, industrial toxicology, pharmacology, behavioral and addiction studies, and neurobiology (Gill *et al.*, 1989; Krinke *et al.*, 2000). Like human, rat thymus is also sexually differentiated (Nunn *et al.*, 1999). The studies on gene expression in rat thymus are very informative and direct way to understand the function of genes. The development of the thymus continues until puberty (around 6 to 8 weeks of age in rats) at which time it reaches its maximum size, containing the highest number of thymocytes. After puberty the thymus undergoes a dramatic loss in weight and volume coupled to an inefficient functioning of the thymus and reduction in thymopoiesis (Lacorazza *et al.*, 1999; Aspinall and Andrew, 2000; Hirokawa *et al.*, 2001).

DEGs in Thymus

Our results showed that bystin-like, TCRB2 and COL5A1 were differentially expressed between P1 and P60 rat thymus. Characteristics of these genes are discussed below.

Bystin has been identified as a cytoplasmic protein which interacts with trophine and tastin, during embryo implantation, and involved in the placental development, the initial blastocyst attachment to the uterus

and perineural adhesion and differentiation of astrocytes during the inflammatory processes following brain injury (Aoki and Fykuda, 2000; Sheng *et al.*, 2004). Recent studies have shown that bystin could be a novel marker for reactive astrocytes in the adult rat brain following injury and an important therapeutic target for neurotrophic cancers (Sheng *et al.*, 2004; Ayala *et al.*, 2006). However, it is not known about the function of bystin in immune system. In our study, bystin-like gene was decreased in developing thymus, suggesting that bystin might involve in age-dependent function of thymus during development.

The T-cell receptor (TCR) is responsible for antigen recognition by T cells. Most mature T cells interact with antigens through the TCR heterodimer consisting of α and β chains. Restricted usage of TCR beta chain variable region (V) contains 65 gene segments was initially observed in animal models of experimentally induced autoimmune diseases (Acha-Orbea *et al.*, 1988; Burns *et al.*, 1989) and prompted the search for non-random TCR V β distribution in clinical samples. Selective usage of V β gene segments or recurrence of unique TCR sequences was found in insulin-dependent diabetes mellitus type I (Conrad and Trucco, 1984). Region of disorders with an immune or autoimmune background such as multiple sclerosis (Ben-Nun *et al.*, 1991; Kotzin *et al.*, 1991; Oksenberg *et al.*, 1993), rheumatoid arthritis (Paliard *et al.*, 1991; Uematsu *et al.*, 1991), and graft versus-host disease (Dietrich *et al.*, 1994) have been attributed to the pathogenesis that might be resulted from changes in assembly of TCR genes that regulated in several important contexts during lymphocyte development (Tillman *et al.*, 2004). Among many segments in TCR beta chain, our results showed that segment 2 was up-regulated in P60 rat thymus, suggesting that segment 2 might regulate pathogenesis of immune or autoimmune disease.

Collagen Type V (COL5) is present in many kinds of tissues and organs as a minor collagen component, and is resistant to mammalian collagenase, but not to trypsin. This gene involves in the control of fibrillogenesis and the partial retention of their N-terminal propeptide of the pro α (I) collagen chain (N-propeptide) extensions in tissue forms, which is unusual for known fibrillar collagens. The COL5 has both cell adhesion and heparin binding sites that could be crucial in physiologic processes such as development and wound healing (Fichard *et al.*, 1997). The COL5 showed molecular abnormalities in patients with Ehlers-Danlos Syndrome (EDS), a group of hereditary disorders which mainly affect the skin and joints (Nicholls *et al.*, 1996). In particularly, COL5A1 was mutated in patients with EDS (Nicholls *et al.*, 1996; Wenstrup *et al.*, 2000), suggesting that COL5A1 affects skin and joints of body. The COL5 occurs as heterotrimers of 3 different polypeptide chains, alpha-1 (A1), alpha-2 (A2), and

alpha-3 (A3). Earlier studies have shown that COL5A1 is regulated in MC3T3-E1 cells, a clonal osteoblastic cell line, treated with TGF- β 1 during the proliferation and differentiation phases of osteogenesis (Kahai *et al.*, 2004), and with advancing years in rat thymus (Dressler *et al.*, 2002).

Our results, in keeping with the previous findings, suggest that bystin-like, TCRB2, and COL5A1 help to regulate functions of thymus such as maturation, differentiation, and development with increasing age.

Age-related immune senescence induces involution of the thymus and alterations in the development of thymocytes. Numerous genes related with thymic involution contribute to many age-related degenerative diseases, and involve in proliferation of thymocytes. We suggest that bystin-like, TCRB2, and COL5A1 may also regulate age-related immune dysfunction.

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