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Quantitative Expression Analysis of Functional Genes in Four Dog Breeds

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One of the domesticated species; the dog has been selectively bred for various aims by human. The dog has many breeds, which are artificially selected for specific behaviors and morphologies. Dogs contribute their life to human as working dogs for guide, rescue, detection or etc. Working dogs requires good personality, such as gentleness, robustness and patience for performing their special duty. Many studies have concentrated on finding genetic marker for selecting the high-quality working dog. In this study, we confirmed quantitative expression patterns of eight genes (*ABAT*; 4-Aminobutyrate Aminotransferase, *PLCB1*; Phospholipase C, Beta 1, *SLC10A4*; Solute Carrier Family 10, Member 4, *WNT1*; Wingless-Type MMTV Integration Site Family, Member 1, *BARX2*; BarH-Like Homeobox 2, *NEUROD6*; Neuronal Differentiation 6, SEPT9; Septin 9 and *TBR1*; T-Box, Brain, 1) among brains tissues from four dog breeds (Beagle, Sapsaree, Shepherd and Jindo), because these genes were expressed and have functions in brain mostly. Specially, *BARX2, SEPT9, SLC10A4, TBR1* and *WNT1* genes were highly expressed in Beagle and Jindo, and Sapsaree and German Shepherd were vice versa. The biological significance of total genes was estimated by database for annotation, visualization and integrated discovery (DAVID) to determine a different gene ontology (GO) class. In these analyses, we suppose to these eight genes could provide influential information for brain development, and intelligence of organisms. Taken together, these results could provide clues to discover biomarker related to functional traits in brain, and beneficial for selecting superior working dogs.

Key words : Brain tissue, dog breeds, functional genes, gene expression profile, qRT-PCR

Introduction

The dog (*Canis lupus familiaris*) had been domesticated from wild gray wolves (*Canis lupus*). Based on archeological data, the dogs had had first artificial selection that from 100,000 to 15,000 years ago in multiple locations, including Europe, the Middle East and East Asia [48, 49]. Nowadays, the dog population is separated into more than 400 breeds exist in worldwide [36]. The dogs evolved through a mutually valuable relationship with human beings, and their abilities have been developed to perform an outstanding variety of working or special roles. These roles include military watch, security guards, shepherds, guides, rescue and pets [45]. Working dogs are required to good personality of gentleness, robustness and patience for performing their special duty. Several animal personality are influenced by the activity of specific genes [17, 42]. Specially, the brain specific genes controls behavior, personality, or aggression, therefore it is needed to study for confirming the gene expression patterns in brain. Microarray studies have been performed to assess changes in behavior with gene expression patterns in the brain [46]. We tried to identify such expression changes of eight genes in brain of four domesticated dog breeds (Beagle, German Shepherd, Sapsaree and Jindo), then compared to their expression patterns.

A major goal in the brain and behavioral sciences is to

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identify genes that influence social behavior and understand how their gene products influence the structure and function of the nervous system [17, 42, 46]. Thus, the aim of this study was to predict the relationships among personality traits (calmness, trainability, dog sociability, boldness, and etc.) of dogs. Gene expression profiles may reflect the complete personality of regulatory pathways; therefore we describe the gene expression patterns of eight genes (*ABAT, BARX2, NEUROD6, SEPT9, SLC10A4, PLCB1, TBR1, WNT1*) in dog breeds.

The domestic dogs display an extraordinary level of phenotypic diversity in personality and behavior, because dog breeding was introduced as various methods by human during the nineteenth century [48, 50]. As the results of artificial selection, each dog breeds have fixed phenotypic traits [12, 47]. In this point, it is important to select the suitable traits of special dog from many dog breeds. In this study, we presented each gene expression patterns in four dog breeds, which provides the clue to study of each dog breeds of specific traits.

The Beagle is one breed of small to medium-sized dog, and developed primarily for tracking wild animals as detection dogs. Their great sense of smell and tracking ability is used to detect food items, drug, or explosive detection [6]. Due to their strength, intelligence, trainability and obedience, German Shepherds are generally used in search-and-rescue, police, or military dogs around the world. German Shepherds have abilities of tracking, patrolling, or detection by training [39]. Therefore, the German Shepherd is the widely used breeds for military, police or scent-work roles.

As the Korean traditional breeds, Sapsaree is known as dauntlessness and loyalty aspects. Jindo is also one of the Korean traditional breeds, and well known for its unwavering loyalty and gentle nature. Because the Sapsaree and Jindo are an active and intelligent dog, it requires frequent interaction with people or another dog in the family [14, 28]. Although they are frequently used as pet dog or guide dog, they are not frequently used as special dogs. In order to use these breeds as special dogs, gene expression patterns in brain could be good clues.

Materials and Methods

Tissue samples, RNA isolation and synthesis of cDNA

One post-mortem brain tissue sample was extracted from four dog breeds (Beagle, German Shepherd, Sapsaree, and Jindo). Beagle and Sapsaree brain tissues were obtained by Chungnam National University with approval by the Animal Ethics Committee (CNU-00199). German Shepherd and Jindo brain tissues by Rural Development Administration (Jeonju, Korea), and the animals received care in accordance with the standard guidelines for the Care and Use of Laboratory Animals provided by the National Institute of Animal Science Animal Care Committee, and the experiment was executed with approval from the animal ethics committee under the operation rule of animal experiment ethics at the National Institute of Animal Science (approval number: 2014-085).

Cellular RNA of dog brain tissues was isolated by TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) to purify total RNA according to the manufacturer's guidelines. After RNA isolation, the quality and quantity of the resulting single-stranded RNAs were assessed using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Total RNA was treated and reverse-transcribed using a PrimeScript RT reagent kit with genomic DNA Eraser (Takara Bio, Shiga, Japan) according to the instructions of the manufacturer. Eight pairs of primer were designed to detect the mRNA of each gene according to the open reading frames (ORFs) in the whole genome (Table 1).

Quantitative real time RT-PCR amplification

Quantitative real-time RT-PCR was performed with the Rotor-Gene Q system (QIAGEN, Hilden, Germany) with a gene-specific primer set (Table 1). Each of amplification reaction mixture (20 μl) contained 7 μl of H_2O, 10 μl of QuantiTech SYBR Green PCR Master Mix (QIAGEN, Hilden, NW, Germany), 1 μl each of forward and reverse primers at 10 nmol/ μl, and 1 μl of cDNA template. In addition, to confirm non-specific background amplification, we amplified a template control without cDNA. Real-time RT-PCR amplifications for target genes and housekeeping genes were conducted as follows: 30 cycles each of 95°C for 15 s, annealing temperature for 15 s, and 72°C for 15 s. Annealing temperature range is set from 54 to 57°C depends on genes. Melting curve analysis was performed for 30 s at 65-99°C. To guarantee reproducibility, we amplified all samples in triplicate and the results were averaged. As a standard control, we used *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) in gene expression, for normalization of real-time RT-PCR amplification.

Table 1. Gene description and the information of primers used in this study for real-time RT-PCR analysis

Gene name	Description	Location (canFam3)	Accession No.	Primer Sequences (5′-3′)	Annealing temp. (°C)	Product size (bp)
ABAT	4-aminobutyrate aminotransferase	chr6:33,388,930-33,425,870	ENSCAFT00000030230	F: TGGAAGAGGTGGAGGATCTGA R: CCTGGAGATGTCTCTCAGCTT	56	143
BARX2	BARX homeobox 2	chr5:5,238,743-5,310,648	ENSCAFT00000045519, ENSCAFT00000016227	F: CTGCAAGTGAAGACTTGGTATC R: GTTCATCTTCTCCTCAGCCT	57	153
PLCB1	Phospholipase C, beta 1	chr24:13,337,234-13,587,167	ENSCAFT00000046106, ENSCAFT00000009472	F: ACCCACTGGAATCTGGAGTT R: ATGAGGGCTCAAACATGCTG	56	171
NEUROD6	Neuronal differentiation 6	chr14:43,914,149-43,915,162	ENSCAFT00000005020	F: CTGAGAATCGGCAAGAGACC R: GCTGTGGTAGGGTGGGTAGA	54	192
SEPT9	Septin 9	chr9:3,497,801-3,626,550	ENSCAFT00000008383, ENSCAFT00000047802	F: CCTCAGAAGGAGTTTGACGA R: CCTCTTCCCATTCACTTGGT	56	117
SLC10A4	Solute carrier family 10, member 4	chr13:44,293,981-44,299,528	ENSCAFT00000003107	F: AAGGTTTCCCTGTGGTCTCTG R: TCCAGGCAGACAGTCCTCTT	56	206
WNT1	Wingless-type MMTV integration site family, member 1	chr27:5,589,117-5,592,060	ENSCAFT00000013918, ENSCAFT00000045709	F: ACGACGGTGTTCTCCGAGA R: GCGGTTGCCATAGAGGAC	56	162
TBR1	T-box, brain, 1	chr36:6,989,703-6,997,934	ENSCAFT00000015885	F: GTCCTTGCACAAGTACCAGC R: ATTGTGTAATGTCGGTGTTCTG	57	155

Gene ontology analysis and functional annotations

Functional annotation of eight genes was analyzed by the DAVID (Database for Annotation, Visualization and integrated Discovery) [16]. DAVID calculates *P*-values to demonstrate GO terms enrichment, where *P*-values less than 0.05 are considered to be strongly enriched in the annotation category after Benjamini multiple test correction. We then performed gene ontology (GO) term analyses and each genes were grouped as GO terms. The results of significant GO terms were queried to the REViGO program in order to construct a scatterplot and interactive graph [44], and then we summarized GO terms on the 2D semantic space by semantic similarities. *P*-values were originated from the Benjamini and Hochberg false discovery rate (FDR), and the color of circles indicates enriched GO terms with FDR <0.05.

Results and Discussion

Gene expression patterns in four breeds

Our gene expression data indicate that similar expression patterns were presented in four breeds of dog. Generally, Beagle and Jindo are dominant expressed patterns, whereas German shepherd and Sapsaree are lower expressed than Beagle and Jindo (Fig. 1).

ABAT has several psychiatry roles, and regulate specially gamma-aminobutyric acid (GABA) in neuronal cells by catalyzing the degradation of GABA. Thus low-expressed ABAT gene induces to enhanced amount of GABA in the synaptic junctions, then increases GABA-mediated signaling by means of the GABA receptors [26]. Specially, GABA has no means to penetrate the blood-brain barrier, so it must be synthesized in the brain. Therefore to elucidate *ABAT* expression patterns in the brain is important for neuronal proc-

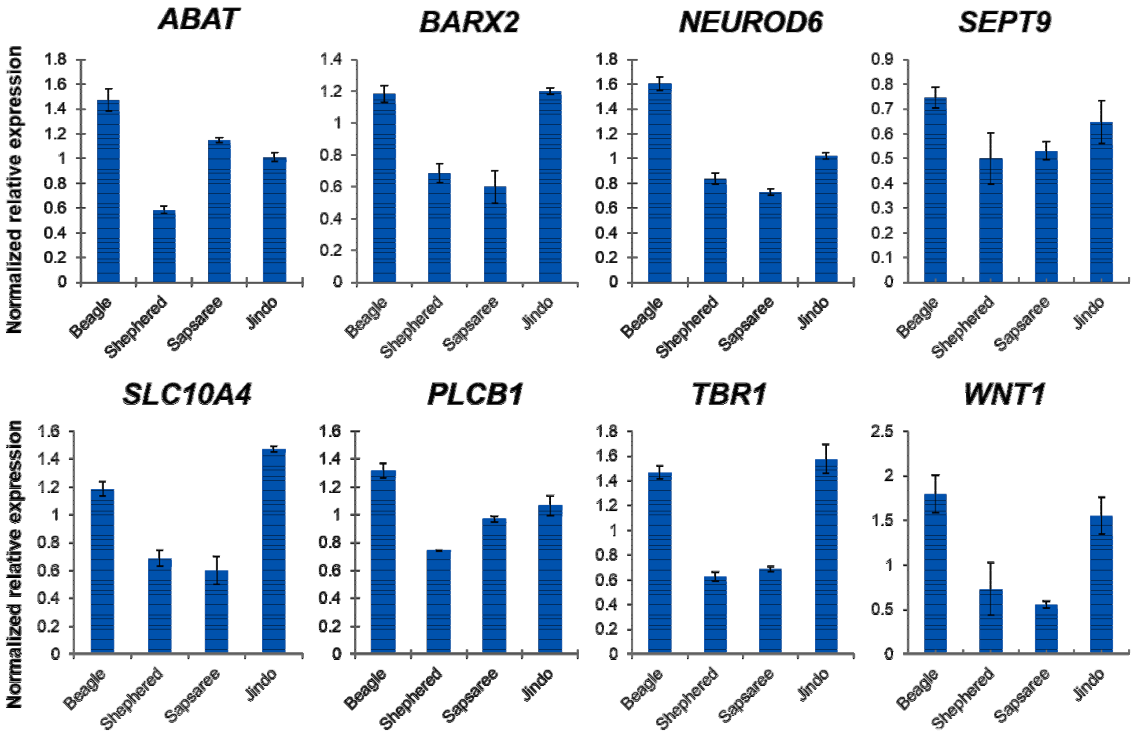

Fig. 1. Quantitative real-time RT-PCR analysis is performed for the comparison of eight genes (*ABAT, BARX2, NEUROD6, SEPT9, SLC10A4, PLCB1, TBR1, WNT1*) of expression levels in four dog breeds. The tanscript copy number of eight genes were normalized to GAPDH housekeeping gene copy number in each sample. The values and their error bars indicate means, and standard deviation (n=3).

esses and brain development [51]. In the brain of four dog breeds, *ABAT* is high-expressed in Beagle, and low-expressed in Shepherd. The two breeds of Sapsaree and Jindo, originated from Korea, have similar expression patterns.

BARX2, *SLC10A4*, *TBR1* and *WNT1* genes have same expression patterns. *BARX2* gene is known as transcription factor of cell adhesion [33]. The mechanisms of cell adhesion is very important for brain morphology, and functions such as learning, signal transduction, and memory [40]. When the early development of the nervous system, neurons maintain synapses by formation of cell-cell adhesions. Differently expressed patterns could provide the formation of brain cell, and the features of personality or intelligence. TBR1 has a role in glutamatergic projection in neuron differentiation. Glutamatergic neurons express receptors for the excitatory neurotransmitter glutamate as opposed to receptors for the inhibitory neurotransmitter GABA [23]. Solute carriers (SLCs) have a role for the transmembrane transport of various materials, such as amino acids, sugars, or inorganic ions. SLC10A4, one of the solute carrier family, has a role of carrying solution in blood or body fluid. *SLC10A1* genes were involved in blood-brain barrier [19], and SLC10A4 also has possible important roles in brain. Therefore, the problems of SLC in the brain could induce mental illness such as ADHD, depression or psychiatric disorders [38]. The study of this gene in the dog could help to elucidate the canine mental illness or behavior disorders.

Proto-oncogene protein WNT1, has been originally considered as a candidate gene for Joubert syndrome, an autosomal recessive disorder with cerebellar hypoplasia as a leading feature. Joubert syndrome is a rare genetic disorder that affects the cerebellum, an area of the brain that controls balance and coordination [31, 32]. Wnt signaling pathway controlled by WNT1, is stimulated by BDNF (Brain-derived neurotrophic factor), then induce the proliferation and differentiation of neural stem cells [13]. These genes same expression patterns have crucial roles in brain, and each gene pathway studies will be performed as further studies.

Three genes, *NEUROD6, SEPT9* and *PLCB1*, were similar expressed patterns and highest expressed in Beagle. NEUROD6 is transcription factors, and associated to differentiation or development of the nervous system. It also regulates fasciculation and targeted axogenesis in the neocortex [8]. SEPT9 (septin 9) is involved in cytokinesis and cell cycle

control, and known as a candidate for the ovarian tumor suppressor gene [20]. Mutations in this gene cause hereditary neuralgic amyotrophy [30], and a chromosomal translocation involving this gene on chromosome 17 and the *MLL* gene on chromosome 11 results in acute myelomonocytic leukemia [29]. SEPT9 is highest expressed in Beagle, but we could not detect to specific expression patterns. SEPT9 is related to not only neural diseases but also diseases such as leukemia or tumor, therefore the specific expression patterns is need to be specified.

PLCB1 catalyzes the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol

Table 2. The function of each genes used in this study

Gene	Function	References
ABAT	• 4-aminobutyrate transaminase activity • Transaminase activity • Pyridoxal phosphate binding • Succinate-semialdehyde dehydrogenase binding • Protein homodimerization activity	[5, 24, 35]
BARX2	• BMP signaling and chondrogenesis • Transcription regulatory region sequence-specific DNA binding • Sequence-specific DNA binding transcription factor activity	[25, 33, 41]
PLCB1	• Phosphatidylinositol phospholipase C activity • Signal transducer activity • GTPase activator activity • Calcium ion binding • Protein binding	[4, 11, 37]
NEUROD6	• Transcription regulatory region sequence-specific DNA binding	[2, 3, 8, 27]
SEPT9	• Filament-forming cytoskeletal GTPase activity • Play a role in cytokinesis	[20, 34]
SLC10A4	• Bile acid:sodium symporter activity	[1, 7, 15]
TBR1	• RNA polymerase II core promoter sequence-specific DNA binding • Sequence-specific DNA binding transcription factor activity	[10, 22, 23]
WNT1	• Ligand for members of the frizzled family of seven transmembrane receptors. • Ligand for the coreceptor RYK, thus triggering Wnt signaling. • Signaling molecule important in CNS development.	[9, 18]

Table 3. The function of eight genes based on GO term analysis

GO ID	GO terms	Count	*P*-value	Genes
GO:0030154	cell differentiation	4	0.038	SEPT9, TBR1, WNT1, BARX2
GO:0048869	cellular developmental process	4	0.042	SEPT9, TBR1, WNT1, BARX2
GO:0032501	multicellular organismal process	6	0.030	ABAT, SEPT9, NEUROD6, TBR1, WNT1, BARX2
GO:0009891	positive regulation of biosynthetic process	3	0.043	TBR1, WNT1, BARX2
GO:0031328	positive regulation of cellular biosynthetic process	3	0.042	TBR1, WNT1, BARX2
GO:0010628	positive regulation of gene expression	3	0.031	TBR1, WNT1, BARX2
GO:0010557	positive regulation of macromolecule biosynthetic process	3	0.039	TBR1, WNT1, BARX2
GO:0051173	positive regulation of nitrogen compound metabolic process	3	0.037	TBR1, WNT1, BARX2
GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3	0.035	TBR1, WNT1, BARX2
GO:0051254	positive regulation of RNA metabolic process	3	0.022	TBR1, WNT1, BARX2
GO:0045941	positive regulation of transcription	3	0.029	TBR1, WNT1, BARX2
GO:0045893	positive regulation of transcription, DNA-dependent	3	0.021	TBR1, WNT1, BARX2

4,5-bisphosphate by using calcium as a cofactor [21]. This reaction plays an important role in the intracellular transduction of many extracellular signals.

The general features of total eight genes in the organisms were listed, and these genes have crucial roles for cellular metabolisms, DNA binding, or cell signaling (Table 2). The variable gene expression patterns in four breeds of dog brain can provide clues for the studies of breed-specific traits.

Function prediction of eight genes

A GO analysis was performed in order to confirm the biological function of the eight genes. The gene set was confirmed as cellular GO terms, such as cell differentiation, multicellular organismal process, and cellular developmental process. The full list of statistically significant GO terms is enumerated (Table 3). Total 12 functions were enriched, and the most significant function is cell differentiation (*P*-value = 0.038) with four genes. Multicellular organismal process includes six of eight genes, except SLC10A4 and PLCB1 genes. BARX2, TBR1, and WNT1 genes were enriched in positive regulation of various biological processes, such as DNA-dependent transcription, RNA metabolic process, and nucleic acid metabolic process. Interestingly, these two genes were high-expressed in Beagle and Jindo, whereas low-expressed in Shepherd and Sapsaree. According to these facts, we summarized that positive regulation of biological process is enhanced in the brain of two breeds, Beagle and Jindo.

We used the REViGO program to get the functional relationship of GO terms in the network structure. The scatter plots and integrated graph show the cell metabolism-related GO terms, including cell differentiation, multicellular organismal process, cellular developmental process, and positive regulation of transcription, DNA-template (Fig. 2). WNT1 gene is included in all GO terms, and crucial factor of each network structure. WNT1 regulate the wnt signaling pathway, then induce neural stem cell differentiation [13]. Therefore, *WNT1* gene can provide a clue for the study of wnt signaling brain cell, and their mental features. SEPT9 gene is also included in the scatter plots and integrated graph. SEPT9 gene is related to cell division, such as cell cytokinesis and cell cycle [20]. SEPT9 is also high-expressed in human lymphoid and malignant brain tumors [43]. We predict to these genes can provide crucial information for brain development, and intelligence of organisms.

In conclusion, we selected eight genes related to brain functions, then confirmed their expression patterns, functions, and network. Total genes have a similar expression patterns, and their function is related to cell differentiation, cellular developmental process and multicellular organismal process. This data provides the fundamental clues for the studies of brain functions.

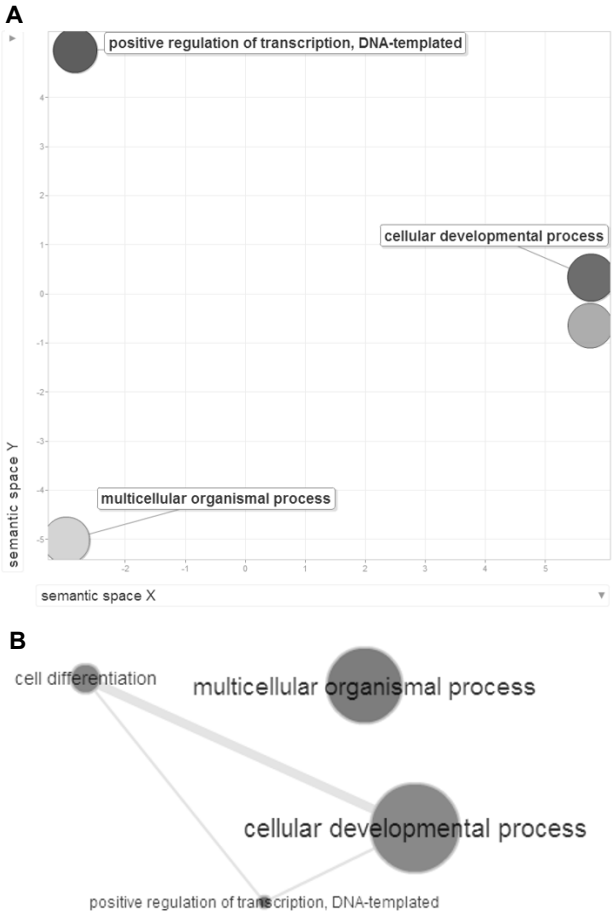

Fig. 2. Molecular functions of eight genes in this study. After DAVID analysis, GO terms were obtained (P-values <0.05). Each GO terms of eight genes were queried to the REViGO program. (A) Scatter plots of GO terms from eight genes are viewered, and circles indicated by color depict significantly enriched GO terms with FDR <0.05. (B) Integrated graph of biological process from eight genes is presented by integrating nodes. Each GO terms (FDR <0.05) of each biological process were depicted in the 2D semantic space as default options from a REViGO. The circle size of each GO terms is relative to statistical significance, and edge thickness depicts between two nodes.

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초록 : 개의 네 품종에서 기능 유전자들에 대한 정량적 발현 분석

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가축화된 동물종 중 하나인 개는, 다양한 목적을 위해 인간에 의하여 선택적으로 육종되었다. 개는 많은 품종을 갖고 있고, 특정한 행동과 형태를 갖도록 인공적으로 선택되어 왔다. 개들은 그들의 삶을 안내, 구조 혹은 탐지 등의 특수 목적에 대하여 인간에게 헌신하고 있다. 특수 목적견에게 요구되는 좋은 품성, 이를테면 온순함, 강건성, 그리고 인내심과 같은 특성은 그들의 특수 임무를 수행하는 데 필요하다. 많은 연구들이 우수한 특수 목적견의 선정을 위한 유전적 마커를 찾는 데 집중되었다. 본 연구에서는, 뇌에서 발현함으로써 기능하는 것으로 알려진 총 8개의 유전자(*ABAT*; 4-Aminobutyrate Aminotransferase, *PLCB1*; Phospholipase C, Beta 1, *SLC10A4*; Solute Carrier Family 10, Member 4, *WNT1*; Wingless-Type MMTV Integration Site Family, Member 1, *BARX2*; BarH-Like Homeobox 2, *NEUROD6*; Neuronal Differentiation 6, SEPT9; Septin 9 그리고 *TBR1*; T-Box, Brain, 1)들의 정량적인 발현 양상을 개의 네 품종의 뇌 조직에서 확인하였다. 특히, *BARX2, SEPT9, SLC10A4, TBR1* 그리고 *WNT1* 유전자들은 비글과 진돗개에서 많이 발현되는데 반하여, 삽살이와 세퍼드에서는 반대되는 발현 양상을 보여 주었다. 본 연구의 유전자들에 대한 Gene ontology (GO) 결정을 위하여 DAVID (Database for annotation, visualization and integrated discovery) 분석이 수행되었고, 이러한 유전자들이 뇌 발생과 개체의 지능에 중요한 기능을 제공할 것이라고 예상하였다. 결론적으로, 이러한 결과들을 통하여, 뇌에서의 기능과 관련된 인자들과 관련된 바이오마커를 발굴하는 데 중요한 단서를 제공해 줌과 동시에, 우수한 특수 목적견을 선발하는 데 도움을 줄 것이라 기대한다.