行政院國家科學委員會補助專題研究計畫成果報告

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視網膜退化在犬模式的候選致病基因之分離、轉植、及表現

The isolation, cloning, and expression of candidate genes for canine retinal degeneration

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計畫主持人：林中天

共同主持人：無

計畫參與人員: 研究生林郁玟、李佳霖

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一、中文摘要

視網膜退化，或稱為"犬漸進性視網膜萎縮(PRA)"，是犬隻最常見的遺傳性眼睛疾病之一，致病基因十分複雜。由於大部分犬視網膜基因仍是未知的，深入研究犬視網膜基因之特性及功能是很重要的。

在本計劃中，採取非染色體定位式候選基因鑑定法 (position-independent candidate gene approach)及 EST (expressed sequence tags)法來篩選犬的重要之視網膜重要基因及 PRA 候選疾病基因。利用分離、選殖、及表現視網膜基因，以了解重要視網膜基因在分子層級的特性。本計畫分成三個主要的部份：自犬視網膜 cDNA 庫鑑定和分離基因;選殖並定序基因;以及視網膜基因的表現。

在第一階段，先建造犬視網膜 cDNA 庫並嵌入λ噬菌載體中，作為犬視網膜基因之來源。此 cDNA 庫中含視網膜特異性表現之基因比率可經由篩減雜交法(subtractive hybridization)增高，以產生犬的篩減視網膜 cDNA 庫(subtractive retinal library)。此兩個 cDNA 庫的視網膜特異性表現之基因可利用差別表現篩檢雜交法(differential plaque/colony hybri-dization)選出。在分離出視網膜特異性表現之基因後，在第二階段，純化並定序基因之 DNA，以獲得之ＤＮＡ序列，並利用 BLAST 和 FASTA 程式作 GenBank 資料庫之搜尋及序列比較分析。在比較資料庫後，在第三階段，選取 6 個重要基因(主要為人視網膜退化致病基因)作表現實驗，利用北氏雜交法(northern blotting hybridization)及 RT-PCR 來分析候選基因在各犬組織的表現情形。

犬視網膜 cDNA 庫之噬菌體量(titer)為 1.5 x 10^9 p.f.u./ml，96%成功地含有犬視網膜 cDNA (inserts)，平均 cDNA 長度為 1.6 kb。自犬視網膜 cDNA 庫，共分析並定序 76 株視網膜特異表現基因及 100 隨機桴樣株。76 株中有 54 株(71%)和資料庫之序列有高相似性，其中意外地首次發現功能很重要之犬 sFRP2 基因，為犬視網膜最豐富存在之特異表現基因，超過過去其他種別視網膜在文獻所述之 opsin 基因。最近發現 sFRP 基因在控制發育、影響腫瘤發生、及調控細胞凋亡具重要之功能。其餘 22 株(29%)為未知之新基因。在 100 隨機株中，53 株和資料庫之序列有高相似性，其餘 47 株為未知之新基因。在基因表現實驗，其中 opsin, transducin 1A, cGMP-PDEA,p61 及 HRG4 為視網膜特異表現之基因，不表現於其他犬組織;而一犬視網膜株 p23 則無任何組織特異性之表現。

關鍵詞：視網膜退化，視網膜基因，犬基因，遺傳疾病，表現基因序列，基因表現

ABSTRACT

Canine generalized progressive retinal atrophies (gPRA) are a group of degenerative retinal diseases that are a major cause of hereditary blindness in a number of dog breeds. Expressed sequence tag (EST) approach was used to identify and characterize potential candidate genes from canine retinal cDNA libraries. A conventional and subtractive canine retinal cDNA libraries were constructed. For the conventional cDNA library, the titer was 1.5 x 10^9 p.f.u./ml. The average insert size was 1.6 kb and 96% of the clones contained inserts. Differential hybridization was performed to identify abundantly retinal expressed cDNA clones. Random and differentially expressed clones were fluorescently labeled and sequenced. The sequences were analyzed using GCG software and searched against GenBank database. For genes of interest isolated from the libraries, northern blotting and RT-PCR were performed to determine mRNA expression of the genes. DNA sequences from 76 differentially expressed clones and 100 random cDNAs and analyzed. 54 out of 76 differentially

expressed cDNA clones (71%) showed homology to known genes in the database. An unexpected and interesting finding is the isolation of a functionally important gene sFRP2, which is the most abundant gene in the canine retina. The remaining 22 cDNA clones (29%) showed no homology to database sequences, representing new sequences. In 100 random canine retinal ESTs, 53 clones (53%) showed homology to database sequences (including known genes and ESTs). The remaining 47 cDNAs (47%) showed no homology to database sequences. Four candidate genes and 2 anonymous retinal ESTs were selected to analyze mRNA expression. The four known genes, namely opsin, cGMP-PDEA, transducin 1A, and HRG4 showed retina-specific expression. In anonymous ESTs, clone p61 revealed retina-specific expression, while p23 showed no tissue specificity. The isolation of sFRP2 and HRG4 is the first finding in the canine retina.

Keywords: retinal degeneration, retinal genes, canine genes, inherited disease, expressed sequence tags, gene expression

二、緣由與目的

Hereditary retinal dystrophies affect a variety of mammals, including humans, dogs, cats, and mice. In canine model of the diseases, canine progressive retinal atrophies (gPRA or PRA) are a group of hereditary retinal diseases causing blindness in a number of dog breeds. In human medicine, the equivalent diseases are termed Retinitis Pigmentosa (RP), a major group of inherited retinal diseases leading to blindness in humans.

gPRA has been reported in more than one hundred breeds to date. Certain subtypes of gPRA are found to segregate in a breed-specific manner and constitute a significantly high incidence of hereditary blindness in particular breeds worldwide. However, no any study or information on PRA is available in Taiwan to date. The vast majority of gPRAs have been described only on clinical signs or/and histopathology, and their genetic causes remains undetermined. Inheritance studies by cross-breeding experiment indicate that most gPRAs are autosomal recessively inherited. The only X-linked form of gPRA was found in the Siberian husky.

Both human and canine retinal degenerations are extremely heterogeneous genetically and clinically, that is, different gene defects cause different subtypes of clinical retinal degeneration in different individual people or dog breeds. In canine gPRA, the only two mutations found to cause gPRA are mutations of cGMP-PDEB in the Irish setter, and mutation of cGMP-PDEA in the Cardigan Welsh corgi breed, both identified by author's previous laboratory at the University of Cambridge. However, these two genes have been excluded as the causes of gPRA in many other dog breeds investigated. The high heterogeniety of the diseases makes the identification of specific gene defects very complex and difficult.

Functional candidate gene approach was used in this project to identify candidate genes. Based on findings in other species, retina-specifically expressed genes are an important group of candidate genes for inherited retinal degeneration. As gene defects causing gPRA are still mostly unknown and little known about the functions and expression of canine retinal geness, we are aiming to identify and characterize more retina-specific genes from the canine retinal gene populations by the construction and analysis of canine retinal cDNA libraries.

三、結果與討論

Please be noted that the following mentioned is the "summary" of all experimental results, not detailed data due to space limit (4 pages) of this report.

1. Titer and insert size of the canine retinal cDNA libraries

The titer of packaged λ phages containing canine retinal cDNAs was 1.5×10^9 p.f.u./ml. The insert sizes of randomly isolated 300 cDNA clones were analyzed following restriction digestion. 96% of the clones contained inserts. The mean insert size was 1.6 kb. For subtracted cDNA library, DNA from 180 clones (abundant 60 retina-specific clones and 120 clones not hybridizing to either total cDNA probe) was checked by restriction digestion and the insert size was also 1.6 kb. The library contains sufficient clones to perform cDNA library-based studies.

2.Clones isolated by differential hybridization

After radiolabelled screen of 2100 plaques of conventional retinal cDNA library and comparison of autoradiographs between total retinal cDNA and cerebellar probes, 16 clones showed abundant retina-specific transcripts. For the subtracted library, 60 differentially expressed clones were isolated and analyzed.

3.DNA sequencing and database comparison

3a. Sequence comparison of 100 random retinal cDNAs

Following database search and sequence comparison of 100 random ESTs, fifty three (53%) of 100 cDNA clones showed homology to sequences (including known genes and anonymous ESTs) deposited in the database. The remaining 47 out of 100 clones (47%) showed no homology to sequences in the database, representing new unknown sequences. The 53 matched clones represented sequences from 6 different species. Among the 53 clones, 31 clones matched to known genes and 22 clones matched to anonymous ESTs in the database. Besides, 34 clones were homologous to human DNA sequences as the biggest group (64.2% of matched population). The second biggest population of cDNA clones was matched to canine sequences (21.4%).

3b. Sequence comparison of 76 abundant retina-specific clones

Sequences of 76 abundant retina-specific cDNA clones (16 clones from conventional library and 60 subtracted clones) were searched against GenBank database. 54 out of 76 clones (71%) showed homology to known genes in the database. Interestingly, the canine homologue of secreted frizzled-related protein 2 (sFRP2) showed the highest abundance in the clones, even more abundant than the known most abundant opsin gene reported in other species. This is the first report to document the presence of sFRP2 in the canine retina. The gene has been recently suggested to have dual roles of apoptosis control and wnt-frizzled signaling. The remaining 22 clones (29%) showed no homology to database sequences, representing new sequences. The number of database matched clones is higher in the prescreened clones than in random clones.

4. Tissue expression analysis by northern blotting hybridization and RT-PCR

4a. Tissue expression of canine homologue of HRG4

Following northern blotting analysis of canine retinal HRG4 with RNA from 12 different canine tissues, a single mRNA species of 1.2 kb was detected in retinal lane only, no signal was visible in other 12 tissues. Thus, it is a retina-specific gene. The function of the new gene is unknown to date. A recent report suggested that the mutation of HRG4 was the cause of the retinal degeneration in mice.

4b. Tissue expression of canine retinal cGMP-PDE ~ and rod transducin ~

Northern blotting analysis of canine cGMP-PDEA showed a mRNA species of 4.3 kb in the retinal track only. The rod transducin clone showed two mRNA species of 1.3 kb and 2.4 kb in the retinal lane only, not expressed in other tissues. These two clones are retina-specific clones and causal genes for human retinal degeneration. A recent report has documented the mutation of the cGMP-PDEA is the cause of PRA in the Cardigan Welsh corgi dog.

4c. Tissue expression of canine retinal opsin

Following northern blotting analysis of canine opsin, three mRNA species of 1.9 kb, 2.6 kb, and 5.5 kb were hybridized to the opsin probe. This is a retina-specific clone and a common causal gene for human retinal degeneration. However, it has been excluded a cause of PRA in many dog breeds.

4d. Tissue expression of a canine anonymous retinal EST p23 and p61

Following several attempts at northern blotting of the canine anonymous EST p23, there was no signal detectable in any tissues Following 15 and 30 cycles of RT-PCR analysis, a positive transcript of expected size was detected in all tissues examined. The random EST did not show tissue specificity. Following hybridization of another anonymous EST p61, a single mRNA species of approximately 0.7kb was detected in the retinal lane only, so it is an anonymous new

retina specific clone and its function remains to be determined.

四、計畫成果自評

In this project, the quality of the constructed canine retinal cDNA library was good containing sufficient clones with good insert sizes (more than 1.6 kb) to perform further analysis of retinal genes of interest. The library provides an excellent resource to study the expression and functions canine retinal genes. There were over two thousand of cDNA clones were isolated and a general picture of the clones has been obtained in this study. Four canine homologues of human causal genes for retinal degeneration were proved canine retina-specifically expressed. An exciting and unexpected finding is the isolation of canine sFRP2, which is the most abundant clone in the canine retina by this study. The gene is recently implicated to have dual roles of apoptosis control and wnt-frizzled signaling and not reported to express most abundantly in the retina in all mammalian species. It is important to know its roles in development and pathogenesis in dogs and other mammalian species. Further investigation of the sFRP gene family (sFRP1 and sFRP2) in canine normal and neoplastic tissues is intensively underway and it is also my this coming year's NSC funded project. To sum up, this project provides a very important basis for further understanding of the functions and expression of important canine genes. The results of this project will be presented at the International FAVA Congress in this coming November.

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