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Isolation of 24 novel cDNA fragments from microdissected human chromosome band

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ABSTRACT

The strategy of isolating the band-specific expression fragments from a probe pool generated by human chromosome microdissection was reported. A chromosome 14q24.3 band-specific single copy DNA pool was constructed based on this probe pool. Using total DNA of the pool as probe to hybridize the human marrow cDNA library, 68 primary positive clones were selected from 5×10^5 cDNA clones. Among these primary clones, 32 secondary clones were obtained after second-round screening and designed as cFD14-1 ~ 32. Finally, 24 band-specific expression fragments were identified from these 32 positive clones by DNA hybridization. Those band-specific clones can hybridize to both 14q24.3 DNA and human genomic DNA but cann't hybridize to 17q11 ~ 12 DNA. Partial sequences of 13 fragments of them were sequenced and identified as novel cDNA sequences, and these sequences were proved to have some homology with known genes in NCBI database. Analysis of expression spectrum of cFD14-1 suggested that the cDNA fragments thus obtained should be used to isolate the genes can not been cloned in 14q24.3 region.

Key words : *Probe pool, chromosome 14q24.3, single* copy DNA pool, expression sequences of genes.

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INTRODUCTION

The procedure of human gene cloning and identification has been greatly propelled by the rapid progress of human genome mapping and sequencing[1]. Positional cloning is the main strategy used in human gene cloning, especially in disease-related gene cloning. Isolating genes from a disease-related chromosome region obtained by microdissection is an efficient way for positional cloning[2, 3].

Chromosome 14q24.3 is an important region for many disease-related genes and cytokine genes such as c-fos, familial early-onset *Alzheimer's* disease related gene, palmoplantar keratoderma related gene and arrhythmogenic right ventricular cardiomyopathy gene (ARVDD) having been mapped in this area. Present paper reported 24 14q24.3 band-specific cDNAs were selected from a human fetal marrow cDNA library. These cDNAs can be used as the starting materials to clone 14q24.3 band-related genes furthermore.

MATERIALS AND METHODS

Band-specific probe pools and primers

The human chromosome 14q24.3 probe pool and 17q11-12 probe pool were constructed separately by State Key Laboratory of Medical Genetics at Human Medical University. X-W primer (5'-CGG GAA TTC CTC TGC GAC ATG-3') was designed according to the probe pool constructing strategy. λgt11 primers are ISA-L 5'-GAC TCC TGG AGC CCG-3' and ISA-R 5'-CGC GGC CAG CGA TGG-3'.

Construction of single-copy DNA pool of 14q24.3

The secondary amplification and cloning of microdissected probe pool were referred to our previous work[4]. Filters containing latticed recombinants were hybridized to human genomic DNA. Positive clones being regarded as repetitive sequences were excluded. The second subtraction of repetitive sequences in negative clones collected after the first screening was performed as described above. The inserts of the negative clones selected after the second screening were cut out with EcoR I. Then the inserts were amplified with probe pool PCR system. A band-specific single-copy DNA pool was thus constructed.

Labelling of single-copy DNA pool

PCR labelling system was composed as follows: 0.4 μl (2U)of Taq polymerase, 1 μ1 of 25 μM X-W Primer, 5 μl of α-^{32}P-dCTP (50 μCi), 1.5 μl of 2 m*M* dATP, dGTP, dTTP respectively, 5 μl of 10 × buffer, and 1 μl of band-specific single-copy DNA pool, which were made into total volume of 50 μl with sterile water. The mixtures were covered with 35 μl of mineral oil. PCR with 27 cycles (93 ℃ 1 min, 60 ℃ 30 sec, 72 ℃ 1 min) were performed, and a final extension of 72 ℃ 5 min. Four same PCR systems were performed.

Blocking of repetitive sequences in labelling probe

The labelled probe purified by Sephadex G-50 (with 500μl total volume) was mixed with 2 μl of human placenta DNA (25 mg/m1), 50μl of 5 *M* NaCl and 1.2 ml of precooled absolute alcohol. The mixture was precipitated at -80 ℃ for 15 min and centrifuged at 15,000 rpm for 10 min. After having been washed with 70% alcohol, the dried precipitant was resolved in 30 μl of TE (pH 8.0). After having been denatured in boiling water bath for 10 min, the DNA was renatured at 55 ℃for 1 h and stored at 4 ℃.

First and second screening of expression sequences

First screening was referred to Lambda Library Protocol Handbook of Clontech Corporation. Ten plates (diameter 150 mm) were spreaded with 50,000 phage plaques on each plate. Double filters were prepared. Prehybridization and hybridization were performed in formamide system[5] at 37 ℃. The filters were washed and autoradiography was performed. The positive plaque regions were transferred to 1000 μl of SM solution with 20 μl of Trichloromethane and stored at 4 ℃.

Plates (diameter 90 mm) were spreaded with 500 plaques on each plate according to the titers of the first screening clones. Double filters were prepared. Prehybridization and hybridization were the same as the first screening procedure. According to the autoradiography results, mono-clone plaques were transferred to 200 μl of SM solution containing 4 μl of Trichloromethane and stored at 4 ℃.

Identification of positive clones by DNA hybridization

Probes were prepared by amplification of phage inserts. The reaction system contained 0.4 μl (2U) of Taq polymerase, 1 μl of 25 μ *M* λgt11 primer, 2 μl of 5 mM dNTP, 5 μl of 10×buffer, and 1 μl of phage solution, which were made into total volum of 50 μl with sterile water. The system was covered with 35 μl of mineral oil. PCR with 35 cycles (93℃ 1 min , 55℃ 1 min, 72℃ 2 min) were performed and a final extension of 72℃ 5 min.

Dot blot membranes loading 14q24.3 DNA, 17q11-12 DNA and human genomic total DNA (HGT DNA) were hybridized with human genomic DNA probe first. Then, after having been washed, these membranes were hybridized with isolated cDNA probes respectively. Eight membranes were prepared and each was hybridized with 4 cDNA probes in turn.

Sequencing and homology comparison of partial cDNAs

cDNA fragments identified by DNA hybridization were cloned into pUC119 vector and sequenced. Homology comparisons were performed with NCBI (National Center of Biology Information) data base.

Northern blot

cFD14-1, which was confirmed to be a 14q24.3-specific expression sequence by DNA hybridization, was hybridized to Multiple Tissue Northern (MTN) membrane as follows: MTN membrane was prehybridized in prehybridization solution (5 × SSPE, 10 × Denhardt's solution, 50% formamide, 2% SDS, and 100 μg/ml CT DNA)at 42 ℃ for 4 h. Then the prehybridization solution was replaced by hybridization solution (5 × SSPE, 10 × Denhardt's solution, 50% formamide, 2% SDS) containing labelled probe and the hybridization was performed at 42 ℃ for 20 h. The membrane was washed at following condition: Washing twice with 2 × SSC/0.05% SDS at 42 ℃ for 15 min each, then twice with 0.1 × SSC/0.1% SDS at 50 ℃ for 30 min. Autoradiography was performed.

RESULTS

Construction of human chromosome 14q24.3 single-copy DNA pool

The secondary amplified product of 14q24.3 probe pool was presented as "smear" in electrophoretic agarose gel with the size ranging from 300bp to 650bp. The amplified product was cloned into pUC119 and the recombinant clones were arranged in lattice on nylon membranes. Colony hybridization was performed with human genomic DNA probe. Results were shown in Tab 1.

Inserts of negative clones selected after two rounds repetitive sequences screening

were cut out with EcoR I. Then single-copy DNA pool was constructed by amplifing the recovered inserts.

Tab 1. Results of repetitive sequence screening by using human genomic DNA as probe

	Clones	Positive clones	Percentage of positive clones
1st screening	7000	1421	21%
2st screening	5579	97	1.8%

Screening of expression sequences

Human marrow cDNA library was screened using labelled 14q24.3 single-copy DNA pool as probe. Sixty-eight primary positive clones were selected after the first screening and 32 secondary positive clones were obtained after the second screening.

Identification of secondary positive clones by DNA hybridization

To identify the 14q24.3 band-specificity of the 32 secondary positive clones, DNA hybridizations were performed. (Fig 1, Tab 2). And, 24 clones hybridizing to 14q24.3 DNA and human total genomic DNA showed strong hybridization signals but no signal was shown to 17q11-12 DNA. Therefore, these clones were confirmed to be the single-copy sequences in the band 14q24.3.

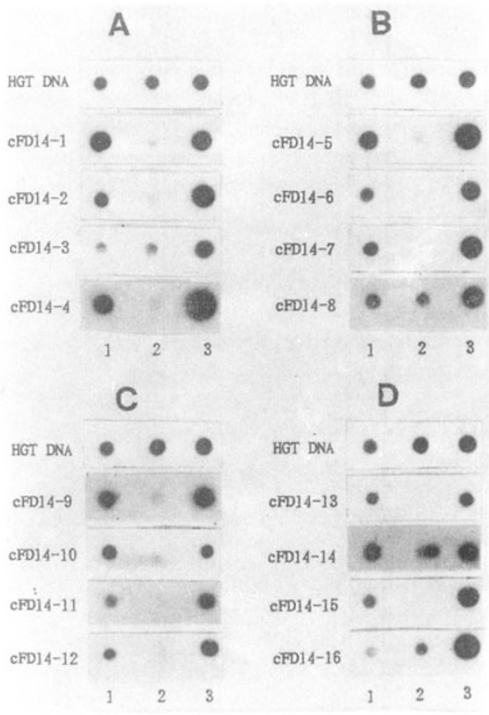

Fig 1. Hybridized identification of candidate clones of human chromosome 14q24.3 band specific expression sequences. A. B.C.D are dot-blot membranes carried with the same DNA samples. These samples are 1: 14q24.3 DNA, 2: 17q11-12 DNA, 3: Human Genomic Total (HGT) DNA. Each membrane was hybridized with HGT and 4 cFD14 probes, the DNAs with hybridized signals only on sample 1 and 3 were regarded as 14q24.3 specific sequences, while on sample 1, 2 and 3 as repetitive sequences.

Tab 2. Identification of the second-round positive clones by DNA hybridization

Name	Length of insert (Kb)	Hybrization signal to human genomic DNA			Results
		14q24.3	17q11-12	Total DNA	
cFD14-1	1.2	++	-	++	S
cFD14-2	0.5	++	-	++	S
cFD14-3	1.8	+	+	++	R
cFD14-4	1.0	++	-	++	S
cFD14-5	0.6	++	-	++	S
cFD14-6	2.5	+	-	++	S
cFD14-7	1.0	++	-	++	S
cFD14-8	1.5	+	+	++	R
cFD14-9	0.8	++	-	++	S
cFD14-10	1.5	++	-	++	S
cFD14-11	1.8	+	-	++	S
cFD14-12	2.0	+	-	++	S
cFD14-13	1.2	+	-	+	S
cFD14-14	1.8	+ +	-	++	S
cFD14-15	2.0	+	-	++	S
cFD14-16	2.5	+	+	++	R
cFD14-17	2.0	+	-	++	S
cFD14-18	1.6	+	+	++	R
cFD14-19	2.5	++	-	++	S
cFD14-20	1.2	++	-	++	S
cFD14-21	1.8	+	+	+	R
cFD14-22	2.0	+	-	++	S
cFD14-23	1.2	++	-	+	S
cFD14-24	1.7	+	-	++	S
cFD14-25	2.2	+	+	++	R
cFD14-26	2.0	+	-	++	S
cFD14-27	0.8	+	+	++	R
cFD14-28	1.4	++	-	++	S
cFD14-29	2.1	++	-	++	S
cFD14-30	0.9	+	+	+	R
cFD14-31	1.8	++	-	++	S
cFD14-32	1.8	+	-	++	S

"S": single-copy expression sequences; "R": repetitive sequences.

Sequencing and homology comparison of partial cDNA fragments

Thirteen cDNAs were partially sequenced and homology comparison of these sequences were performed with NCBI database. Results are shown in Tab 3.

Northern blot of cFD14-1

The expression pattern of cFD14-1 is shown in Fig 2. The size of transcripts in 8 tissues has some differences. For an example, 4 transcripts with different size are shown in kedney while only 2 transcripts (1.0 kb and 2.4 kb) in heart.

Tab 3. Homology comparison of 14q24.3 band-specific expression fragments in NCBI database

cDNA		Higher homology gene			Homologous range (bp)	Homology
Name	Length (bp)	Name	Length (bp)	Aceession No.		
1 cFD14-1	171	Mouse antigen Ly-9 mRNA	2345	gb/M84412	53	82%
2 cFD14-2	171	Schistosoma japonicum sj23 protein	1158	emb/X31401	58	79%
3 cFD14-4	163	Human factor IX gene (exon 2, 3)	599	gb/K02402	57	74%
4 cFD14-5	213	Human calmodulin (CALM1) gene	6581	gb/U12022	110	81%
5 cFD14-6	215	Human estrogen receptor-related protein mRNA	2315	gb/M69297	215	94%
6 cFD14-7	197	B.tauras Cyt C reductase	1622	emb/Z59693	63	65%
7 cFD14-9	214	Mouse L1 encoded ORF	219	gb/M29621	189	95%
8 cFD14-10	154	Human bone sialoprotein (BNSP) gene	2415	gb/L24756	132	82%
9 9cFD14-11	373	Human Z-lens crystallina protein/quionone reductase gene	5546	gb/L31521	140	70%
10 cFD14-12	295	Human chromosomal protein HMG1 related gene	2140	dbj/D14718	232	80%
11 cFD14-13	252	Reverse transcriptase like protein	1202	emb/X07857	216	84%
12 cFD14-14	293	Patent EP 0150735	15155	gb/L04391	240	80%
13 cFD14-15	175	Human D3 periodicity pseudogene	2022	gb/M90815	50	92%

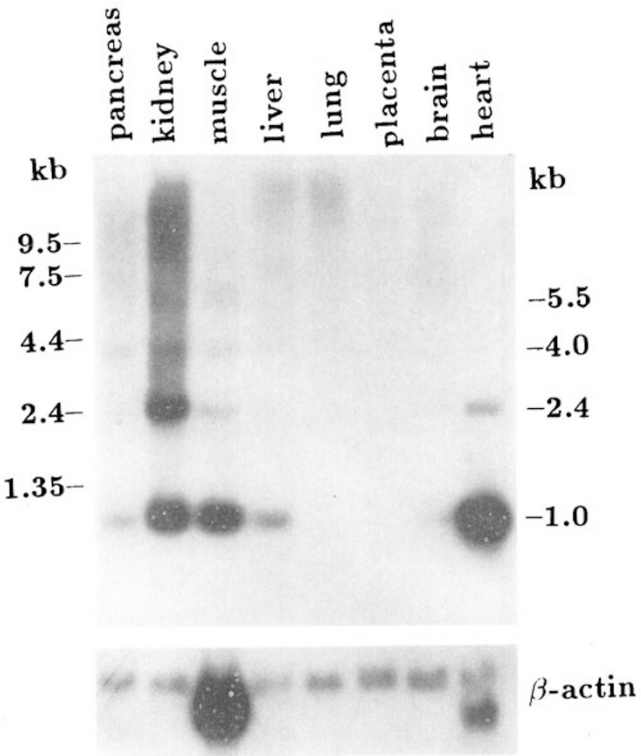

Fig 2. Northern hybridized analysis of cFD 14-1. β-actin mRNA is included as a loading control

DISCUSSION

Subtraction of repetitive sequences from chromosome band probe pool

Human genome contains about 35% ~ 40% repetitive sequences[6] and so does microdissected chromosome band. In cDNA library there is also about 3% ~ 5% repetitive sequences[7]. If hybridized signals of repetitive sequences can not be blocked efficiently, it is difficult to isolate cDNA from cDNA library. So the keypoint of isolating expression sequences from a band specific probe pool is to get rid of the interference of the repetitive sequences.

Improvements in two facets are reported here. The first is that construction of a single-copy DNA pool can remove most of the repetitive sequences in microdissected band. The second is to block low copy-number repetitive sequences with human placenta DNA in a small reaction volume[8].

The advantages of microdissected probe pool in cloning disease-related genes

There are about 50,000 ~ 100,000 genes in a human genome[6]. Using microdissected chromsome probe pool, band-specific cDNAs which might be the candidate of disease-related genes can be batchly isolated. In addition, since there are many gaps in the YAC-contigs map of human genome[9], microdissected chromosome probe pool is useful for cloning the genes located in the gaps.

For there are several important functional genes mapped in chromosome 14q24.3 (GDB database), we chose this band as the target area to perform positional cloning of genes. The microdissected human chromosome 14q24.3 probe pool was afforded by State Key Laboratory of Medical Genetics at Hunan Medical University. The probe pool was proved to be 14q24.3 precise by flouorescence in situ hybridization (FISH) (Data not shown). And 24 band-specific cDNAs were isolated. After sequencing and homology comparison of these cDNA clones, 13 of 24 band-specific cDNAs were proved to have some homology with known genes (Tab 3). For example, cFD14-5 has had 8l % homology with human *CALM1* gene[10] in 110bp, the homology between cFD14-6 and human estrogen receptor protein related gene[11] reaches 93 % in 231bp and cFD14-10 has 82 % homology with human *BNSP* gene[12] in 132bp. All these known genes have been mapped on other chromosomes, which infers that the cDNA fragments reported here may represent the gene having the similar structure or function as those genes. But in these cDNA clones, no one is the same to these known genes. The proper reason is the 14q24.3 band is too large (about 12.8 Mb) and the number of known genes located in this band is small (GDB database). In addition, we have recently isolated a novel STR marker D14S1435 from this microdissected probe pool in another project[13] and relocalized this STR on 14q24.3 accurated by FISH. This result further confirmed the good quality of the probe pool and the band-specific nature of cDNAs. Furthermore, the expression patterns of cFD14-1 showed this fragment was differentially expressed in many tissues (i.e. the hybridizing signal in heart is much stronger than that in the other

tissues and the signal in brain is too weak to detect) and the phenomenon of RNA alternative splicing or homologous genes might be existed. All these suggest that the cDNAs obtained in this way should be helpful to isolate uncloned genes to be mapped in the specific chromosome 14q24.3 band.

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