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Original Article

Human *BRAL1* and *BCAN* Genes that belong to the Link-module Superfamily are Tandemly Arranged on Chromosome 1q21-23

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We herein determined by fluorescence *in situ* hybridization the chromosomal localization of 2 human genes, *BRAL1* and *BCAN*, both of which belong to the link-module superfamily, *i.e.* to the same band of chromosome 1q21-23. Further analysis of the genomic organization of *BRAL1* and *BCAN* revealed that the *BRAL1* gene was located 20-kb upstream of the *BCAN* start site. We isolated a polymorphic dinucleotide (CA) repeat sequence from a genomic clone containing the *BCAN* gene. High heterozygosity (0.79) makes this polymorphism a useful marker in the study of genetic disorders. Knowledge of the structure of the genes and the marker provides essential information for further analysis of the gene locus at chromosome 1q21-23.

Key words: *BRAL1*, *BCAN*, FISH, schizophrenia, polymorphic marker

An increasing number of hyaluronan (HA)-binding extracellular matrix and membrane molecules have been identified and are now referred to as the link-module superfamily [1]. This superfamily possesses a common structural domain of about 100 amino acids, from which the family name was derived. The link-module superfamily includes the lecticans and the link proteins. Lecticans are a family of chondroitin sulfate proteoglycans (CSPGs), encompassing aggrecan, versican/PG-M, neurocan, and brevican (*BCAN*); they display a high degree of homology in their N- and C- terminal globular domains [2]. Aggrecan and versican are rather widely expressed, whereas neurocan and brevican are present specifically in the central nervous system [2-4]. cDNA cloning for human *BCAN* was recently reported and mapped on human chromosome 1q31 by radiation hybrid screening [5].

Cartilage link protein-1 (CRTL1) is a major structural component of cartilage and is also present in various connective tissues. The major function of CRTL1 protein found to date is to stabilize HA/aggrecan aggregates by binding to both molecules, the proteoglycan monomer and HA. The complex has been clearly visualized in glycerol spraying/rotary shadowing studies [6]. In the presence of CRTL1 protein, the aggeregate was much more stable and was also larger than that in the absence of CRTL1 protein [7]. Analysis of *Crtl1* knockout mice has demonstrated that the Crtl1 protein is important for the formation of proteoglycan aggregates and the normal organization of hypertrophic chondrocytes [8]. In spite of the fact that the lectican proteoglycan family contains at least 4 members having distinct forms, expression patterns, and probably functions, until recently only one link protein has been identified. Last year, we reported the cDNA cloning, genomic structure, and characterization of a novel brain-specific link protein gene, *BRAL1* [9].

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The developmental expression pattern of *BRAL1* mRNA suggested the possibility of a functional relationship between *BRAL1* protein and brain-specific lectican such as *BCAN* [9].

In the present study, dual-color fluorescence *in situ* hybridization was used to determine the chromosomal localization of 2 human genes, *BRAL1* and *BCAN*, to the same band of chromosome 1q21-23, and to elucidate the genomic organization and structure of both genes. Furthermore, we isolated and characterized a highly informative dinucleotide repeat polymorphism at the *BCAN* locus. The data shown here contribute to the ongoing search for links to genetic disorders that can be established by positional candidate approaches.

Materials and Methods

Isolation of genomic clones. Two human brevican EST (expressed sequence tag) cDNA clones (GenBank accession No. R21312 and R13037) were supplied by Research Genetics. A cDNA fragment encoding the GAG (glycosaminoglycans)-attachment domain from the EST clone was randomly labeled with [α-^{32}P]-dCTP (Amersham Pharmacia, Uppsala, Sweden) and used to screen a human genomic library constructed in the EMBL3 SP6/T7 vector (HL1067j: Clontech). Two overlapping 13 kb and 16 kb genomic clones (HN1 and HN2) were isolated and further characterized by restriction mapping analysis and by Southern blot hybridization. The *BRAL1* genomic clone (MH322) was already cloned as previously described [9].

Long-distance genomic polymerase chain reactions (PCRs) were carried out by using LA Taq (Takara, Kyoto, Japan) according to the manufacturer's protocol. Briefly, 50-μl volumes of reaction mixture contained 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 2.5 mM MgCl2, 500 ng of template, and 2.5 U of LA Taq DNA polymerase (Takara). Amplification was carried out with an initial denaturation at 94 °C (1 min) followed by 14 cycles of 98 °C (20 s), 68 °C (20 min), and 16 cycles of 98 °C (20 s), 68 °C (20 min + 15 s/cycle), and final extension at 72 °C (10 min). The primers used were
hLP2-LA1, 5'-TCTGCTACCTTGAAACCTGCACC CTCTAGG-3';
hLP2-LA2, 5'-AGTCAGAGCCCCAGAAAGCCAAG ATACTGG-3';
hBR-LA1, 5'-CACTGGCAGAGCTGGAAGCATGTA GATCAG-3';
hBR-LA2, 5'-GCCATTAACACCAATGATGTCTGC CCCTGG-3'.

Sequence analysis. Clones were inserted into a pBluescript SK vector (Stratagene, La Jolla, CA, USA), and both DNA strands were sequenced by using an ABI BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed with an ABI 310 capillary automatic sequencer (Applied Biosystems).

Dual color-fluorescece in situ hybridization (FISH) of chromosomes. Chromosome spreading and hybridization were carried out as described previously [10]. The isolated genomic DNA probe HN2 was biotinylated at 15 °C for 1 h by using dATP and a BioNick labeling kit (Gibco BRL, Rockville, MD, USA). The genomic DNA probe (MH322) was labeled with digoxigenin. The procedure for FISH detection was performed according to previously described methods [10, 11]. Briefly, the slides were baked at 55 °C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 2 × SSC for 2 min at 70 °C, followed by dehydration with ethanol. Probes were denatured at 75 °C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulphate. Probes were loaded on the denatured chromosomal slides. After overnight hybridization, the slides were washed and the fluorescent signals were detected as well as amplified. FISH signals were observed under a fluorescence microscope equipped with FITC and rhodamine filters. Images of FISH signals and 4', 6-diamidino-2-phenylindole (DAPI) banding patterns were captured separately and combined by a CCD camera. The assignment of the FISH mapping data to chromosomal bands was achieved by superimposing FISH signals on DAPI-banded chromosomes [11].

Repetitive sequence and allele detection. To examine the *BRAL1* and *BCAN* genes for CA repeats, we screened 3 genomic clones, already isolated for the respective genes, *i.e.*, MH322 for the former and HN1 and HN2 for the latter, with a ^{32}P-labeled $(CA)_9$ probe [12]. Restriction fragments containing CA repeats were subcloned into pBluescript SK vectors and sequenced by the dye-terminator method. PCR primers were designed based on sequences at the flanking regions of the CA repeats. PCRs were carried out in 10-μl volumes of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM CA strand primer: 5'-GTTTCTTCTGACACTCTATGAGGGGCCAATT

CC-3', 0.5 μM 6-FAM labeled GT strand primer: 5'-TCC AGAGGTAGACAGGCTGAAGCTGG-3', 0.2 mM dNTPs, 10 ng of genomic DNA, and 0.25 U of Ampli Taq Gold DNA polymerase (Applied Biosystems,). Amplification was carried out with an initial denaturation at 95 °C (10 min) followed by 35 cycles of 95 °C (15 s), 62 °C (15 s), and 72 °C (30 s), and final extension at 72 °C (2 min). One-microliter aliquots of samples were denatured at 95 °C and electrophoresed on a 310 Genetic analyzer (Applied Biosystems). Allele sizes were determined by comparison with internal lane 400HD ROX size standards (Applied Biosystems) by using Gene Scan analysis software (Applied Biosystems). Expected heterozygosity was calculated by the formula, $1-\Sigma\ (Pi)^2$, whereby the frequency of allele *i*. PIC (polymorphism information content) value was calculated as previously described [13].

Results and Discussion

***Dual-color fluorescence in situ* hybridization.** Perveen *et al.* determined that the genes encoding human cartilage link protein-1 (*CRTL1*) and versican (*CSPG2*) localize to a region of chromosome 5q14.3, to which Wargner syndrome (*WGN1*; MIM 143200) is linked [14]. Rauch *et al.* reported the localization of the mouse brevican gene (*Bcan*) to a region on chromosome 3 having synteny with the human chromosome interval 1q25-1q31 [15]. Gary *et al.* reported that human *BCAN* is localized to chromosome 1q31 by radiation hybrid mapping [5]. To determine the chromosomal localization of the human *BRAL1* gene, we screened by PCR a panel of Human/Rodent somatic cell hybrids retaining individual human chromosomes (NIGMS Coriell Cell Repositories). The 251 bp PCR product indicating *BRAL1* was detected in human chromosome 1 hybrid DNA, whereas no amplification product was seen in any of the other samples, including rodent genomic DNA (data not shown). A previous result reported by Perveen *et al.* suggested that the lectican family gene and the link protein family gene can co-localize on the same band of chromosome 1 [14]. In order to identify the human *BRAL1* and *BCAN* gene loci cytogeneticaly, we performed dual-color FISH using the individual genomic clones (MH322 and HN2) as the specific probes (Fig. 1 A and B). Both *BRAL1* and *BCAN* were mapped to human chromosome 1, region q21-23.

Structure and organization of human BRAL1 and BCAN genes. Since the FISH analysis revealed that *BRAL1* and *BCAN* were co-localized at the same band of chromosome 1q21-23, we attempted the long-distance genomic PCR with several primer-pairs shown in Fig. 1C. One of the primer-pairs (hLP2-LA2 and hBR-LA1) could amplify a prominent band of 15 kb indicating the *BRAL1* gene to be located 20-kb upstream of the *BCAN* start site (Fig. 1D, lane 3). The genomic Southern blot reacted with a specific probe for exon 7 of the *BRAL1* gene demonstrated the restriction enzyme map constructed by the overlapping clones (Fig. 1E). The 2 overlapping genomic clones (HN1 and 2) included all coding sequences of the *BCAN* gene. The *BCAN* was found to contain 13 coding exons for the full-length (secreted) isoform, and it spanned approximately 13 kb. Thus, together with the data of dual-color FISH, we could relocate the human *BCAN* gene and could demonstrate that human *BRAL1* and *BCAN* genes are tandemly arranged on chromosome 1q21-23.

Two genes, *CRTL1* and *CSPG2*, which belong to the link-module superfamily, were mapped to a 2-cM region of chromosome 5q14.3 [13]. Together with the data presented here concerning the genomic organization of *BRAL1* and *BCAN*, the diverse chromosomal co-localization of the lecticans and link protein genes suggests an early divergence of the link-module superfamily genes during evolution. The functional relationship between *BRAL1* and *BCAN* has not been clarified yet and remains to be determined.

Repetitive sequence in BCAN. The brain- and nerve-tissue specificity displayed by *BRAL1* and *BCAN* marks its abnormal form as a potential candidate for involvement in neurological diseases. Among the human phenotypic mutations mapped to this region, schizophrenia 9 (SCZD9; OMIM No. 604906; ref. 16) is an allele with such potential. Recently, a genome-wide scan for schizophrenia susceptibility loci in 22 extended families with high rates of schizophrenia provided highly significant evidence for such loci at chromosome 1q21-q23, with a maximum heterogeneity logarithm of the likelihood of linkage (Lod) score of 6.50 [16].

To look for repetitive CA repeats within the *BRAL1* and *BCAN* genes, we hybridized the 3 phage clones, MH322, HN1, and HN2, which cover the entire coding exons of both *BRAL1* and *BCAN* genes (Fig. 1C), with a $(CA)_9$ probe. The *BCAN* clone HN2 was the only positive one. Further analysis revealed 2 repetitive

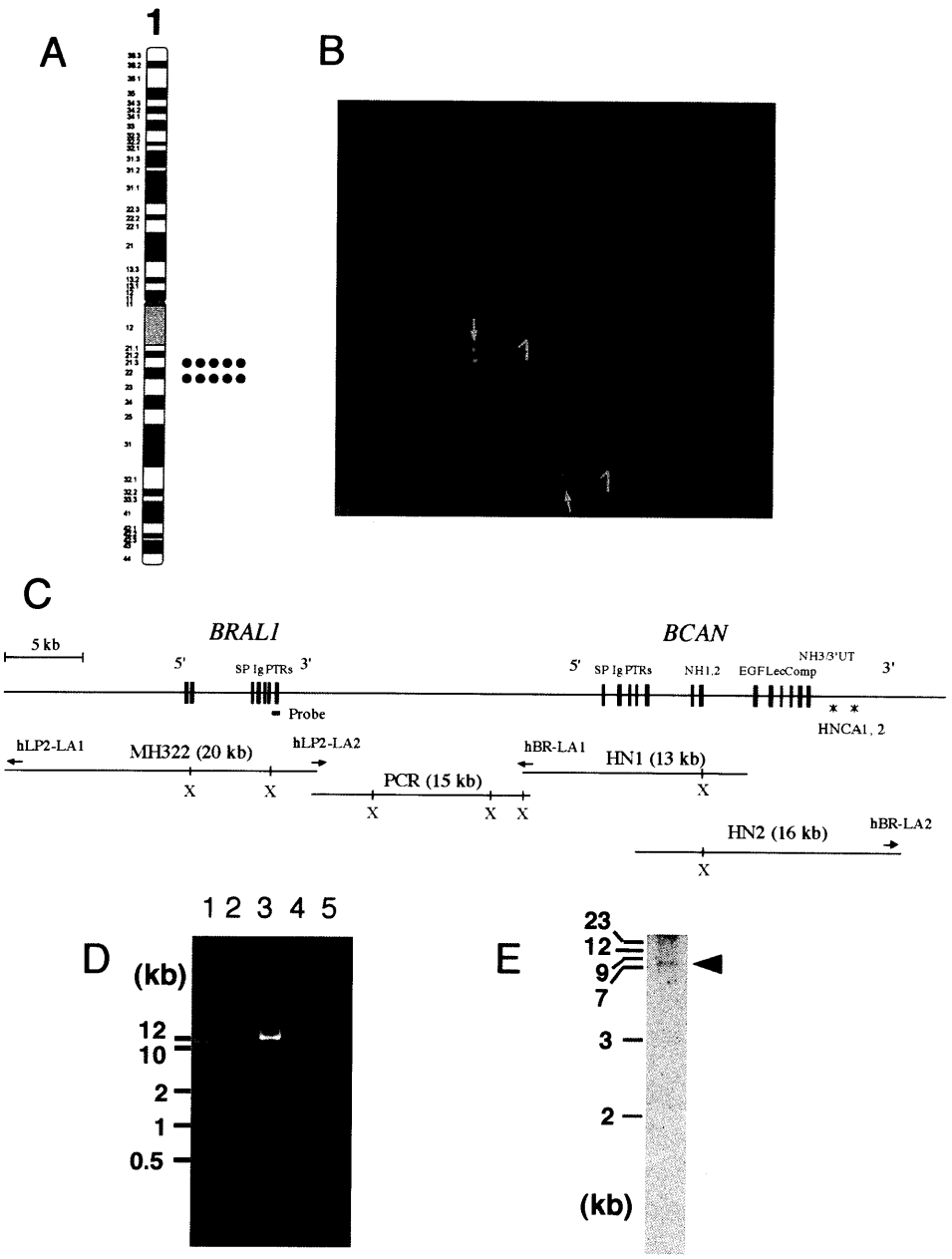

Fig. 1 Chromosomal mapping and genomic organization of *BRAL1* and *BCAN*. (**A**) Diagram of FISH mapping results with probes MH322 and HN2. Each dot represents one pair of FISH signal detected from one out of ten images analyzed. (**B**) Example of a dual-color FISH mapping with probes MH322 and HN2: the 2 color FISH signals from probes MH322 and HN2 (green represents probe HN2, labeled with biotin and detected by FITC, whereas red represents the probe labeled with digoxigenin and detected by rhodamine) were located on human chromosome 1, which was identified by staining with DAPI. Note that both probes were overlapped and seen as yellow (arrows). (**C**) Comp, complement regulatory protein-like domain; EGF, epidermal growth factor-like domain; IG, immunoglobulin-like; Lec, C-type lectin-like domain; NH, nonhomologous domains; PTRs, proteoglycan tandem repeats; SP, signal peptide. Asterisks in the 3' part of *BCAN* indicate the locations of the CA repeats. Xho I sites are indicated by capital letters: X. (**D**) Long-distance genomic PCRs. Four primers were designed in the phage clones, as indicated by small arrows. PCRs were carried out using the following 4 different primer-pairs: Lane 1, 1 Kb DNA Ladder (Gibco BRL); Lane 2, hBR-LA1 and hLP-LA1; Lane 3, hBR-LA1 and hLP-LA2; Lane 4, hBR-LA2 and hLP-LA1; and Lane 5, hBR-LA2 and hLP-LA2. (**E**) Genomic Southern hybridization. Total genomic DNA was digested with Xho I. The blot was hybridized with a probe specific for exon 7 of *BRAL1*. The single 7.5-kb band detected is indicated by the arrowhead.

sequences, in clones HNCA1 and HNCA2, which were located in the 3' part of the *BCAN* gene. To determine the polymorphic variations of the 2 microsatellites in the general population, we analyzed genomic DNAs isolated from 25 unrelated Japanese individuals. PCR products obtained with the flanking primers were analyzed on an ABI 310 Genetic analyzer (Applied Biosystems). The HNCA2 marker, which turned out to be identical to the CA-repeat marker D1S2624, was found to be useful for linkage analysis in the Japanese population because at least 6 alleles were present. The observed heterozygosity was 0.79, and the PIC value was 0.76. The size and frequencies of the 6 alleles are shown in Table 1. The highly informative CA-repeat marker HNCA2 presented here would facilitate the investigation of the possibility that *BRAL1* and *BCAN* genes may be involved in inherited schizophrenia.

Table 1 Sizes and frequencies of the alleles of the CA repeat polymorphism in the *BCAN* locus

Allele	CA (n)	Frequency
A1	16	0.24
A2	17	0.22
A3	18	0.18
A4	19	0.24
A5	20	0.10
A6	21	0.02

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