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The Specific Expression of Three Novel Splice Variant Forms of Human Metalloprotease-like Disintegrin-like Cysteine-rich Protein 2 Gene in Brain Tissues and Gliomas

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We have previously identified 67 exons on a yeast artificial chromosome contig spanning 1.5 Mb around the multidrug resistance 1 gene region of human chromosome 7q21.1. In this study, we identified three novel cytoplasmic variants (MDC2-γ, MDC2-δ, and MDC2-ε) of the human metalloprotease-like disintegrin-like cysteine-rich protein 2 (MDC2) among these exons by screening a human brain cDNA library and also by using a reverse transcription polymerase chain reaction. Genomic sequence analysis strongly supported the idea that the variations in the cytoplasmic domain were generated by alternative splicing. The expression of MDC2 variant forms in human brain tissue and gliomas was examined by reverse transcription polymerase chain reaction and RNase protection assay. MDC2-ε was expressed only in the cortical and hippocampal regions in human brain, but not in gliomas. In contrast, MDC2-γ was a major form expressed in human gliomas. Specific expression of these cytoplasmic variants of MDC2 in human brain and its malignancies is discussed.

Key words: MDC — ADAM — Alternative splicing — MDR region

The mammalian ADAM (a disintegrin and metalloprotease) family is a recently identified gene family encoding membrane proteins, and has an extensive sequence similarity to snake venom disintegrin and metalloprotease.[1)] Snake venom disintegrins are a family of anticoagulant peptides with a high cysteine content.[1)] Typical ADAMs are cell-surface proteins that have multiple domains; pro, metalloprotease-like, disintegrin-like, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains. The ADAM family is expected to be involved in cell-cell interactions. For example, fertilin, the first ADAM described, has been implicated in integrin-mediated sperm-egg binding,[2, 3)] and meltrin is required for myotube formation.[4)] In contrast, some members of this ADAM protein family have metalloprotease-like domains that are catalytically active and degrade specific substrates. An example of this type of ADAM is human tumor necrosis factor-α converting enzyme, which releases soluble tumor necrosis factor-α by proteolysis.[5, 6)] Because cell-cell interaction and protease activity have important roles in metastasis and invasion of cancer cells, the ADAM family could be associated with malignant phenotype in cancer cells.

We previously assembled a contig of 21 non-chimeric yeast artificial chromosomes (YAC) across 1.5 Mb of the multidrug resistance gene (*PGY1* and *PGY3*) region on human chromosome 7q21.1.[7)] Exon-trapping directly on the YACs resulted in an exon collection that includes 21 exons identical to the cDNA sequences of *PGY1*, *PGY3*, and sorcin, and 43 exons homologous or similar to human cDNA sequences.[8)] In these 43 exons, nine exons were homologous to a cDNA coding a human metalloprotease/disintegrin-like cysteine-rich protein (MDC), which is a member of the ADAM family.[9, 10)] These exons specifically detected a prominent 10 kb mRNA in the brain among various human tissues examined.[8)] Sagane *et al.*[11)] reported that two novel *MDC* genes, *MDC2* and *MDC3*, are highly expressed in the brain. We observed that the MDC2 sequence includes our nine MDC-related exons.[8)] In this study, we identified splicing variants of the *MDC2* gene, and determined the partial exon-intron structure. Structures of the MDC2 splicing variants are compared with that of the MDC2 reported by Sagane *et al.*[11)] Furthermore, the expression of these splicing variants was examined in the human brain and glioma cells.

MATERIALS AND METHODS

Materials Total RNA from the human brain cortex was obtained from OriGene Technologies, Inc. Surgical specimens of human hippocampus and tumor tissues were obtained from patients with epilepsy and glioma. All human samples were obtained under an Institutional

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Review Board (IRB)-approved protocol after subjects had given informed consent. Histologic diagnosis was obtained in all cases. According to the revised WHO classification, the tumors included 2 pilocytic astrocytomas, 1 anaplastic astrocytoma, 1 anaplastic oligoastrocytoma, and 3 glioblastomas. All glioma cell lines used in this study were obtained and cultured as previously described.[12)]

Isolation of MDC2 cDNA and its variant forms Short cDNA fragments (3Y-23, 3Y-36, 3Y-248, 3Y-15, 3Y-59, 3Y-50, 3Y-69, 3Y-52 and 3Y-62), which were obtained by exon amplification,[8)] were used as probes to clone full-length cDNA. The cDNA clones were obtained by screening a pCMVSPORT human brain cDNA library (GIBCO BRL, Rockville, MD). The largest plasmid clone, B5-2, is thought to lack the 5′ end of the coding sequence, because this B5-2 clone does not contain the sequence included in the clones Y3-23 and Y3-36. We performed PCR to isolate the 5′ part of the MDC2 clone. Primer pairs were designed between the 5′ end of the B5-2 clone 5′-GGTATGTACAACGGAAGCC-3′ and the vector sequence 5′-GCAAAAAGCTATTTAGGTGAC-3′. A 1 kb fragment containing the Y3-23 and Y3-36 sequences was amplified by PCR using the pCMVSPORT human brain cDNA library as a template, and was subcloned into pMOSblue. To detect splice variant forms in the cytoplasmic domain, PCR was performed using the following primer pairs: downstream 5′-AATGGAGTTTGCAGTAATGAGC-3′ and upstream 5′-TAGACCCAGTTTGATGAAGAAGG-3′ for MDC2-γ, δ, ε, and downstream 5′-AATGGAGTTTGCAGTAATGAGC-3′ and upstream 5′-ACAGTTCTTTGACTATACTGC-3′ for MDC2-α, -β. The PCR conditions were the same as described below in the reverse transcription-polymerase chain reaction (RT-PCR) section.

RT-PCR Single-strand cDNA was prepared from 1 μg of total RNA and 2.5 μmol/liter random primer (TaKaRa Shuzo, Tokyo) in a total volume of 20 μl containing Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and RNase inhibitor (TaKaRa Shuzo). PCR was performed in a final volume of 5 μl containing 10 ng of total RNA, 1 μmol/liter each of the sense and antisense primers and 1 U of *Taq* Gold DNA Polymerase (Perkin-Elmer, Foster City, CA). Amplification was performed using a DNA thermal cycler (Perkin-Elmer) according to the following protocol: initial denaturation for 9 min at 95°C, 45 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 57°C, polymerization for 30 s at 72°C, and a final extension for 10 min. The PCR products were separated by electrophoresis on 5% acrylamide gels, which were then stained with ethidium bromide.

RNase protection assay We performed PCR to obtain a fragment as a template to synthesize an antisense RNA probe. The PCR primers were 5′-AATGGAGTTTGCAGTAATGAGC-3′ and 5′-TAGACCCAGTTTGATGAAGAAGG-3′. The cDNA from human glioma cell line IN157 was used as the PCR template. We obtained a 441-bp fragment and subcloned it into the pGEM-T Easy vector. The plasmid containing the 441-bp fragment, designated RPA-γ, was linearized by *Nco*I, and an RNase protection assay was run using a Maxiscript Kit (Ambion, Austin, TX) and an RPA II kit (Ambion) according to the manufacturer's protocol. Fifteen micrograms of RNA was hybridized with 2×10^5 cpm of antisense RNA probe.

Determination of genomic organization Using an RPA-γ fragment as a probe, we screened the lambda Fix II human genomic library (Stratagene, La Jolla, CA). Sequencing analysis was performed directly from a phage clone.

DNA sequencing DNA was sequenced using a Dye terminator kit and an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

RESULTS AND DISCUSSION

Two splicing variant forms of MDC2, MDC2-α and -β, were previously reported[11)] (Fig. 1, A and B). We independently isolated MDC-related fragments from the *PGY1* region on human chromosome 7q21.1 by exon amplification.[8)] We further isolated full-length cDNA corresponding to the MDC-related fragments as described in "Materials and Methods." We found that this cDNA was identical to base pairs 1–2469 of MDC2-β cDNA[11)] (sequence numbering starts at the initiation codon ATG), and designated the cDNA as MDC2-γ. The reading frame of MDC2-β ended at 2469 and was followed by a stop codon, TAA, but the reading frame of MDC2-γ was extended by 162 bp (54 amino acids) (Fig. 1, A and B).

MDC2-α, -β and -γ were all different from each other in their cytoplasmic domains (Fig. 1, A and B), and we examined if further splice variant forms exist in the cytoplasmic domain using PCR. Transcripts MDC2-δ and -ε were thus detected as well (Fig. 1, A and B). MDC2-δ had an 87-bp (29 amino acids) insertion at position 2468 of MDC2-γ, and MDC2-ε had a 108-bp (36 amino acids) insertion of the corresponding region of the MDC2-α sequence at position 2300 of MDC2-γ (Fig. 1, A and B). We confirmed that these differences in the transcripts were due to alternative splicing of transcripts by genome sequence analysis. The genomic region corresponding to positions 2400–2750 bp of the MDC2-δ cDNA was extensively studied. DNA sequence analysis of this region showed the presence of 3 exons (X, X+1, X+2) (Fig. 2A). The genome sequence following exon X was identical to the 3′ non-coding sequence of MDC2-β, and thus MDC2-β was expected to be produced by retaining exon X, and MDC2-γ, -ε were expected to lack a single exon, X+l, which was included in MDC2-δ. The nucleotide sequences at the exon-intron junctions analyzed were consistent with the GT-AG rule, and the consensus sequence of the

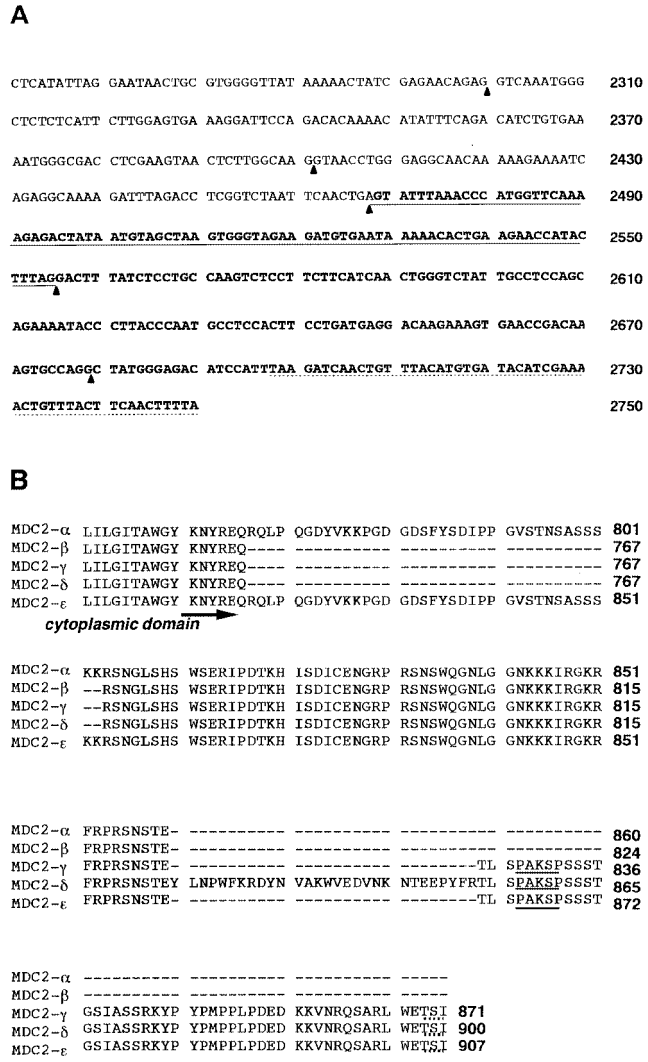

Fig. 1. Nucleotide and amino acid sequences of MDC2 splicing variant forms. A. Partial nucleotide sequences of cDNA for MDC2 variants. Sequences in bold letters are additional cDNA sequences to MDC2-α and -β, and underlined sequences are deleted from MDC2-γ and -ε. MDC2-α and -ε has a 108-bp (36 amino acids)[11] insertion at position 2300 of MDC2-γ. The dashed underline indicates 3′ non-coding sequence. Sequence numbering starts from the start codon ATG. Arrowheads indicate the positions split by introns. B. Deduced amino acid sequences of cytoplasmic domain of MDC2 variants. The underlined sequence is a putative target sequence recognized by ERK1 and ERK2. The dashed underline indicates a PDZ binding motif.

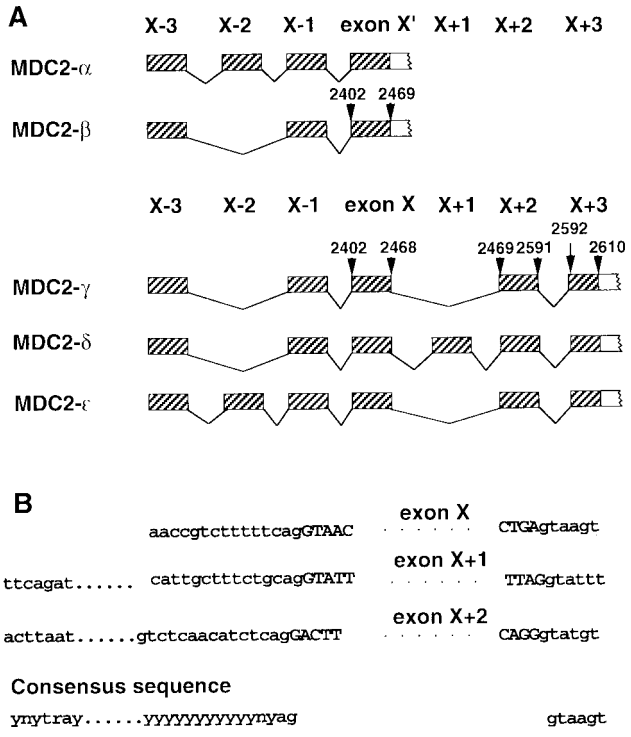

Fig. 2. Schematic representation of genomic structures and relevant nucleotide sequences around exon-intron junctions of MDC2 variant forms. A. Shaded boxes are coding exons of MDC2. White boxes are non-coding exons. Exon X corresponds to positions 2402–2468 of MDC2-γ. The genomic region corresponding to exons X, X+1, X+2 was examined in this study, and that corresponding to exons X–1, X–2, and X–3 has been examined.[11] B. Exon-intron junction sequences are shown. Small letters indicate intron sequences, and large letters indicate exon sequences. The consensus sequence of the branching point is also shown.

branching point[13, 14] was also found at an appropriate position (Fig. 2B), further confirming the generation of mRNA variants by alternative splicing. The nucleotide sequence at the branching point of intron X+1 was highly homologous to the consensus sequence (Fig. 2B), in accordance with the presence of splicing between exon X and exon X+2.

Our previous study showed the location of MDC2-related exons on 7q21.1, and their specific expression in the human brain.[8] We examined the expression of the MDC2 variant forms in human brain tissues, gliomas, and human glioma cell lines by RT-PCR. We prepared primer pairs that specifically amplified MDC2-α, -β and MDC2-γ, -δ, -ε, respectively. Products of 486 bp corresponding to MDC2-β were detected only in one glioma cell line, IN157, and two clinical glioma samples, case 3 and case 7. Products of 594 bp corresponding to MDC2-α were not observed in any sample (Fig. 3A). By contrast, MDC2-γ fragments (440 bp) were expressed in all samples except for a glioma cell line, CCF-STTG1 (Fig. 3A). MDC2-δ fragments (527 bp) were observed in some clinical glioma samples and cell lines, but not in the cortex and the hip-

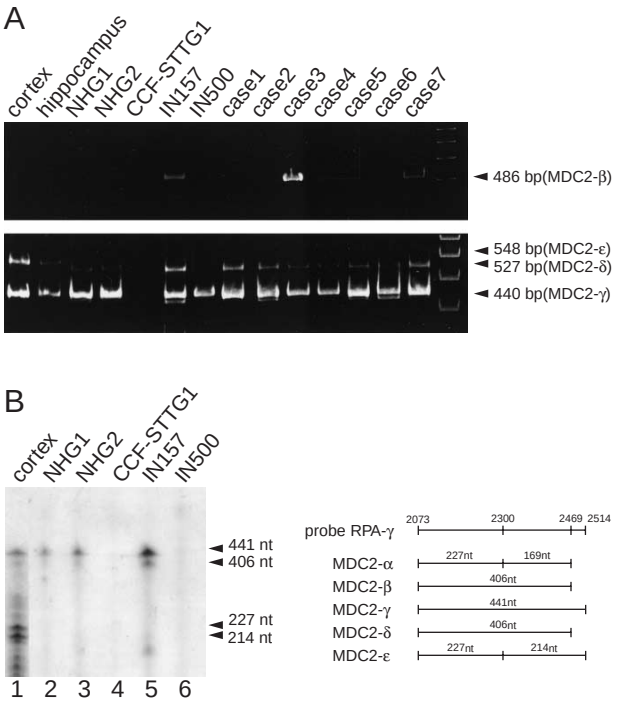

Fig. 3. Expression of MDC2 variant forms. A. The upper panel shows the results of a PCR analysis using MDC2-α and -β specific primer pairs. Product sizes were 594 bp (MDC2-α) and 486 bp (MDC2-β). The lower panel shows the results of a PCR analysis using MDC2-γ, -δ, and -ε specific primer pairs. Product sizes were 440 bp (MDC2-γ), 527 bp (MDC2-δ) and 548 bp (MDC2-ε). Normal brain tissue of cortex region, brain tissue of hippocampus region from an epilepsy patient, glioma cell lines (NHG1, NHG2, CCF-STTG1, IN157, IN500),[12] and resected clinical glioma samples, including pilocytic astrocytomas (cases 1, 7), anaplastic astrocytoma (case 4), anaplastic oligoastrocytoma (case 6), and glioblastomas (cases 2, 3, 5), were examined. B. Expression of MDC2 variant forms analyzed by RNase protection assay. Antisense RNA probe RPA-γ corresponded to positions 2073–2514 of MDC-γ. Fragments and their lengths are indicated. This probe could not distinguish MDC2-β and MDC2-δ.

pocampus. MDC2-ε fragments (548 bp) were observed only in the cortex and in the hippocampus. The intensities of MDC2-γ were clearly stronger than those of MDC2-δ and MDC2-ε in epileptic hippocampal tissue with marked gliosis, clinical glioma samples, and cell lines, suggesting that MDC2-γ might be a dominant form of MDC2. However, MDC2-ε showed approximately the same intensity as MDC2-γ in the cortex.

To confirm quantitatively which transcript was the major form in the central nervous system, an RNase protection assay was performed using antisense RNA probes corresponding to MDC2-γ (Fig. 3B). The probe RPA-γ detected a 441-nt fully protected fragment, and 227-nt and 214-nt digested fragments in the RNA from the cortex (Fig. 3B). The results of the RT-PCR showed that the 441-nt fragment corresponded to MDC2-γ, and the 227-nt and 214-nt fragments corresponded to MDC2-ε. We measured the radioactivity of these fragments and standardized their lengths. MDC2-ε appeared to be the major form, though MDC2-γ was expressed at one-third the level of MDC2-ε in the human cortex. By contrast, MDC2-ε fragments were not observed in any glioma cell line. A 441-nt fully protected fragment (MDC2-γ) was detected in three out of five glioma cell lines. The 406-nt fragment, which could be generated from MDC2-β and MDC2-δ, was detected only in IN157 cells,

Table I. Expression of MDC2 Variants in Cortex, Epileptic Hippocampus and Gliomas

Human tissues	Constituent cells	Expression of MDC2
Cortex	Astrocytes Neurons	ε>γ
Epileptic hippocampus	Reactive astrocytes Degenerated neurons	ε<γ
Gliomas	Displastic astrocytes	γ>δ

Table I illustrates the differential expression of splicing variants in human brain tissues and gliomas. Considering that epileptic hippocampus tissue contains many reactive astrocytes and sparse degenerated neurons,[15–18] the splice variant MDC2-ε, a major form of MDC2 in the human cortex (Fig. 3B), might be specifically expressed in neurons. On the other hand, MDC2-γ was expressed mainly in human gliomas (Fig. 3, A and B), suggesting that MDC2-γ is specifically expressed in astrocytes. The expression of MDC2-ε and -γ in the hippocampus might reflect the presence of a mixture of both reactive astrocytes and sparse and degenerated neurons. MDC2-δ was only expressed in gliomas, but not in the cortex and hippocampus, in which the major constituents are normal astrocytes and neurons (Fig. 3A and Table I). Glia cells consist of mainly astrocytes and all gliomas used in this study were generated from astrocytes as described in "Materials and Methods." Although there appeared to be no correlation between the expression of MDC2-δ and the WHO classification of gliomas, MDC2-δ could be a useful tumor marker for gliomas. MDC, another member of the ADAM family, appeared to be involved in the malignant phenotype of breast cancer.[9] It remains unclear whether or not MDC2 plays an important role during the acquisition of malignant phenotype in gliomas.

The metalloprotease-like domain of MDC2 lacks the HEXXHXXGXXH amino acids for zinc binding,[11] which are critical for protease activity,[19] suggesting that MDC2 may not have catalytic potential. On the other hand, the

amino acid sequence around the disintegrin-like domain of MDC2 has a marked similarity to other *ADAM* genes, suggesting that MDC2 selectively binds to integrin or extracellular matrix via a conserved disintegrin-like domain. In this study, we identified three splicing variant forms of MDC2, all in the cytoplasmic domain. Because the consensus sequence of the target amino acids for MAP kinase is P-(X)-X-S/T-P,[20] amino acid residues (P-A-K-S-P) in the extra amino acid sequences specific for MDC2-γ, -δ and -ε of the cytoplasmic domain could be recognized and phosphorylated by ERK1 and ERK2 protein kinase[20] (Fig. 1B). On the other hand, the amino acid residues T-S-I at the carboxyl terminus (Fig. 1B) are consistent with the consensus recognition sequence of the PDZ domain (S/T-X-V/I),[21] and this motif could be recognized by proteins containing the PDZ domain. The cytoplasmic domain of the MDC2 protein probably interacts with other proteins and variations of the domain among the MDC2 variants thus might generate biological variations through modification of the MDC2 protein or the signal-transduction process from the MDC2 protein. Further functional analysis of MDC2 is required.

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