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Brain Specific Human Genes, NELL1 and NELL2, Are Predominantly Expressed in Neuroblastoma and Other Embryonal Neuroepithelial Tumors

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Abstract

***NELL1* and *NELL2* encode cysteine-rich amino acid sequences including six epidermal growth factor-like motifs, which contain signal peptides at the N-terminals. The deduced amino acid sequences of both genes are 55% identical and their cysteine stretch structures are conserved. *NELL1* is expressed in the brain and kidney, whereas *NELL2* is expressed specifically in the brain. The cell lineage expressing *NELLs* in the nervous system was investigated in established cell lines and central nervous system tumor tissues obtained from patients by Northern blot and reverse transcriptase-polymerase chain reaction analyses. *NELL1* and *NELL2* were predominantly expressed in neuroblastoma cell lines and little expressed in glioblastoma cell lines. *NELL1* and *NELL2* were also expressed in central neurocytoma, medulloblastoma, and some astrocytic tumors. Immunohistochemical analysis revealed that NELL2 protein was localized in the cytoplasm of neurons. These results suggest that *NELL2* is predominantly expressed in the neuronal cell lineage in the human nervous system. *NELL1* is expressed mainly in tumors in the neuronal cell lineage.**

Key words: *NELL1*, *NELL2*, epidermal growth factor-like motif, brain tumor, neural differentiation

Introduction

Epidermal growth factor (EGF)-like repeat structures have been found in membrane proteins such as lin-12/Notch family members and their ligand proteins, Delta, Serrate, Jagged, and APX-1.[7,8] The lin-12/Notch signaling systems are important in cell fate control and the development of many systems.[2] Recently, we isolated a nel gene encoding a 91 kd cysteine-rich protein which includes six EGF-like repeats from a chick embryonic complementary deoxyribonucleic acid (cDNA) library.[14] Although the nel gene was expressed ubiquitously in the early stage chick embryos, expression increased in neural tissues and decreased in non-neural tissues during embryonic development, and expression of the nel gene was restricted in neural tissues in the adult chick.[13]

A human nel (*NELL2*) and its homolog (*NELL1*) were isolated from a human fetal brain cDNA library.[20] The deduced amino acid sequence of *NELL2* was approximately 88% identical to chick nel and that of *NELL1* about 55% identical to *NELL2*. Through Northern blot analysis expression of *NELL1* was observed in the adult brain and kidney, whereas that of *NELL2* was observed specifically in the brain. *NELL1* and *NELL2* were mapped to chromosome 11p15.1–p15.2 and 12q13.11–q13.12, respectively.[20]

Determination of NELL expression in the cells of the human brain is difficult to achieve, but the cell lineage that expresses the genes can be investigated

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in surgically obtained tumor tissues and established cell lines. This study investigated the expression of *NELL1* and *NELL2* in neuroblastomas, medulloblastomas, and other astrocytic tumors, as well as human peripheral blood cells.

Materials and Methods

I. Tumor tissues and cell lines

Human brain tumor tissues were obtained at surgery from patients treated at Saga Medical School Hospital and stored in nitrogen liquid until use. Peripheral blood mononuclear cells were obtained from a healthy volunteer. Peripheral blood mononuclear cells containing CD34-positive cells were obtained from a patient with leukemia who underwent peripheral blood stem cell transfusion. The neuroblastoma cell lines NB9, NB6, NB19, and NB69, and MRC5sv1tg1 were obtained from the RIKEN Cell Bank (Tsukuba Science City, Ibaraki). MRC5 and HT1376 were obtained from Flow Lab. (McLean, Va., U.S.A.) and Widr was obtained from the Health Science Research Resources Bank (Tokyo). The neuroblastoma cell lines IMR32 and SKNSH, all glioma cell lines, and other cell lines including HeLa, A431, and CEM were used from laboratory stocks. All cell lines were cultured in Dulbecco's Minimum Essential Medium containing 10% fetal bovine serum.

II. Northern blot analysis

Northern blot of adult human tissues used the product of Clontech (Palo Alto, Calif., U.S.A.). Extraction of total ribonucleic acid (RNA) from tumor tissues and cell lines was performed according to the manufacturer's protocol using an Isogen kit (Wako Pure Chemical Ind. Ltd., Osaka). Northern blot analysis was carried out according to the method described in the instruction manual using a Genescreen Hybridization Transfer Membrane (PerkinElmer Life Sciences Inc., Boston, Mass., U.S.A.). DNA probes, nucleotides 170–1820 (1650 bp) of *NELL1* (Genbank accession No. D83071) and nucleotides 770–polyA tail (2500 bp) of *NELL2* (Genbank accession No. D83018), were labeled with ^{32}P-deoxycytidine triphosphate using the Wako DNA labeling kit.

III. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed using the Perkin Elmar kit (Part No. N808–0178; Applied Biosystems, Foster City, Calif., U.S.A.), with 20 μl solution which contained 500 ng of total RNA. Primers 5′AGACTCCCTCCCAGTACACA3′ (1050–1060nt, forward) and 5′GTCTCCCTGGACAGAGATGT3′ (1367–1386nt, reverse) of *NELL1*, and 5′TTAAGTCGGCTCTTGCGTATG3′ (1040–1360nt, forward) and 5′ATCCTCTCGAAGAGCCCTAA3′ (1379–1398nt, reverse) of *NELL2* were used to produce RT-PCR products of 337 bp and 359 bp from NELL1 and NELL2 messenger RNA (mRNA), respectively. The bands 337 bp and 359 bp were not produced by PCR from human genomic DNA using the NELL primers.

IV. Immunohistochemical staining of the brain

The brain of a 65 year-old man, who died of cancer, was fixed in 10% formalin. Frozen sections of the fixed brain 5 μm thick were prepared and stained with anti-NELL2 C-terminal antibodies[15] and M1 antibody[14] using the ABC system with a rabbit S-HRP staining kit (Seikagaku Ind. Co., Tokyo). The anti-NELL2 C-terminal protein antibody was prepared by immunizing rabbits with the synthetic peptide, SVDPQCLQEL of the C-terminal sequence, which is identical in human, murine, and chick species, and the M1 antibody was prepared against a chick nel peptide, 101-GVIFSIHHLDHRYLELESSGHRNE-124, which is identical in the three species. An exception is the 104F which is I in human NELL2 and murine Nel2 (Genbank accession No. RNU48245 for rat and MMU59230 for mouse). Both antibodies were purified by affinity chromatography using peptide columns as described elsewhere.

Results

I. Expression of *NELL1* and *NELL2* in established cell lines

Both *NELL1* and *NELL2* were expressed in the adult brain, although the levels of *NELL1* were much lower than those of *NELL2* (Fig. 1). *NELL1* was also highly expressed in the kidney, whereas *NELL2* expression was restricted to the brain.

Total RNA derived from established cell lines of neuroblastoma and glioblastoma was examined by Northern blot and RT-PCR analyses. NELL2 mRNA expression was observed in five of the six neuroblastoma cell lines, except for SKNSH (Fig. 2, lane 5). A clear NELL1 mRNA band was detected in IMR32 and NB19 (lanes 1 and 6, respectively), and a very weak band was detected in NB16 and NB69 (lanes 3 and 4, respectively). No band was detected in NB9 and SKNSH (lanes 2 and 5, respectively). However, RT-PCR found expression of both genes in all neuroblastoma cell lines (Fig. 3A, Table 1). In contrast, no expression of either gene was detected in the glioma cell lines U373, U138, U87, T98G, and A117 by Northern blot analysis, and quite low expression of *NELL2* was observed in a few glioma cell lines. No

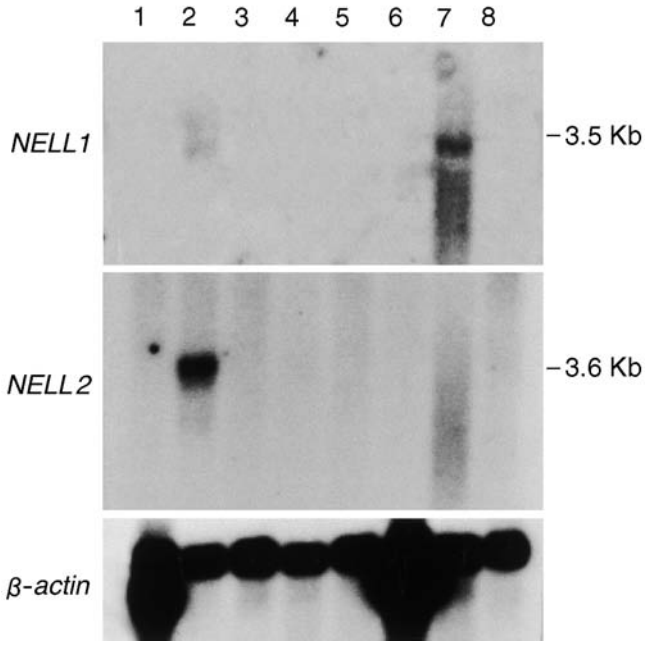

Fig. 1 **Northern blot analysis of *NELL1* and *NELL2* in human tissues. PolyA ribonucleic acid (2 μg) derived from heart (1), brain (2), placenta (3), lung (4), liver (5), skeletal muscle (6), kidney (7), and pancreas (8) tissues was hybridized with ^{32}P-labeled NELL1 and NELL2 probes. The spleen, peripheral blood mononuclear cells, prostate, and ovary tissues gave negative results (not shown).**

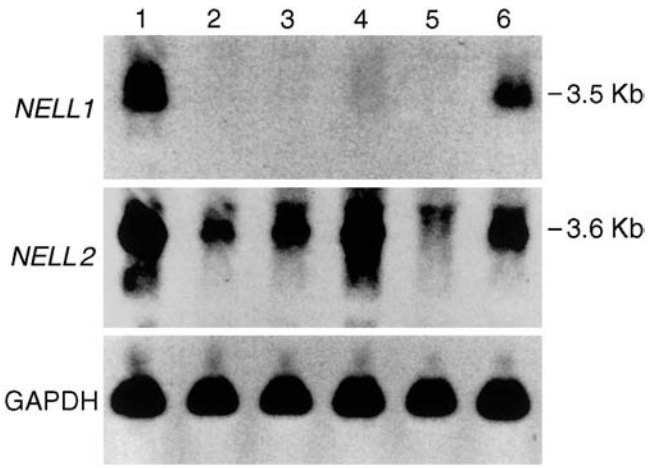

Fig. 2 **Northern blot analysis of *NELL1* and *NELL2* in human neuroblastoma cell lines. Total ribonucleic acid (20 μg) obtained from the cell lines, IMR32 (1), NB9 (2), NB16 (3), NB69 (4), SKNSH (5), and NB19 (6), was hybridized with ^{32}P-labeled NELL1 and NELL2 probes. GAPDH shows the same blot hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe as a control.**

expression of *NELL1* was observed in these cells on RT-PCR (Table 1).

The following non-neural cell lines were also examined: HeLa, MRC5 (normal fetal lung fibroblast), MRC5sv1tg1 (SV40 transformed MRC5), A431 (epidermoid carcinoma), HT1376 (bladder cancer), CEM (T-lymphoma), and Widr (colon cancer). Weak *NELL2* expression was observed in MRC5sv1tg1 and A431 by RT-PCR (Table 1).

II. Expression of *NELL1* and *NELL2* in tumor tissues

The analytical results of the established cell lines indicated that NELL genes may be preferentially expressed in the neuronal cell lineage in the nervous system. Surgical specimens of one neuroblastoma tissue and four glioma tissues excised from patients were analyzed by Northern blot analysis. *NELL2* expression was observed in neuroblastoma tissue but not in any of the glioma tissues (Fig. 4). *NELL1* expression was not observed in these tissues. Various types of other tumor tissues were analyzed by RT-PCR using NELL1 and NELL2 primers (Fig. 3B). The results of both RT-PCR and Northern blot analysis were summarized in Table 2. The mRNA amounts of both genes obtained by RT-PCR are in agreement with the results of Northern blot analysis. A relatively clear band of *NELL1* was observed in medulloblastoma 600 and astrocytoma 508, and a clear band of *NELL2* in medulloblastomas 568, 636, and 676, and astrocytoma 508 (Fig. 3B) in addition to neuroblastoma tissue. There was no band in central nervous system lymphoma tissues (lanes 5 and 6). Peripheral blood mononuclear cells containing CD34-positive hematopoietic stem cells obviously expressed both NELL1 and NELL2 mRNAs, although peripheral blood mononuclear cells obtained from a normal volunteer showed no expression (lanes 7 and 14).

III. Expression patterns of NELL2 protein in the brain

Sections of human cerebral cortex, hippocampus, and cerebellum were stained with two antibodies, anti-NELL2 C-terminal antibody and M1 anti-chick nel synthetic peptide antibody. Western blot analysis of the COS-7 cells expressing rat NELL2 protein showed that anti-NELL2 C-terminal antibody specifically recognized NELL2 protein without cross reactivity.[15] Western blot analysis of adult chick brain also confirmed that M1 antibody recognized nel protein specifically (Maeda et al., manuscript in preparation).

The cytoplasm of nerve cells was positively stained in the sections of cerebral cortex and hip-

pocampus with both antibodies (Fig. 5). The staining patterns with anti-C-terminal antibody and M1 antibody were essentially the same, although the staining intensity with M1 was higher than that with anti-C-terminal antibody. No clear staining patterns were obtained in the cerebellum with either of the antibodies, probably due to the lower expression of *NELL2* in the cerebellum which was expected based

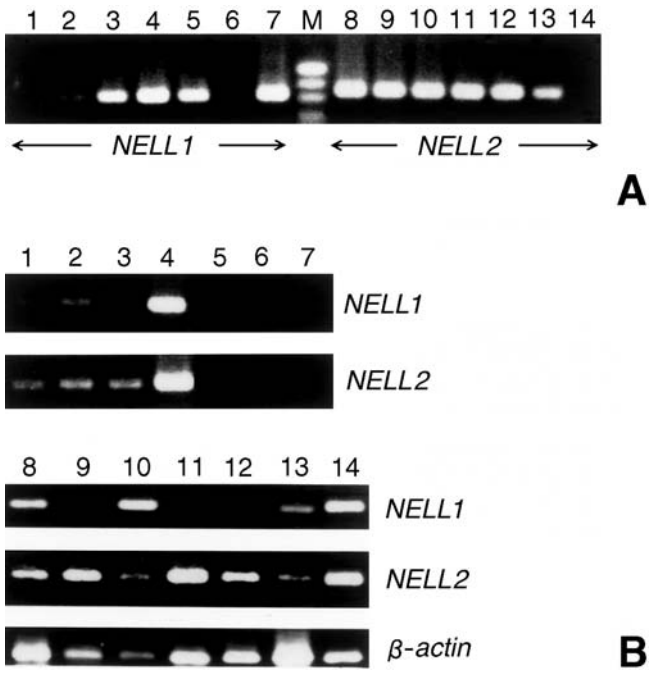

Fig. 3 **Reverse transcriptase-polymerase chain reaction (RT-PCR) of total ribonucleic acid (RNA) derived from neuroblastoma cell lines, and brain tumor tissues and human blood samples with NELL1 and NELL2 primers. A: Total RNA obtained from neuroblastoma cell lines, NB9 (2 and 9), NB16 (3 and 10), NB19 (4 and 11), NB69 (5 and 12), SKNSH (6 and 13), IMR32 (7 and 8), and no RNA controls (1 and 14), was analyzed with NELL1 primers (1–7) and NELL2 primers (8–14). M: deoxyribonucleic acid size marker; 501 + 489, 404, 331, and 242 bp from upper band, respectively. B: Total RNA obtained from astrocytoma 146 (1), glioblastoma 467 (2) and 470 (3), astrocytoma 508 (4), lymphoma 463 (5) and 504 (6), central neurocytoma 598 (8), medulloblastoma 568 (9), 600 (10), 636 (11), 676 (12), and 726 (13), human peripheral blood mononuclear cells (7), and human peripheral blood mononuclear cells containing CD34-positive hematopoietic stem cells (14) was subjected to RT-PCR using NELL1 and NELL2 primers.**

Table 1 **Expression of *NELL1* and *NELL2* in established cell lines**

Cell lines	*NELL1*		*NELL2*	
	Northern blot	RT-PCR	Northern blot	RT-PCR
Neuroblastoma:				
IMR32	+	+	+	+
NB9	−	±	+	+
NB16	±	+	+	+
NB19	+	+	+	+
NB69	±	+	+	+
SKNSA	−	−	−	+
Glioma:				
A117	−	Nd	−	Nd
T98G	−	Nd	−	Nd
U87	−	−	−	±
U118	Nd	−	Nd	±
U138	−	Nd	−	Nd
U373	−	−	−	−
Non-neural:				
Hela	Nd	−	−	−
MRC5	Nd	−	Nd	−
MRC5sv1tg1	Nd	−	Nd	±
A431	Nd	−	Nd	±
HT1376	Nd	−	Nd	−
CEM	Nd	−	−	−
Wider	Nd	−	Nd	−

+: positive, ±: equivocal, −: negative, Nd: not done. RT-PCR: reverse transcriptase-polymerase chain reaction.

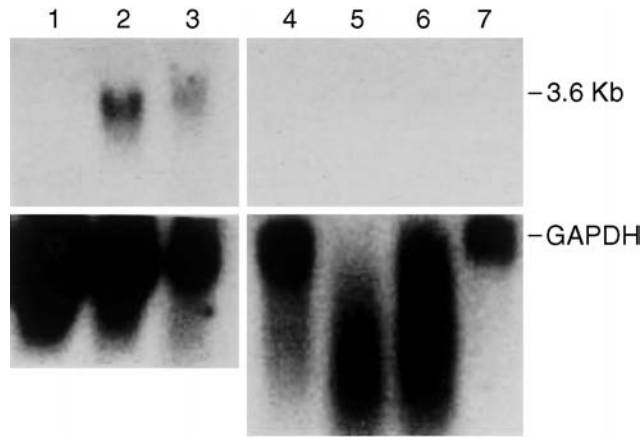

Fig. 4 **Northern blot analysis of *NELL2* in established cell lines and brain tumor tissues. Total ribonucleic acid (10 μg) obtained from established cell lines, CEM (1) and IMR32 (2), and from surgical samples of neuroblastoma (3), glioblastoma 467 (4), anaplastic astrocytoma 216 (5), astrocytoma 146 (6), and glioblastoma 180 (7) was hybridized with ^{32}P-labeled NELL2 probe. GAPDH shows the same blot hybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe as a control.**

Table 2 ***NELL1*** **and** ***NELL2*** **expression in brain tumor tissue samples and peripheral blood samples**

Samples		*NELL1*		*NELL2*	
		Northern blot	RT-PCR	Northern blot	RT-PCR
Neuroblastoma		−	Nd	+	Nd
Central neurocytoma	598	Nd	+	Nd	+
Medulloblastoma	568	Nd	−	Nd	+
Medulloblastoma	600	Nd	+	Nd	+
Medulloblastoma	636	Nd	−	Nd	+
Medulloblastoma	676	Nd	−	Nd	+
Medulloblastoma	726	Nd	+	Nd	+
Astrocytoma	146	−	−	−	+
Astrocytoma	508	Nd	+	Nd	+
Anaplastic astrocytoma	216	−	Nd	−	Nd
Glioblastoma	180	−	Nd	−	Nd
Glioblastoma	467	−	+	−	+
Glioblastoma	470	Nd	−	Nd	+
Malignant lymphoma	463	Nd	−	Nd	−
Malignant lymphoma	504	Nd	−	Nd	−
PBMNC		Nd	−	Nd	−
PBSC		Nd	+	Nd	+

+: positive, −: negative, Nd: not done. PBMNC: peripheral blood mononuclear cells, PBSC: peripheral blood stem cells, RT-PCR: reverse transcriptase-polymerase chain reaction.

on results of in situ hybridization analysis in other species (Maeda et al., manuscript in preparation).[10]

Discussion

Northern blot analysis showed *NELL2* was abundantly expressed in neuroblastoma cell lines but little expressed in glioblastoma cell lines. Immunohistochemical study of the human cerebral cortex showed NELL2 protein was accumulated in the cytoplasm of nerve cells. These results indicated that *NELL2* is expressed predominantly in neuronal cells and decreased in glial differentiation. *NELL1* expression was restricted in some neuroblastoma cell lines and was not observed in glioma cell lines. *NELL1* is thought to be expressed in the neuronal cell lineage. The results of RT-PCR supported these assumptions. Tissue samples of embryonal neuroepithelial tumors expressed both genes. All five medulloblastoma samples expressed *NELL2* and one or two of the five expressed *NELL1*. Central neurocytoma also expressed both genes, although only one sample was available. Astrocytic tumors rarely expressed both genes. Some glioma tissues expressed NELL1 and NELL2 genes by RT-PCR, possibly as a result of contamination of normal neuronal cells or their primitive transformation.

Chick *nel* (*NELL2 homolog*) expression begins in the mantle layers of the neuroepithelia in early chick embryos[14] and is restricted in neurons in adults as revealed by in situ hybridization analysis of the brain (Maeda et al., manuscript in preparation). NELL2 mRNA was detected most abundantly by in situ hybridization in the hippocampus, and moderately in the cerebral cortex, amygdaloid complex, thalamus, and ventromedial hypothalamic nucleus of adult rat brain. These areas are considered to exhibit high neuronal plasticity.[10] These observations are compatible with the above assumption that *NELL2* is expressed in neuronal cells but not in glial cells in the human brain.

NELL1 is expressed in calvarial osteoprogenitor cells which originated from the neural crest, and expression is upregulated in the premature fusing and fused coronal sutures of unilateral coronal synostosis.[18] However, *NELL1* is not expressed in the mesodermal origin cartilage. Our Northern blot examination revealed abundant expression of *NELL1* in the adult kidney, although expression was not observed in the adult rat kidney. Such high expression was also observed in an another kidney sample by Northern blot and by RT-PCR which revealed 325 bp band of *NELL1*. Therefore, NELL1 mRNA is very likely to be abundantly present in the human kidney tissue. Determining which kinds of cells express the gene in the tissue is an interesting subject for future study.

Neural stem cells can differentiate into both cells of the central nervous system and blood cells.[4,5] We observed abundant *NELL1* and *NELL2* expression in peripheral blood mononuclear cells containing CD34-positive hematopoietic precursors. Expression of NELL genes in some hematopoietic progenitors might indicate they contain properties of neuronal progenitor cells. Further investigation is needed.

We conclude that both NELL genes are preferentially expressed in the neuronal cell lineage, that *NELL2* is predominantly expressed in neurons and their progenitors, and that *NELL1* is expressed in diverse kinds of cells including neuroblasts.

Both NELL amino acid sequences contain signal peptide at the N-terminal. The chick nel protein and rat Nel proteins were secreted when the genes were expressed in NIH3T3 cells (Matsuhashi et al., unpublished data) and in COS-7 cells,[11] respectively. The anti-NELL2 antibodies reacted with the cytoplasm of neurons but not with plasma membrane or matrix of the human brain. Similar results were also obtained in the chick brain and rat brain (Maeda et al., manuscript in preparation). Accumulation of the antigen was observed in the endoplas-

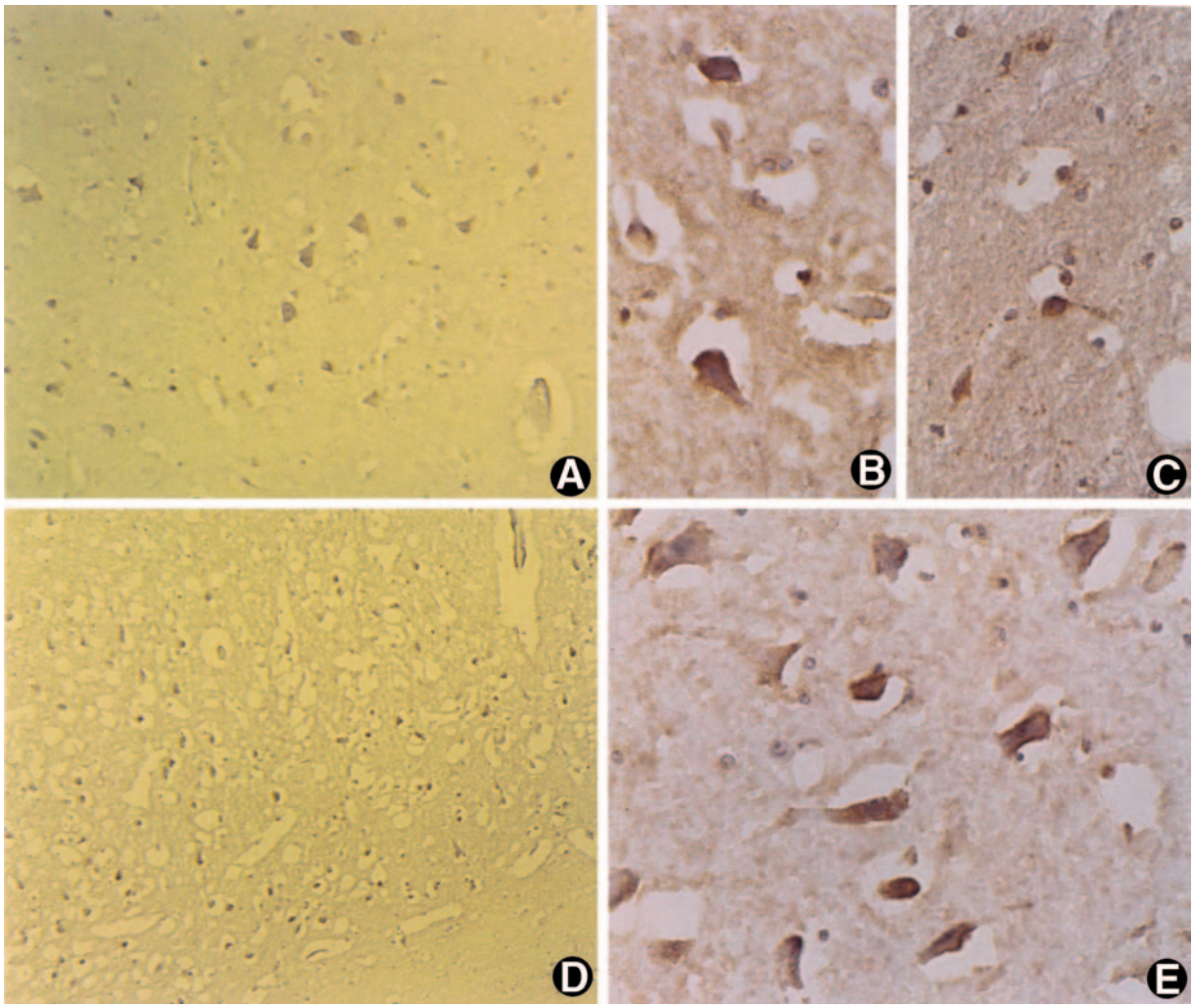

Fig. 5 Immunohistochemical staining of nerve cells with anti-NELL2-protein antibody. A: Cerebral cortex giant pyramidal cells positively stained with anti-NELL2 C-terminal antibody. ×100. B: Cerebral cortex giant pyramidal cells stained with M1 antibody. ×400. C: Cerebral cortex small pyramidal cells stained with M1 antibody. ×400. D: Hippocampus nerve cells stained with anti-NELL2 C-terminal antibody. ×100. E: Hippocampus nerve cells positively stained with M1 antibody. ×400.

mic reticulum lumen of CL8c4.7 neuroblastoma cells (Matsuhashi et al., unpublished data), and of rat hippocampus neurons.[15] The antigen was observed in the varicose fibers of the neuritis in the chick brain (Matsuhashi et al., unpublished data) and in the rat brain,[15] so secretion of NELL2 protein might be regulated in the endoplasmic reticulum, or the protein might be transported as particles to the axons and dendrites through the cytoplasm.

EGF-like proteins such as Delta, Serrete, or Jagged function as ligands of the Notch signaling system. The nel proteins may also function as ligands, although their functions are not yet known. The Notch signaling pathway is essential for the appropriate differentiation of many cell types in various organs.[1,3,8,19] Differentiation abnormalities are implicated in a variety of human diseases including neoplasms such as leukemia, cervical cancers, small cell lung carcinoma, and neuroendocrine tumor, and the two hereditary syndromes Alagille and CADASIL.[6,8,9,12,16,17,21] NELL genes may be active in controlling the neural differentiation pathway and thus important in the pathogenesis of the human nervous system.

Acknowledgments

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Commentary on this paper appears on the next page.

Commentary

As the authors mentioned, NELL1 and NELL2 are members of the epidermal growth factor (EGF) gene family that have previously been shown to be expressed almost exclusively in brain tissue. In this manuscript, they described that NELL1 and NELL2 are expressed in the neuronal cell lineage although the degree are different between them. Luce and Burrows reported that NELL1 and NELL2 were also expressed in hemopoietic cells.[1] The function of NELL proteins is not yet known. EGF-like domains of NELL proteins are reported to be the target of protein kinase C that mediates various signal pathways (ref. 11 of this article). Further studies concerning functions and distribution of NELL proteins are warranted.

Reference

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The analysis of tumor specific markers expressed in central nervous system tumor is a study that is connected with the development of 21st century medicines such as gene medicine, reproductive medicine, or new drug design. In this paper, the expression of brain specific human genes, NELL1 and NELL2, in neuroblastoma and other embryonal neuroepithelial tumors is examined. This is very timely and has priority. Unfortunately, the gene expression is analyzed at the mRNA level only (Northern blot and RT-PCR) but not the protein level in the case of the tumors. Furthermore, the authors use a normal RT-PCR technique, not a microdissected RT-PCR one. If they are improved, all data must become much more clear-cut. In the future, I would like to know the relationship between NELL1 and NELL2 gene expression and tumorigenicity, because amino acid sequences of NELL1 and NELL2 include epidermal growth factor-like motifs. If any correlation could be discovered, this study would have developed to become more powerful and attractive. Anyway, I expect that more studies like this paper will be performed.

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