EMP3, a Myelin-Related Gene Located in the Critical 19q13.3 Region, Is Epigenetically Silenced and Exhibits Features of a Candidate Tumor Suppressor in Glioma and Neuroblastoma

Miguel Alaminos,1,2 Verónica Dávalos,1 Santiago Ropero,1 Fernando Setién,1 Maria F. Paz,1 Michel Herranz,1 Mario F. Fraga,1 Jaume Mora,2 Nai-Kong V. Cheung,4 William L. Gerald,1 and Manel Esteller1

1Cancer Epigenetics Laboratory, Molecular Pathology Programme, Spanish National Cancer Centre, Madrid, Spain; 2Department of Histology, Unidad Mixta de Investigaciones Médicas, University Hospital San Cecilio, Granada, Spain; 3Department of Oncology, Hospital Sant Joan de Deu, Barcelona, Spain; and Departments of 4Pediatrics and 1Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York

Abstract

The presence of common genomic deletions in the 19q13 chromosomal region in neuroblastomas and gliomas strongly suggests the presence of a putative tumor suppressor gene for these neoplasms in this region that, despite much effort, has not yet been identified. In an attempt to address this issue, we compared the expression profile of 89 neuroblastoma tumors with that of benign ganglioneuromas by microarray analysis. Probe sets (637 of 62,839) were significantly down-regulated in neuroblastoma tumors, including, most importantly, a gene located at 19q13.3: the epithelial membrane protein 3 (EMP3), a myelin-related gene involved in cell proliferation and cell-cell interactions. We found that EMP3 undergoes hypermethylation-mediated transcriptional silencing in neuroblastoma and glioma cancer cell lines, whereas the use of the demethylating agent 5-aza-2-deoxycytidine restores EMP3 gene expression. Furthermore, the reintroduction of EMP3 into neuroblastoma cell lines displaying methylation-dependent silencing of EMP3 induces tumor suppressor–like features, such as reduced colony formation density and tumor growth in nude mouse xenograft models. Screening a large collection of human primary neuroblastomas (n = 116) and gliomas (n = 41), we observed that EMP3 CpG island hypermethylation was present in 24% and 39% of these tumor types, respectively. Furthermore, the detection of EMP3 hypermethylation in neuroblastoma could be clinically relevant because it was associated with poor survival after the first 2 years of onset of the disease (Kaplan–Meier; P = 0.03) and death of disease (Kendall τ, P = 0.03; r = 0.19). Thus, EMP3 is a good candidate for being the long-sought tumor suppressor gene located at 19q13 in gliomas and neuroblastomas. (Cancer Res 2005; 65(7): 2565-71)

Introduction

The most prevalent tumors of the nervous system are gliomas and neuroblastomas. Neuroblastoma, a tumor arising from precursor cells of the peripheral sympathetic nervous system, is the most common extracranial malignant solid tumor of childhood (1, 2), whereas gliomas affect the central nervous system, particularly in adults, usually with a poor prognosis (3). It is thought that both neuroblastomas and gliomas might arise from neuroectodermal precursor cells (4).

With an overall survival rate of <50%, the prognosis of neuroblastoma patients is highly variable and is associated with many biological and clinical features, including the stage of the disease (5), patient age, tumor ploidy (6), and a series of genetic and epigenetic molecular features of the tumor, such as MYCN amplification (5), 1p deletions, 1q gains, and HOXA9 and RARB2 CpG island hypermethylation (7). Children (>12 months old at diagnosis) with stage IV or stage III tumors with amplification of the oncogene MYCN are at high risk of mortality (>60%), whereas infants (<12 months at diagnosis) with stage IVS disease have the highest survival (nearly 100%) even without treatment (8, 9). On the other hand, gliomas are heterogeneous central nervous system neoplasms. The major subtypes of glioma are astrocytomas and oligodendrogliomas; each subtype has characteristic histologic features and different clinical behaviors. Collectively, these lesions are the most common central nervous system tumors of adults, with nearly 15,000 diagnosed annually in the United States and a mortality approaching 80% within 1 year following diagnosis (10).

One of the common genetic aberrations found in neuroblastoma and glioma is 19q13 loss of heterozygosity (3, 9, 11–16). The heterozygous deletion of 19q13 is particularly characteristic of aggressive neuroblastoma, especially in local-regional recurrent neuroblastoma (9). For gliomas, 19q13 deletions are the only known common genetic alterations shared by all pathologic types (11, 13); gross deletions occur in ~75% of oligodendrogliomas, 45% of mixed oligoastrocytomas, and 40% of astrocytomas (15). Furthermore, 19q13 allelic loss is associated with malignant progression and a poorer prognosis in astrocytomas (14, 16). All these observations suggest that 19q13 harbors at least one tumor suppressor gene that is important for the development of human primary gliomas and neuroblastomas. However, this putative tumor suppressor gene (or genes) remains unidentified. In this study, we have found that the myelin-related gene epithelial membrane protein 3 (EMP3), located in the critical 19q13.3 region, undergoes CpG island promoter-associated silencing in neuroblastomas and gliomas and that their reintroduction into cancer-deficient cell lines has growth inhibitory effects. Furthermore, the presence of EMP3 CpG island hypermethylation in human primary neuroblastomas is associated with a poor prognosis in these patients. Thus, our findings imply that EMP3 is a likely candidate for the long-sought tumor suppressor gene in the 19q13 chromosomal region.

Requests for reprints: Manel Esteller, Cancer Epigenetics Laboratory, Spanish National Cancer Centre, Melchor Fernandez Almagro 3, 28029 Madrid, Spain. Phone: 34-912328000; Fax: 34-91246923; E-mail: mesteller@cnio.es.

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Materials and Methods

Cell lines and tumor samples. Nine neuroblasticoma, one glioma, and one medulloblastoma cancer cell lines were studied. The neuroblasticoma lined used were: LAI-SS (MYCN amplified, S type), LAI-SSN (MYCN amplified, N type), LAN-1 (MYCN amplified, N type), SHIN (MYCN nonamplified, I type), SH-EP1 (MYCN nonamplified, I type), and SK-N-BE(2) (a I-type, MYCN-amplified variant of neuroblasticoma established from a relapse tumor sample) kindly provided by R. Ross and B. Spengler of Fordham University (Fordham, NY); SK-N-JD (MYCN amplified, I type) derived in one of our laboratories (Nai-Kong V. Cheung, Memorial Sloan-Kettering Cancer Center, New York, NY), and finally, SK-N-AS (MYCN nonamplified, S type, derived from a bone marrow metastasis) and IMR-32 (N-type and MYCN-amplified, derived from a primary tumor) were purchased from the American Type Culture Collection (Manassas, VA). "N," "I," and "S" stands for neuroblastic, undifferentiated, and Schwannian cell types, respectively. The glioma cell line used was U-87 and the medulloblastoma cell line D283 was also analyzed. Cells were cultured and passaged in RPMI or DME-HG with 10% FCS.

For human primary tumors, we first analyzed 89 neuroblastoma tumors (41 SS stage I, 13 stage II, 10 stage III, 52 stage IV, and 10 stage IVS) and 10 benign ganglioneuromas by using Affymetrix GeneChip Human Genome U95 Set oligonucleotide arrays (62,839 probe sets). For the EMP3 methylation studies, we used DNA from samples corresponding to 116 neuroblastic primary tumors and 41 gliomas obtained at the time of surgery and immediately frozen in liquid nitrogen. The Institutional Review Boards at the Memorial Sloan-Kettering Cancer Center and the Spanish National Cancer Centre approved this research. The neuroblastic tumors corresponded to five ganglioneuromas, six stage IVS, seven stage I, 16 stage II, 16 stage III, and 66 stage IV neuroblastoma tumors. At the mean follow-up time of 76.5 months, 73 patients were alive and 43 patients had died of the disease. Histologic sections of the frozen samples were reviewed by a pathologist, and the areas of high tumor content (>70%) were manually dissected.

Microarray gene expression analysis. Total RNA corresponding to the tumors and cell lines was extracted by using TRizol reagent (Life Technologies, Inc., Gaithersburg, MD) and purified with the Qiagen RNeasy System (Qiagen, Mississauga, Ontario, Canada), according to the manufacturer's recommendations. Quality and integrity of the RNAs was verified by checking 28S and 18S rRNA after ethidium bromide staining of total RNA samples on 1.2% agarose gel electrophoresis. Total cDNA was synthesized in vitro with 2 μg of RNA using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD). Two micrograms of RNA were reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and amplified using specific primers for EMP3 5'-CTTACTGTTTTTGTGGCCAC-3' (forward) and 5'-GATGAGAGAGAGAGAGAGAGAGAGAGGAGGAGGAC-3' (reverse). PCR was done for 35 cycles (94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds) in a final volume of 25 μL containing 1× PCR buffer (Life Technologies, Gaithersburg, MD), 1.5 mM MgCl₂, 0.3 mM/L deoxynucleotide triphosphate, 0.25 mM/L of each primer, and 2 units Taq polymerase (Life Technologies, Gaithersburg, MD). Reverse transcriptase-PCR (RT-PCR) primers were designed between exons 1 and 3, encompassing large introns to avoid any amplification of genomic DNA. Glyceroldehydes 3-phosphate dehydrogenase was used as an internal control to ensure cDNA quality and loading accuracy. The amplification products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The cell lines were treated with 2 μg/mL 5-aza-2’-deoxycytidine (Sigma, St. Louis, MO) for 3 days to achieve demethylation, as previously described (18).

EMP3 transfection and colony formation assay. The EMP3 expression vector pCMV-EMP3 was constructed by cloning the cDNA corresponding to the gene EMP3 in pCMV/4sg3A vectors (Strategene, La Jolla, CA) under the control of a cytomegalovirus (CMV) immediate-early promoter. Primers used for amplification of the four exons of the EMP3 gene from total RNA of the EMP3-expressing neuroblastoma cell line SK-N-AS were 5'-GGCGGATCCGATGAGATGAGATGAGAAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
GAPDH expression as loading control.

...cell line SK-N-JD as a positive control for EMP3 expression.

CpG dinucleotides (short vertical lines). Location of bisulfite genomic sequencing PCR primers (black arrows) and methylation-specific PCR primers (white arrows). Results of bisulfite genomic sequencing of 12 individual clones for five human cancer cell lines and normal lymphocytes (NL). Presence of a methylated cytosine (black square) and presence of an unmethylated cytosines (white square).

A, analysis of EMP3 CpG island promoter methylation status in human cancer cell lines. Top, schematic depiction of the EMP3 CpG island around the transcription start sites (long black arrows). EMP3 unmethylated (in vitro methylated DNA (IVD) are used as negative and positive control for unmethylated and methylated genes, respectively. Lane M, methylated genes; lane U, unmethylated genes. Normal lymphocytes (NL) and EMP3 unmethylated cell line SK-N-JD as a positive control for EMP3 expression. GAPDH expression as loading control.

The right flank was always used for EMP3-transfected LAN-1 cells and the left for empty vector LAN-1 control cells. Tumor development at the site of injection was evaluated daily. Animals were sacrificed at 30 days. The tumors were then excised and weighed.

Statistical analysis. For the analysis of the microarray data, average expression corresponding to neuroblastomas or ganglioneuromas was calculated for each probe set. The mean expression ratio was then calculated by dividing the mean expression of ganglioneuroma by that of neuroblastoma. The Mann-Whitney test was used to identify probe sets that were differentially expressed in the compared groups. Fisher’s exact test was used to examine the association of EMP3 CpG island methylation and clinical subgroups. The Kaplan-Meier log-rank test was used to determine the association between methylation and survival in primary tumors. Correlation between the methylation status of EMP3 and survival was estimated by Kendall’s τ. Values of \( P < 0.05 \) were considered significant for all tests.

Results

EMP3 gene expression is down-regulated in primary neuroblastoma tumors. Our goal was to find a candidate gene located in the neuroblastoma and glioma critical 19q13 region undergoing methylation-associated silencing. To this end, we first studied the microarray expression pattern of 89 neuroblastoma tumors (4 INSS stage I, 13 stage II, 10 stage III, 52 stage IV, and 10 stage IVS) and 10 ganglioneuromas by using Affymetrix Genechip Human Genome U95 Set oligonucleotide arrays (62,839 probe sets), looking for underexpressed genes, only six were located at the 19q13 chromosomal locus: EMP3, related ras viral oncogene homologue (RRAS), Fc fragment of the IgG receptor transporter (FCGRT), NR1H1, nucleobindin 1 (NUCB1), nuclear receptor subfamily 1 group H member 2 (NR1H2), and selenoprotein W1 (SEPW1). Most importantly, the EMP3 gene was the only one with over 3-fold down-regulation that contained a CpG island. The EMP3 gene is a member of the peripheral myelin protein 22 family (19), encoding a four-transmembrane domain protein involved in cell proliferation and cell-cell interactions (19, 20) and has been mapped at the critical region 19q13.3 (21).

EMP3 CpG island hypermethylation in neuroblastoma and glioma cell lines and its association with transcriptional gene silencing. EMP3 is a candidate gene for hypermethylation-associated inactivation in human cancer because a 5′-CpG island is located around the transcription start site of each gene (Fig. 1A). To analyze the methylation status of the promoter-associated CpG island of EMP3, we screened nine neuroblastoma and two glioma cell lines using bisulfite genomic sequencing and methylation-specific PCR targeted to the area surrounding its transcription start site, as described in Materials and Methods. EMP3 CpG island promoter hypermethylation was found in three neuroblastoma cell lines, SK-N-BE(2)C, IMR-32, and LAN-1 and one glioma cell line, U-87. The remaining neuroblastoma and medulloblastoma cell lines, LAI-5S, LAI-55N, SHIN, SH-EP1, SK-N-JD, SK-N-AS, and D283, revealed 637 transcripts in neuroblastoma that were significantly down-regulated relative to benign tumor ganglioneuromas. Of these underexpressed sequence genes, only six were located at the 19q13 chromosomal locus: EMP3, related ras viral oncogene homologue (RRAS), Fc fragment of the IgG receptor transporter (FCGRT), NR1H1, nucleobindin 1 (NUCB1), nuclear receptor subfamily 1 group H member 2 (NR1H2), and selenoprotein W1 (SEPW1). Most importantly, the EMP3 gene was the only one with over 3-fold down-regulation that contained a CpG island. The EMP3 gene is a member of the peripheral myelin protein 22 family (19), encoding a four-transmembrane domain protein involved in cell proliferation and cell-cell interactions (19, 20) and has been mapped at the critical region 19q13.3 (21).
were unmethylated at the EMP3 CpG island. All normal tissues analyzed, including lymphocytes and adrenal medulla, were completely unmethylated at the EMP3 CpG island promoter. These results were confirmed by bisulfite genomic sequencing and methylation-specific PCR (Fig. 1A and B).

Having noted EMP3 promoter hypermethylation in the described cancer cell lines, we assessed the association between this epigenetic aberration and the putative transcriptional inactivation of the EMP3 gene at the RNA level. The neuroblastoma and glioma cell lines SK-N-BE(2)C, IMR-32, LAN-1, and U-87, hypermethylated at the EMP3 CpG island, did not express any EMP3 RNA transcript, as determined by RT-PCR (Fig. 1C). However, the remaining neuroblastoma and glioma cell lines and normal lymphocytes, unmethylated at the EMP3 promoter, strongly expressed EMP3 (Fig. 1C). We established a further link between EMP3 CpG island hypermethylation and its gene silencing by the treatment of the methylated neuroblastoma and glioma cell lines with a DNA demethylating agent. The treatment of the IMR-32, LAN-1, and U-87 cell lines with the demethylating drug, 5-aza-2’-deoxycytidine, restored expression of the EMP3 RNA transcript (Fig. 1C).

Reintroduction of EMP3 in deficient neuroblastoma cell lines has tumor suppressor-like properties in colony formation assays and mouse tumor xenografts. Although the potential features of EMP3 in cell proliferation have been proposed before, we assayed the ability of EMP3 to function as a suppressor of tumor growth in our model, using IMR-32 and LAN-1, two neuroblastoma cell lines with EMP3 methylation-associated silencing.

We first tested the inhibitory abilities of EMP3 in IMR-32 cells using a colony-focus assay with G418 selection after transfection with the EMP3 gene (pCMV-EMP3) or the empty vector, as described in Materials and Methods. EMP3 expression was monitored by RT-PCR. EMP3 reexpression in IMR-32 cells showed tumor-suppressing activity with markedly less colony formation than with the empty vector (Fig. 2A). An average of 11 colonies formed when the gene EMP3 was transfected, compared with an average of 78 cell colonies formed in the control empty vector–transfected cells (Mann-Whitney test, \( P < 0.001 \); Fig. 2B).

Additionally, as internal controls, we used vectors for the bonafide tumor suppressor gene \( p16^{INK4a} \) and an inactive, mutant form of this gene in the transfection assays. An average of five colonies grew after transfection of the tumor suppressor gene \( p16^{INK4a} \), whereas 60 colonies appeared when IMR-32 cells were transfected with the mutant form of the gene (Fig. 2B). This implies that the growth inhibitory effect of EMP3 transfection is similar to that of \( p16^{INK4a} \) transfection.

We next tested the ability of the other EMP3-methylated neuroblastoma cell line, LAN-1, to form tumors in nude mice
EMP3 is hypermethylated in primary neuroblastomas and gliomas and is a predictor of poor outcome. Once the functional consequences of EMP3 CpG island hypermethylation had been determined, we considered the relevance of EMP3 methylation in human primary tumors by studying a large set of human primary neuroblastomas and gliomas. EMP3 CpG island hypermethylation was found in 24.1% of neuroblastomas (28 of 116) and in 39% of gliomas (16 of 41; Fig. 3A). For gliomas, EMP3 hypermethylation was independent of the age of onset and sex of the patient and the histologic type, being present in glioblastomas and anaplastic astrocytomas. We had previously characterized neuroblastoma tumors for loss of heterozygosity at 19q13, 1p36, 1p22, and 11q, gain of 17q and MYCN amplification (9, 22–24), all of which are putative molecular prognostic factors for this tumor type. Most importantly, the only genetic defect associated with EMP3 hypermethylation was the loss of heterozygosity at 19q13.3 (Fisher’s exact test, \( P = 0.004 \)), which is the exact chromosomal region where EMP3 is located, once again suggesting a role for EMP3 as the putative tumor suppressor gene confined to this locus.

We next determined whether or not there was a relationship between the hypermethylation status of the gene EMP3 and the outcome of neuroblastoma patients (this clinical information was not available for the gliomas). First, we confirmed that our studied cases were representative of the general population of neuroblastic tumors. Indeed, noninfant patients of the cohort had poorer survival than infant patients (Kaplan-Meier log-rank, \( P = 0.006 \); Fig. 3B) and patients with MYCN-amplified tumors had poorer survival than patients without MYCN amplification (Kaplan-Meier log-rank, \( P < 0.001 \); Fig. 3B), as expected (1, 5, 25, 26). With respect to EMP3, we found that EMP3 promoter-associated CpG island hypermethylation was significantly associated with death of disease (Kendall \( \tau, P = 0.030; r = 0.190 \); Fig. 3B). In this context, mortality was higher in the group of tumors with methylated EMP3 than in the group with the unmethylated gene (53.6% deceased cases when EMP3 was methylated versus 31.8% when the gene was unmethylated). This association was also found when the cases were stratified by stage: for both local-regional and metastatic neuroblastomas, the percentage of deceased patients was higher in tumors with methylated EMP3 (33.3% for local-regional and 78.6% for stage IV) than in tumors with unmethylated EMP3 (13.3% for local-regional and 46.1% for stage IV). Furthermore, EMP3 promoter hypermethylation was significantly associated with poor survival in patients that remained alive after 2 years follow-up (Kaplan-Meier, \( P = 0.030 \)) for all stages (Fig. 3B), and for all stage IV patients (Kaplan-Meier, \( P = 0.030 \); Fig. 3B). Thus, EMP3 CpG island hypermethylation is a likely predictor of poor outcome in neuroblastoma patients.

**Discussion**

Loss of 19q13 is commonly found in human malignant gliomas (1, 3, 11–16) and neuroblastomas (9, 23, 27) associated with a specific clinical behavior and survival rate for both tumor types. Indeed, our own previous data indicated an association between specific 19q13.3 loss of heterozygosity and progressing neuroblastoma tumors with poor survival and lack of response to standard cytotoxic therapy (9). Thus, it has been of considerable interest to identify the putative tumor suppressor gene (or genes) from this candidate region of the long arm of chromosome 19 (27–29). So far, the numerous studies based on classic genetic techniques have failed to localize the tumor suppressor gene of the 19q13 region. In the present study, we adopted a double strategy to address this matter, combining the use of genetic expression approaches using microarrays and epigenetic analysis of CpG island hypermethylation. A comprehensive expression analysis in a large cohort of neuroblastic tumors revealed that only one gene located at the critical region 19q13.3 was consistently down-regulated in neuroblastomas, the myelin-related gene EMP3.

EMP3 belongs to the peripheral myelin protein 22-kDa (PMP22) gene family (also known as the TMP gene family), which includes four closely related members, PMP22, EMP3, EMP2, and EMP1, and an additional, more distant, member, MP20 (19, 20). The EMP3 gene encodes a 163-amino acid protein of 18 kDa containing four transmembrane domains and two N-linked glycosylation sites in the first extracellular loop. The EMP3 protein sequence is similar to that of PMP22, EMP1, and EMP2 in 33% to 43% of amino acids, with the greatest homology found in the transmembrane domains. PMP22 is the best-characterized member of the family. It is a major component of myelin and is produced predominantly by Schwann cells (30). PMP22 is most highly expressed in peripheral nerves, where it plays a crucial role in physiologic and pathologic processes in the peripheral nervous system. Genetic alterations in the PMP22 gene, including duplications, deletions, and point mutations, are responsible for several forms of hereditary peripheral neuropathies, including Charcot-Marie-Tooth disease type 1A, Dejerine-Sottas syndrome, and hereditary neuropathy with liability to pressure palsies (30). In contrast, the functions of the product of the gene EMP3 are not fully known. However, based on the homology with PMP22, which is strongly expressed during growth arrest (20), EMP3 is thought to be involved in cell proliferation and cell-cell interactions (19, 20). Our demonstration that EMP3 reexpression in deficient cancer cells reduces colony formation and tumor growth in xenografts also implies a tumor suppressor function for the EMP3 gene.

Transcriptional inactivation of tumor suppressor genes by associated promoter CpG island hypermethylation is now recognized as a common feature of human tumors (31–33). Hypermethylation of these loci is known to give rise to particular profiles according to the tumor type (34, 35). For gliomas, several genes are known to undergo this epigenetic alteration, including the DNA repair gene, MGMT, and the cell cycle inhibitor, p16\(^{INK4a} \) (36–38), whereas for neuroblastoma CpG island hypermethylation, among others, have been described for the tumor necrosis factor receptors (39) and the developmental gene HOXA9 (7). In the particular case of neuroblastic tumors, a particular clustering of CpG island hypermethylation may allow...
the classification of these patients into clinically relevant subgroups (7). However, only one other gene located at 19q13 has been reported to have methylation-associated silencing in a subtype of glioma (oligodendroglioma), the ZNF342 gene that encodes a putative zinc finger protein (40). This is a very interesting observation and suggests that the high rate of loss of heterozygosity observed in these tumors could be related to the presence not only of one putative tumor suppressor gene but at least two: ZNF342 and EMP3.

Finally, from a clinical standpoint, it is worth emphasizing the relevance of EMP3 hypermethylation as a marker of poor outcome in neuroblastoma patients. The analysis of the cell lines provided the first indication of this: EMP3 hypermethylation was restricted to those neuroblastoma cell lines of great malignant potential, with a high proliferation rate, MYCN gene amplification and belonging to the undifferentiated (I) or neuroblastic (N) cell types, whereas none of the less malignant cell lines with the Schwannian cell-type phenotype (S) or without MYCN amplification had EMP3 hypermethylation. Most importantly, in the primary neuroblastoma tumors, EMP3 hypermethylation is one of the first markers to predict poor prognosis after the first 2 years of onset of the disease. This finding is especially significant because most of the currently available clinical factors and molecular markers of poor prognosis in neuroblastoma (such as MYCN amplification, advanced age or stage, and diploidy), are associated with a rapid tumor progression (2) and an increased short-term mortality, and currently it is difficult to predict the outcome of patients lacking these markers of poor prognosis after 2 years of the diagnosis.

In conclusion, our findings indicate a role for CpG island hypermethylation-silencing of the myelin-related gene EMP3 in the development of neuroblastomas and gliomas. EMP3 also exhibits tumor suppressor features and is located in the 19q13 critical region of loss of heterozygosity in both tumor types. Furthermore, for neuroblastomas, the presence of EMP3 hypermethylation is a prognostic factor of poor outcome in these patients. These observations warrant further research to elucidate EMP3 function in normal cells and dysfunction in cancer cells.

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EMP3, a Myelin-Related Gene Located in the Critical 19q13.3 Region, Is Epigenetically Silenced and Exhibits Features of a Candidate Tumor Suppressor in Glioma and Neuroblastoma

Miguel Alaminos, Verónica Dávalos, Santiago Ropero, et al.


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