

**CASE REPORT****Genetic Alterations of E-cadherin and Beta-Catenin in Germinoma and Teratoma: Report of Two Central Nervous System Cases**Nives PEĆINA-ŠLAUS,<sup>1,2</sup> Tamara NIKU EVA-MARTIĆ,<sup>1,2</sup> Vili BEROŠ,<sup>3</sup> Davor TOMAS<sup>4</sup><sup>1</sup>Laboratory of Neurooncology, Croatian Institute for Brain Research, School of Medicine, University of Zagreb,<sup>2</sup>Department of Biology, School of Medicine, University of Zagreb, <sup>3</sup>Department of Neurosurgery, University Hospital "Sisters of Charity", <sup>4</sup>Ljudevit Jurak Department of Pathology, University Hospital "Sisters of Charity", Zagreb, Croatia

The genetic basis as well as mechanisms of development of germ cell tumors of the CNS are still unexplained. In the present article changes of E-cadherin (CDH1) and beta-catenin (CTNNB1) genes in two CNS germ cell tumors are reported. Both gene products are components of adherens junctions, but are also involved in the Wnt signaling pathway. A case of germinoma of the central nervous system and a case of spinal channel teratoma were tested for loss of heterozygosity (LOH) of E-cadherin gene by PCR amplification of tetranucleotide polymorphism (D16S752). Changes of beta-catenin were tested by heteroduplex

method. Both germ cell tumors analyzed demonstrated LOH of the CDH1 gene. Analysis of exon 3 of the CTNNB1 gene showed additional band in the germinoma, suggesting that this sample harbors mutation in the beta-catenin gene. Immunostaining showed that LOHs in our samples were accompanied with the absence of E-cadherin protein. We also investigated E-cadherin expression in four other germinomas, of which three were negative and one was mildly positive. Our findings may contribute to better understanding of the genetic profile of germ cell tumors. (Pathology Oncology Research Vol 13, No 4, 370–374)

*Key words:* germinoma, teratoma, E-cadherin gene (CDH1), beta-catenin gene (CTNNB1), Wnt signaling pathway

**Introduction**

Primary germ cell tumors (GCTs) of the central nervous system (CNS) are rare neoplasms that occur predominantly in children and adolescents. It has been suggested that GCTs arise from developmental nests of primitive germ cells in midline structures, including the pineal and suprasellar regions of the brain.<sup>19,20</sup> GCTs consist of a variety of histologic entities including germinoma, teratoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma and mixed germ cell tumor types.<sup>19</sup> The genetic basis of germinoma as well as teratoma is

unclear, and much work is still required to determine the final list of genes involved. With this in mind we investigated two genes, molecular components of the Wnt signaling pathway, E-cadherin (CDH1) and beta-catenin (CTNNB1).

E-cadherin is a glycoprotein that functions as a cell-to-cell adhesion molecule, interacting with groups of proteins called catenins.<sup>9,14</sup> Deletion of the intracellular catenin-binding domain of E-cadherin or alterations in the functionally active catenins results in the loss of its ability to promote cell adhesion.<sup>17</sup> Beta-catenin plays important roles in both cell-cell interactions<sup>18</sup> and transcriptional regulation in the Wnt signaling.<sup>13</sup> Failure to properly degrade beta-catenin results in beta-catenin accumulation and migration to the nucleus where it can up-regulate transcription of a number of growth-promoting genes, including c-myc and cyclin D1.<sup>6</sup>

Our interest in genes of the Wnt pathway in the formation of GCTs stemmed from several findings. Wnt proteins regulate critical developmental processes of normal

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brain development.<sup>11,15</sup> Beta-catenin was identified as a critical factor for dendritic morphogenesis.<sup>24</sup> Mutations of beta-catenin gene have been reported in sporadic medulloblastoma.<sup>4,23</sup> Finally, it has been well documented that E-cadherin and beta-catenin are implicated in cancer.<sup>10,16</sup>

### Materials and methods

#### Tumor specimens and DNA extraction

A case of germinoma of the CNS and corresponding blood sample were obtained from a 15-year-old male patient admitted to University Hospital Sisters of Charity, Zagreb. Using magnetic resonance imaging (MRI) we found the lesion in the pineal region. Another single case of spinal channel teratoma and corresponding blood sample were obtained from a 43-year-old female patient. By MRI we found a tumor lesion at level CI-CII. Neither of the patients had undergone chemotherapy or radiotherapy prior to surgery. The local ethical committee approved our study, and the patients gave informed consent.

Genomic DNA was isolated from unfixed frozen tumor samples and peripheral blood leukocytes by standard methods.

#### Polymerase chain reaction

The D16S752 polymorphic region (GATA tetranucleotide repeat) linked to the E-cadherin gene was amplified in a volume of 25  $\mu$ l: 5 pmol of each primer (5'-AATTGACGGTATATCTATCTGTCTG-3' and 5'-GATTGGAGGAGGGTGATTCT-3'), 200 ng DNA, 2.5  $\mu$ l PCR buffer, 1.5 mM MgCl<sub>2</sub>, 2.5 mM of each dNTP, 0.5 U Taq polymerase (Eppendorf, Germany).

The reaction mixture (25  $\mu$ l) for CTNNB1's exon 3 amplification was: 10 pmol of each primer (5'-CCA ATC TAC TAA TGC TAA TAC TG-3' and 5'-CTG CAT TCT GAC TTT CAG TAA GG -3'), 200–400 ng template DNA, 2.5  $\mu$ l PCR buffer, 2.5 mM MgCl<sub>2</sub>, 2.5 mM of each dNTP, 0.5 U Taq polymerase.

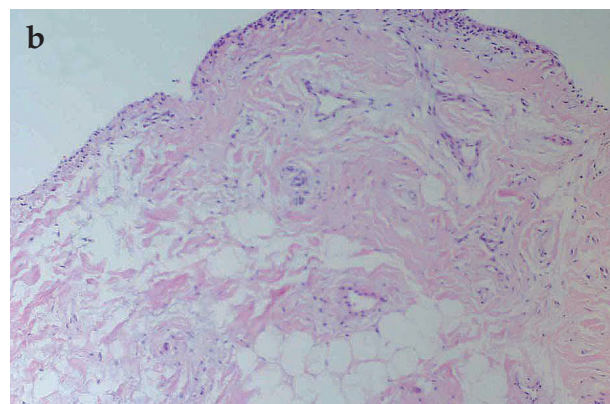
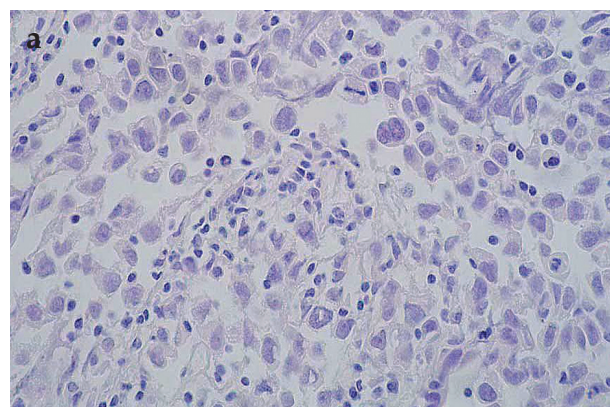
PCR conditions: initial denaturation, 3 min/96°C; denaturation, 30 s/96°C; annealing, 35 s/55°C; extension, 30+1 s/72°C; final extension, 72°C/10 min; 35 cycles. PCR products were analyzed on 2% agarose gels.

#### Loss of heterozygosity

To discover LOH of the E-cadherin gene, D16S752 heterozygous samples were visualized on Spreadex EL 300 Mini gels (Elchrom Scientific, Switzerland) stained with SyberGold (Molecular Probes, Netherlands). We performed analysis on Spreadex gel and confirmed it on 13% polyacrylamide gel. Absence or significant decrease of one allelic band in the tumor compared with autologous blood sample was considered as LOH of CDH1 gene.

#### Heteroduplex analysis

Exon 3 of the CTNNB1 gene was screened for mutations. Heteroduplexes were formed by heating 3  $\mu$ l of PCR products (tumor mixed with normal DNA) at 95°C for 3 min, followed by incubation on ice for 20 min. About 3  $\mu$ l



**Figure 1.** (a) Germinoma. Tumor cells with abundant clear cytoplasm, vesicular nuclei and visible nucleoli admixed with lymphocytes (H&E, 400x). (b) Teratoma. Tumor tissue composed of vascularized connective and mature fat tissue covered with few layers of epithelial cells (H&E, 100x) and (c) moderately cellular, incompletely mature cartilage (H&E, 200x)

of each sample was mixed with 7  $\mu$ l of mixture of formamide and 10 mM NaOH (1:100) prior to loading to a gel. Electrophoresis was performed on the GMA gels in the SEA 2000 submarine electrophoresis apparatus (Elchrom Scientific). The temperature of the running buffer was kept at 9°C.

#### Immunohistochemistry

Immunohistochemistry was performed in order to establish the level of protein expression of E-cadherin. The samples were formalin-fixed, paraffin-embedded, and 4- $\mu$ m-thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). The sections were immunostained using the biotin-avidin-horseradish peroxidase method. Deparaffinized and rehydrated sections were microwaved in Dako Target Retrieval Solution (Dako Corporation, USA) three times for 5 min at 800 W to unmask epitopes. To block endogenous peroxidase activity, we fixed the cells in methanol containing 3% H<sub>2</sub>O<sub>2</sub>. Non-specific binding was blocked by the application of normal mouse serum for 30 min in a humid chamber. Slides were blotted and the primary antibody at optimized dilution of 1:100 was applied for 30 min at room temperature. The antibody used was monoclonal mouse anti-human E-cadherin NCH-38 (Dako Corporation). After incubation, the slides were washed three times in phosphate-buffered saline/goat serum. Secondary LINK antibody was applied for 25 min. The washing was repeated, and the slides were incubated with streptavidin horseradish peroxidase for another 25 min. All chemicals were from DakoCytomation. Negative control in the experiment was a sample that underwent the same staining procedure with the exclusion of the primary antibody. Normal skin and normal fetal brain served as positive internal controls. The analysis of the labeling was performed on an Olympus BH-2 microscope by two independent observers, i.e. blinded pathologists, experts in the field.

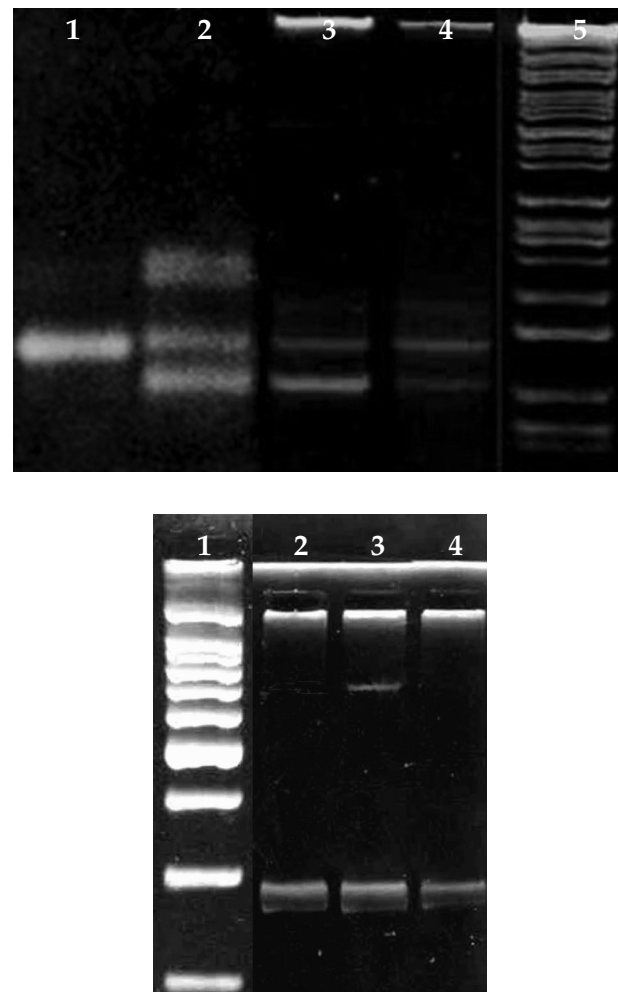
#### Results and Discussion

Tumors were studied by pathologists and classified according to the WHO criteria.<sup>19</sup> Microscopically, the germinoma was composed of sheets and lobules of relatively uniform cells with large vesicular nuclei, visible nucleoli and a clear cytoplasm admixed with small lymphocytes (Fig. 1a). Tumor cells showed up to 4 mitoses per 10 high-power fields and immunohistochemically positive reaction for placental alkaline phosphatase (PLAP).

During operative procedure many small samples of the teratoma measuring between 0.2 and 0.6 cm were submitted for frozen sections analysis. The frozen sections showed mature fat tissue and smooth muscle bundles, small samples of vascularized connective and mature fat tissue covered with one or few layers of epithelial cells

(Fig. 1b), and poorly to moderately cellular incompletely mature cartilage (Fig. 1c). Despite its bland histology, incompletely mature cartilage was suggestive of aggressive behavior.

The analysis of both our probands' blood samples for marker D16S752 showed that patients were heterozygous for this polymorphism. Moreover, the germinoma and teratoma both demonstrated LOH of the CDH1 gene as shown in Fig. 2a. This finding led us to conclude that both samples comprised gross deletion of the E-cadherin gene.



**Figure 2.** (a) Loss of heterozygosity (LOH) at CDH1 in a 15-year-old-patient with CNS germinoma and in a 43-year-old patient with CNS teratoma. Polymorphic marker D16S752 shown on Spreadex gel stained with Sybergold. Lane 1, LOH of the E-cadherin in germinoma; lane 2, corresponding blood sample; lane 3, informative blood sample; lane 4, LOH of the E-cadherin gene in teratoma; lane 5, M3 standard. (b) Heteroduplex analysis of the germinoma sample. Exon 3 of the CTNNB1 gene was screened for mutations on GMA gels. Lane 1, M3 standard; lane 2, corresponding blood DNA sample; lane 3, additional bands showing heteroduplexes when tumor and normal sample are mixed; lane 4, tumor DNA

The germinoma case we analyzed showed yet another alteration. Analysis of exon 3 of CTNNB1 gene showed an additional band when the tumor and normal blood DNA were mixed, suggesting that the tumor sample harbors mutation in the CTNNB1 gene (Fig. 2b).

The germinoma and teratoma in which we detected LOH of the E-cadherin gene were negative for E-cadherin protein expression, indicating that gross deletions were accompanied with the absence of the protein. We also investigated E-cadherin expression in four other CNS germinoma samples, of which three were also negative, while one showed mildly positive immunostaining detectable diffusely in the cytoplasm (Fig. 3).

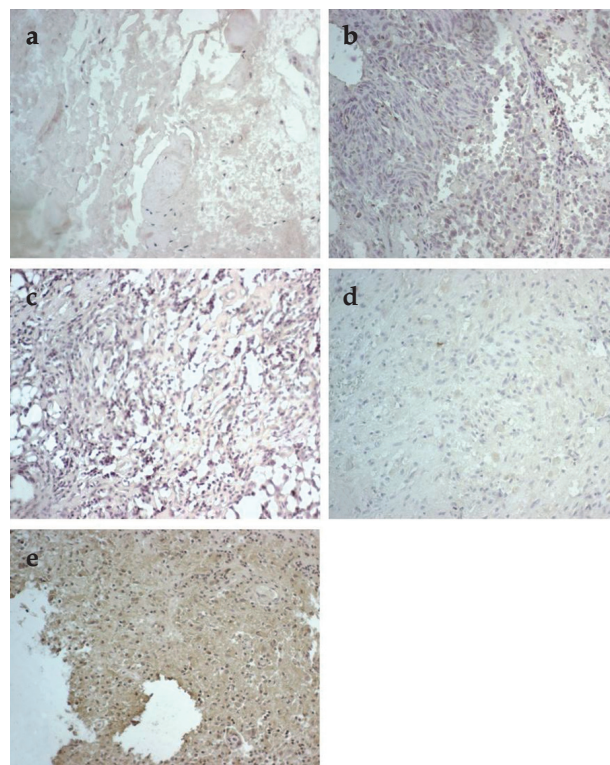
Our knowledge on the genetic background of specific histopathologic types of CNS germ cell tumors still needs to be extended, although great progress has been achieved along with the advances in molecular genetics.<sup>3</sup> Novel molecules are being proposed as diagnostic tools<sup>12,21</sup> and responsible signaling pathways are being identified. It is still questionable whether the molecular etiology of GCT of the CNS is similar to other types of brain tumors or to GCTs at other sites. The established belief of common origin of GCT of the brain and those that occur in the gonads is nowadays questioned, since recent evidence demonstrated that neural stem cells can also give rise to many different cell types. Scotting<sup>22</sup> argues in favor of endogenous neural progenitor cells as likely origins of GCT of the CNS.

On the other hand, there are views<sup>1</sup> that GCTs arise due to acquired genetic instability, a consequence of dysregulation of normal mitotic and meiotic control, rather than being dependent upon dysregulation of specific signaling pathway.

One of the most important hallmarks of malignant tumors is their invasive behavior. The invasive phenotype of human tumors is thought to be associated to the cadherin group of adhesion molecules. Although E-cadherin molecule is a well-known suppressor of invasion, little is known on the role of cell-cell adhesion and cellular migration in GCTs.

Mature teratomas are composed of fully differentiated tissue elements with low mitotic activity and are considered as the benign forms. Immature teratomas, on the other hand, are aggressive tumors having primitive, undifferentiated components resembling “fetal” tissues which tend to recur frequently.<sup>2,7</sup> Even though our teratoma case was characterized as benign, some elements indicated that it may conceal a more aggressive phenotype. There is evidence that E-cadherin expression is absent from fetal germ cells of the second and third semester.<sup>8</sup> A study by Honecker et al<sup>8</sup> also tested E-cadherin in germ cell neoplasia. Although they did not investigate tumors of the CNS, their finding that E-cadherin was not expressed in GCTs supports our results.

Our findings on changes of CDH1 and CTNNB1 genes in CNS cases may contribute to better understanding of the



**Figure 3.** Teratoma and germinoma samples immunohistochemically stained for protein expression of E-cadherin. Teratoma (a) and germinoma samples (b) with LOHs negative for E-cadherin expression. Additionally analyzed germinoma samples negative for E-cadherin (c, d) and one mildly positive sample (e)

genetic profile of germinoma and teratoma. These findings are in accordance to a recent article by Fritsch et al<sup>5</sup> who demonstrated activation of Wnt signaling in immature teratomas and endodermal sinus tumors by microarray analysis.

Further investigation regarding these genes on a larger sample of GCTs should be performed in the future to confirm our suggestion.

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