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Changes of AXIN-1 and Beta-Catenin in Neuroepithelial Brain Tumors

Tamara Nikuševa Martić · Nives Pećina-Šlaus · Vesna Kušec · Tomislav Kokotović · Hana Mušinović · Davor Tomas · Martina Zeljko

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Abstract In the present study changes of components of Wnt signaling pathway—axin (AXIN1) and beta-catenin (CTNNB1) in a sample of 72 neuroepithelial brain tumors were investigated. AXIN-1 gene was tested by PCR/loss of heterozygosity (LOH). Immunostaining and image analysis revealed the quantity and localization of relevant proteins. Polymorphic marker for AXIN-1, showed LOH in 11.1% of tumors. LOH was distributed to 6.3% of glioblastomas, one was found in neuroepithelial dysembrioplastic tumor and one in medulloblastoma. Down regulation of axin expression and up regulation of beta-catenin were detected in the analyzed tumors. Axin was observed in the cytoplasm in 68.8% of samples, in 28.1% in both the cytoplasm and nucleus and 3.1% had no expression. Beta-catenin was observed mainly in the nucleus and cytoplasm (59.4%). Expression in 34.4% of samples was in the cytoplasm and 6.3% showed no expression. Comparison of mean values of relative increase of axin and beta-catenin showed that they are significantly reversely proportional (P=0.014). Relative quantity of beta-catenin in patients with gross deletion of AXIN1 was significantly higher in comparison to patients without LOH (P=0.040). Our results demonstrate that changes of key components of the Wnt signaling play a role in neuroepithelial brain tumors.

Keywords Axin · Beta-catenin · Image analysis · Loss of heterozygosity · Neuroepithelial brain tumors · Wnt signaling

T. Nikuševa Martić · N. Pećina-Šlaus (✉) · T. Kokotović · H. Mušinović · M. Zeljko
Laboratory of Neurooncology, Croatian Institute for Brain Research, School of Medicine University of Zagreb,
Šalata 12,
10000 Zagreb, Croatia
e-mail: nina@mef.hr

T. Nikuševa Martić · N. Pećina-Šlaus
Department of Biology, School of Medicine,
University of Zagreb,
Šalata 3,
10000 Zagreb, Croatia

V. Kušec
Clinical Institute of Laboratory Diagnosis,
Clinical Hospital Centre Zagreb,
Kišpatićeva 12,
10000 Zagreb, Croatia

D. Tomas
Ljudevit Jurak Department of Pathology,
University Hospital "Sisters of Charity",
Vinogradska 29,
10000 Zagreb, Croatia

Introduction

Neuroepithelial brain tumors are central nervous system (CNS) neoplasms that embody a series of primary brain tumors including astrocytic, oligodendroglial, ependymal, choroid plexus, pineal parenchymal and embryonal tumors [1]. Tumors of neuroepithelial origin account for approximately 49% of CNS tumors with glioblastomas comprising the majority at 23%. Intracranial tumors of neuroepithelial origin show distinct features that control their ontogeny, pattern of invasion, clinical outcome, and prognosis. These features may reflect the complexity of the molecular and genetic alterations in pathways involved in the onset, maintenance and progression of CNS tumors [2].

The Wnt signaling pathway plays an essential role in cancer development [3], and lately it has been shown in brain tumorigenesis as well [4].

Tumor suppressor gene AXIN-1 is an inhibitor of Wnt signaling. It down-regulates beta-catenin, pathway's main

signaling molecule, by facilitating its phosphorylation by glycogen synthase kinase (GSK)-3beta [5]. It binds directly to adenomatous polyposis coli (APC), beta-catenin, GSK-3beta and dishevelled [6]. In response to Wnt signaling, or under the circumstances of mutated axin or APC, beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus, where it stimulates the expression of target genes including c-myc, c-jun, fra-1 and cyclin D1 [3, 7].

There are several reasons why we propose studying axin and beta-catenin in neuroepithelial brain tumors. New knowledge on Wnt signaling shows that wnt proteins and other components of the Wnt signaling cascade, namely beta-catenin and axin, regulate critical developmental processes of normal CNS development [8–10]. Processes that include cellular adhesion, synaptic rearrangements, embryonic cell patterning, proliferation, differentiation, and apoptosis require the expression of molecular components of the Wnt pathway [11]. All these findings suggest that molecular components of Wnt signaling perform important functions in CNS tissues.

The molecular mechanisms and candidate genes involved in development and progression of neuroepithelial brain tumors still need investigation and elucidation. Our work offers two new candidates, axin and beta-catenin, to fill in the puzzle of genetic basis of human brain tumors.

Materials and Methods

Tumor Specimen

Samples of 72 neuroepithelial tumors of the brain and 72 autologous blood tissues were collected from the Department of Neurosurgery, and Department of Pathology University Hospital "Sisters of Charity", Zagreb, Croatia. Using magnetic resonance imaging (MRI), tumor lesions were found in different cerebral regions (predominantly in the temporal and parietal region), with the surrounding zone of perifocal oedema. During surgery, the tumor was removed using a micro neurosurgical technique. The patients had no family history of brain tumors, nor had they undergone chemotherapy or radiotherapy prior to surgery. Collected tumor tissues were frozen in liquid nitrogen and transported to the laboratory, where they were immediately transferred at −75°C. The peripheral blood samples were collected in EDTA and processed immediately. All tumors were studied by pathologists and classified according to the WHO criteria [1]. Thirty-four patients were female (47.2%), and 38 were male (52.8%). Patient age ranged from 13 to 80. The mean age at diagnosis for both females and males was 52.5 years.

The local Ethical Committee approved our study, and patients gave their informed consent.

DNA Extraction

Tumor samples for DNA isolation were evaluated by a neurosurgeon on the basis of macroscopic appearance, tissue color, density, and consistency on gross section. The sample was also evaluated for the percentage of tumor cells by pathologist and consisted of more than 85% of tumor cells. Approximately 0.5 g of tumor tissue was homogenized with 1 ml extraction buffer (10 mM Tris HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (100 μg/ml; Sigma, USA; overnight at 37°C). Phenol chloroform extraction and ethanol precipitation followed.

Blood was used to extract leukocyte DNA. Five ml of blood was lysed with 7 ml distilled water and centrifuged (15 min/5,000 *g*). The pellet was then processed as for DNA extraction from the tissue samples.

Polymerase Chain Reaction and Loss of Heterozygosity

The D16S521 polymorphic region (CA dinucleotide repeat) linked to the AXIN1 gene was amplified in a volume of 25 μl: 5 pmol of each primer (5′- GAGCGAGACTCCGT CTAAA-3′ and 5′-CAGCAGCCTCAGGGTT-3′), 200–400 ng DNA, 2.5 μl PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 mM $MgCl_2$, 2.5 mM of each dNTP, 5 U/μl Taq polymerase (Promega, USA). Polymerase chain reaction (PCR) conditions were initial denaturation, 2 min/95°C; denaturation, 45 s/95°C; annealing, 45 s/63°C; extension, 1 min/72°C; final extension, 72°C/10 min; 30 cycles. PCR products were analyzed on 2% agarose gels and minimal length of the repeat was 168 bp.

To discover loss of heterozygosity (LOH) of the AXIN1 heterozygous samples were visualized on Spreadex EL 300 Mini gels (Elchrom Scientific, Switzerland) stained with SyberGold (Molecular Probes, Netherlands) and on 15% polyacrylamide gels stained with silver. Absence or significant decrease of one allelic band in the tumor compared with autologous blood sample was considered as LOH of AXIN1 gene.

Immunohistochemistry

Immunohistochemistry was performed in order to establish the level of axin and beta-catenin protein expression. The samples were formalin-fixed, paraffin-embedded, and 4-μm thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). The sections were immunostained using the peroxidase-anti-peroxidase method as described previously [12]. The primary antibodies at optimized dilutions of 1:100 for axin and 1:200 for beta-catenin were applied for 30 min at room temperature. The antibodies used were rabbit polyclonal anti-human AXIN1 antibody (Zymed Laboratories, San Francisco, CA, USA)

and mouse anti-human beta-catenin monoclonal antibody (Dako Corporation, Carpinteria, USA). All chemicals were from DakoCytomation. Negative controls were samples that underwent same staining procedure with the exclusion of the primary antibodies. Cortex of the frontal part of the normal brain [13], as well as normal skin, served as positive controls. The analysis of the labeling was performed by two independent observers, *i.e*. blinded pathologists, experts in the field on an Olympus BH-2 microscope.

Image Analysis

Protein expression was then quantified with the aid of Image Analyzer. For each sample, the intensity of staining in a well-defined area was evaluated using image-analyzing software manufactured by Vamstec (Zagreb, Croatia). The region of interest was chosen as one representative field (200,000 μm^2) under × 100 magnification in the center of malignant tissue. All density measurements were "calibrated" against its slide characteristics, i.e. transparency. Density was depicted as the intensity of light retained by tissue or tissue transparency and expressed in grey scale pixels ranging from 0 to 222 for axin protein, zero representing no transmission of light and 222 total transparency and 0 to 495 for beta-catenin protein. Density in the area of tumor location was compared with density of the normal brain tissue sections.

Statistical Methods

All individuals were analyzed for the following features: PHD status, sex, age, AXIN1 LOH, AXIN1 and beta-catenin protein expression. Differences in the frequencies of the analyzed features were tested with the T-student test and Pearson's correlation when appropriate.

Results

The pathohistological status of analyzed neuroepithelial brain tumors was as follows: glioblastomas were seen in 66.6% of patients; astrocytomas in 12.5%; oligoastrocytomas in 4.16%; oligodendrogliomas in 5.5%; ganglioglioma, medulloblastoma and anaplastic ependymoma in 2.8% each; and one patient suffered from neuroepithelial dysembrioplastic tumor. The glioblastomas were considered primary because the diagnosis was made at the first biopsy, without clinical or histopathologic evidence of a less malignant precursor lesion.

Of 72 neuroepithelial brain tumor samples 62.5% were informative for D16S521 polymorphic dinucleotide marker. The results of our analysis showed LOH of AXIN-1 in 11.1% of total informative tumors. It was distributed to 6.3% of glioblastomas, 1 LOH was found in neuroepithelial dysembrioplastic tumor and one in medulloblastoma (Fig. 1).

Immunohistochemical method revealed localization and quantity of proteins axin and beta-catenin. Down regulation of axin expression was detected in 65.6% of our total sample when compared to the levels of axin in healthy brain tissues (glial cells and neurons). In 31% of glioblastomas, 22% of astrocytomas and all of the investigated oligodendrogliomas intensively lower expressions of axin were detected.

Axin was observed in the cytoplasm in 68.8% of samples, in 28.1% in both the cytoplasm and nucleus and 3.1% of neuroepithelial brain tumors had no expression. The majority of glioblastomas 69% had axin localized solely in the cytoplasm and the same localization was noted in 56% of astrocytomas.

Beta-catenin was observed mainly in the nucleus or cytoplasm and nucleus (59.4%). Expression in 34.4% of samples was in the cytoplasm and 6.3% showed no expression.

Higher levels of expression of beta-catenin were noted in 53.1% of our total tumor sample when compared to the levels of beta-catenin in healthy brain tissues.

In 21.4% of glioblastomas beta-catenin was accumulated exclusively in the nucleus, while in 38.1% of them the protein was localized both in the cytoplasm and nucleus. 33.3% of astrocytomas had the protein localized in the nucleus.

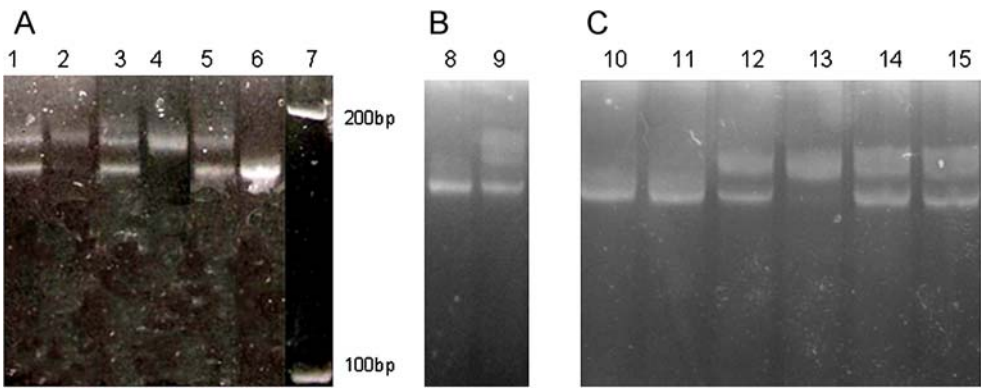

Fig. 1 **a** Loss of heterozygosity of AXIN-1 gene in 3 glioblastomas, lanes 2, 4 and 6; **b** loss of heterozygosity of AXIN-1 gene in neuroepithelial dysembrioplastic tumor, lane 8; **c** loss of heterozygosity of AXIN-1 gene in medulloblastoma, lane 13. Lanes 1, 3, 5, 9, 12 show corresponding blood samples

The obtained results were then evaluated by image analysis as staining density, i.e. light permeability (LP). Density was depicted as the intensity of light retained by tissue or tissue transparency, and it is reversely proportional to the protein quantities. Mean values for light permeabilities for axin in normal brain tissue was 63, and for beta-catenin 150. These numbers enabled us to calculate relative LP increase or decrease of axin and beta-catenin proteins in tumor tissue. We introduced variables relative increase of protein expression in tumor tissue defined with the following equations: *Axinrela = axinpro/axinnv × 100*; *Betarela = betapro/betanv × 100* where *Axinrela* and *Betarela* represented relative increase or decrease of LP in tumor tissue for axin/beta-catenin; *axinpro* and *betapro* denoted measured LP in tumor tissue; *axinnv* and *betanv* mean values of LP for axin/beta-catenin in normal tissue.

In our total tumor sample the values of light permeability for axin were lower than values for beta-catenin indicating that relative quantity of expressed axin was higher than quantities of beta-catenin in our sample.

Comparison of mean values of relative increase of protein axin and beta-catenin shows that they are significantly reversely proportional (P=0.014). Pearson's correlation is shown in Table 1.

We demonstrated that there was no difference in axin protein levels in patients with AXIN1 LOH (LP = 160.4) and in patients without it (LP = 164.9), (F=1.862; P=0.956). Contrary to this finding, relative quantity of beta-catenin in patients with AXIN1 LOH was significantly higher in comparison to patients without LOH (F=4.566; P=0.040).

The highest relative LP value of axin was measured when the protein was in the nucleus and it was accompanied with the lowest relative LP value for beta-catenin. The lowest relative LP value of axin was measured when the protein was localized in the cytoplasm and nucleus, and it was accompanied with the highest relative LP value for beta-catenin. This means that the quantity of axin is lowest in the nucleus, and is accompanied with the highest quantity of beta-catenin, while the quantity of axin is highest when located in the cytoplasm and nucleus, and is accompanied with the lowest quantity of beta-catenin (Table 2).

Table 1 Comparison of mean values of relative increase of protein axin and beta-catenin

	Axinrela	Betarela
Axinrela Pearson's correlation	1.000	−0.379
significant		0,014
N	41	41
Betarela Pearson's correlation	−0.379	1.000
significant	0,014	
N	41	41

Table 2 Comparation of relative LP values for axin and beta-catenin to cellular localization

Cellular localization	LP for beta-catenin[a]	LP for axin[a]
Nucleus and cytoplasm	140.86	158.81
Cytoplasm	83.25	176.04
Nucleus	80.19	184.74

[a] The numbers represent LP = light permeability, so the protein levels are reversely proportional

Discussion

The events that are involved in initiation, early development and progression of neuroepithelial brain tumors are still inadequately explained. The evolution of specific histopathological type differs significantly with respect to the molecular genetic alterations underlying the oncogenesis and progression.

Our findings on AXIN-1 gross deletions are novel reports on genetic instabilities in neuroepithelial brain tumors. All cases of LOHs of AXIN1 gene were found in very aggressive tumor types designated as grade IV, except for one found in tumor grade I. It is interesting that less malignant grades did not demonstrate LOHs, and we can assume that changes of this tumor suppressor are not the initial events but rather assigned to later stages of tumor progression. Down regulation of axin's gene expression was observed in 65.6% of our total sample indicating classical behavior of tumor suppressor protein expression. This finding may also indicate axin's inability to negatively regulate beta-catenin's destruction. The majority of analyzed glioblastomas had axin distributed in the cytoplasm, showing that nuclear location of axin is not necessarily associated to progression.

Surprisingly, no difference in axin protein levels was observed in patients with AXIN1 LOH and in patients without it. It seems that inactivation of both alleles did not occur. One allele may have suffered gross deletion, leaving the other one do the job of protein expression.

Up regulation of expression of beta-catenin was noted in 53.1% of our total tumor sample. Moreover, beta-catenin's location in the nucleus was found in 59.4% of investigated tumors. Immunolocalization of beta-catenin in the nucleus and genetic alteration of its exon 3 were reported in human malignant tumors by many authors [7] and nuclear location is an indicator of beta-catenin's acquisition of oncogenic activity. We can speculate that in our sample beta-catenin might be accumulated to a level above the threshold for the regulation of axin and APC. The amounts of beta-catenin observed in our study, and the fact that its levels are higher than the levels in healthy brain tissues favors this suggestion.

Findings on significantly higher relative quantity of beta-catenin (P=0.040) in patients with AXIN1 LOH in comparison to patients without LOH may suggest that beta-catenin changes precede axin changes. Moreover, findings on beta-catenin's accumulation in the nucleus in 21.4% of glioblastomas but also in 33.3% of astrocytomas may also suggest that changes in beta-catenin are assigned to earlier stages in neuroepithelial tumor evolution.

The results on significantly reversely proportional (P= 0.014) mean values of protein axin and beta-catenin in our tumor sample are very interesting. It is well known that axin protein can shuttle between the cytoplasm and nucleus accompanying beta-catenin [5, 6]. Nucleo-cytoplasmatic shuttling under normal circumstances suggests existence of possible "salvage pathway" that would be activated by axin translocation to the nucleus in order to reduce beta-catenin' oncogenic activity by exporting and degrading it in the cytoplasm [14]. Recent experiments [14] indicate that axin promotes the cytoplasmic localization of beta-catenin and that the two proteins co-localize in cellular compartments. Our findings on significantly reversely proportional quantities of the two proteins may indicate that the normal shuttling is impaired in the tumors we investigated.

Distribution of axin and beta-catenin proteins was reported by Anderson et al. [15]. Although this group was studying the distribution in neoplastic colon tissue, on the subcellular level the obtained data are very similar to ours. The results obtained on medulloblastoma are in accordance with the reported data [16–19]. Our previous results demonstrated that other Wnt molecular components were also changed in CNS tumors [20–22].

In case of neuroepithelial brain tumors, little is known of alterations of molecules which contribute to Wnt pathway irregular activation. Our results indicate for the first time the relation of beta-catenins' and axins' quantities and locations. The quantity of axin was lowest in the nucleus and was accompanied with the highest quantity of beta-catenin, while the quantity of axin was highest when located in the cytoplasm and nucleus, and is accompanied with the lowest quantity of beta-catenin. We could assume that dynamic changes of axin's subcellular expression and spatial regulation are relevant for tumor development. The levels of studied proteins and the balance between them are very important in physiological circumstances. Losing this balance might be responsible for tumor development.

Our findings on changes of the Wnt molecular components may contribute to better understanding of brain tumor formation and offer improvement in comprehension, diagnosis and treatment of this disease.

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