Wnt signaling and astrocytic brain tumors

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In modern high-income societies, the battles are fought within cells. Human cancer cell is a modern day battlefield and so is the transformed astrocyte. Following this line of thought, the knowledge on cellular signaling pathways and their altered behavior in tumor cells is an important step forward in understanding astrocytic brain tumor etiology. Astrocytic brain tumors are the most common primary CNS neoplasms and are classified according to their lineage of origin and behavior into four WHO grades. However, despite many recent advances [1], the molecular blueprint of development and progression of astrocytic brain tumors is still largely unexplained. Astrocytes, the specialized glial cells that fivefold outnumber neurons, play a major role in the evolution of primary brain tumors [2]. Although the cells of origin of astrocytic gliomas are still unknown and under intense investigation [3], what is known is that astrocytomas exhibit great molecular heterogeneity. Histological research established that glioblastoma in particular is extremely heterogeneous demonstrating both intertumor and intratumor heterogeneity [4]. The signaling pathways that have been implicated primarily not only in glioblastoma but also in astrocytomas include p53, Rb and RTK/Ras signalling [1,4]. The TCGA group identified that these pathways are interconnected and their deregulation plays vital role in glioblastoma pathogenesis. Nevertheless, the results of our previous and continuous research [5–7] suggest that molecular changes of Wnt signaling are also implicated in astrocytic branch of brain tumors.

The canonical Wnt signaling pathway is one of the basic mechanisms of the cell signaling widely conserved in the animal kingdom. It is critical for embryonic development and adult tissue homeostasis, and is controlling events ranging from cellular adhesion, cell motility, embryonic cell patterning, proliferation, differentiation, apoptosis and synaptic rearrangements [8,9]. The misregulation of the Wnt pathway plays important roles in tumorigenesis. The pathway is activated by binding of different Wnt ligands to specific receptors. As a consequence, through several cytoplasmic relay components, β-catenin levels raise,

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and it translocates to the nucleus where it finds a partner – a member of the DNA-binding transcription factor family LEF/TCF. When bound to specific promoter sequences together, they activate transcription of Wnt target genes. This results in the activation of transcription of Wnt target genes, among which are *c-myc*, *N-myc*, *c-jun*, *MMP-7* and *cyclin D1* [9,10]. From the list of the target genes, it is obvious why constitutive activation of this pathway can lead to cancer [11]. A critical step in the inactivation of Wnt signaling involves the control of β-catenin degradation meaning that the pathway is inactive when the levels of β-catenin are kept low. Multiprotein destructive complex consisting of scaffold protein AXIN, adenomatosis polyposis coli (APC) tumor suppressor protein, CK1 and GSK3β performs this task. In the absence of factors that activate Wnt signaling, the complex binds to β-catenin with subsequent β-catenin phosphorylation, ubiquitination and degradation by proteasomes. In this scenario, TCF/LEF repress target genes through a direct association with corepressors [12]. The activation of Wnt signaling also happens in case APC, AXIN and other components of β-catenin destruction complex are mutated and nonfunctional [9,13].

Our research group has been devoted to studies of basic research in the field of genetics of brain tumors, especially focusing on the role of molecular components of Wnt signaling in the formation and progression of astrocytic brain tumors. We investigated key players, β-catenin (CTNNB1), TCF1 and LEF1, adenomatous polyposis coli (APC), AXIN1 and dishevelleds (DVL1, DVL2 and DVL3) in human astrocytomas.

The main signaling effector molecule of the Wnt pathway is β-catenin and it has been characterized as a potent oncogene product. Activating mutations of β-*catenin* gene are found in a variety of tumors including sporadic medulloblastoma where this molecule was proposed as prognostic factor. Our investigations on β-*catenin* in astrocytomas revealed 10% of samples with potential activating mutations. The results on protein levels demonstrated that 50% of glioblastomas (grade IV) and 56% of astrocytomas (grades II and III) showed upregulation of β-catenin. Nuclear localization, indicative of the activation of Wnt signaling, was found in 52.1% of glioblastomas [5]. Central mediators of transcription of the Wnt pathway are TCF/LEF transcription factors. We found that they were also

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upregulated. Strong TCF1 and LEF1 expression was observed in 51.6 and 71% of glioblastomas. Astrocytoma grade I (which we used as benign reference) showed almost opposite expression levels with weak or no expression in the 63.2% for TCF1 and 68.2% for LEF1. Statistical analysis additionally confirmed significant differences in protein expression levels. Analysis of variances performed on the total sample indicated significant differences in the values of TCF1 weak expression ($p = 0.045$), LEF1 weak ($p = 0.008$) and LEF1 strong expression ($p = 0.002$) with regard to malignancy grade. The F-ratios for two variables (LEF1 strong and LEF1 weak) indicated that differences between astrocytomas (II,III) and glioblastomas were statistically significant ($p < 0.02$). Discriminant function analysis further showed that just one variable – the strong expression of LEF1, emerged to discriminate between astrocytomas and glioblastomas. This suggests that LEF1 may serve as potential diagnostic marker distinguishing glioblastoma from astrocytoma group [7].

The components of β-catenin destruction complex represent negative regulators of the pathway and in the circumstances of tumorigenic process they function as tumor suppressor genes. Consistently, allelic losses of *APC* gene were frequent with glioblastomas showing 60% and diffuse astocytomas (grade II) 20%. Our interest in elucidating the role of APC stemmed principally from the findings that wild-type APC protein is expressed in the CNS and is critically involved in the initiation of neuronal differentiation [14]. Furthermore, its involvement in particular syndromes, such as the Tourcot syndrome, which include the development of primary brain tumors, suggests that it performs important functions in these tissues also.

In this complex signaling network AXIN1, named for its role in the inhibition of vertebrate axis formation during embryonic development, is another interesting tumor suppressor. Bearing in mind the known function, AXIN1 is expected to be located in the cytoplasm of normal cells where it promotes β-catenin phosphorylation and degradation. Nevertheless, AXIN1 has been known to shuttle from cytoplasm to nucleus and nowadays it is considered a scaffold protein [12,15]. Our studies found allelic losses of *AXIN1* in 10% of glioblastomas and downregulation of AXIN1 proteins in 31% of glioblastomas and 22% of astrocytomas. In 31% of glioblastomas AXIN1 was localized in the nucleus [5]. Our results also

showed the relation of β-catenin's and AXIN's quantities and locations. Comparison of mean values of relative expression levels of AXIN1 and β-catenin showed that they are significantly reversely proportional (p = 0.014). Comparison of their relative expression values to cellular localization showed that the quantity of AXIN1 is lowest in the nucleus, and is accompanied with the highest quantity of β-catenin, while the quantity of AXIN1 is highest when located in the cytoplasm and is accompanied with the lowest quantity of β-catenin. We could assume that dynamic changes of AXIN's subcellular expression and spatial regulation are relevant for tumor development. Our novel research focuses on Dishevelleds (Dsh or DVL), highly conserved multifunctional phosphoproteins with three members in humans (DVL1, DVL2 and DVL3). DVL forms large molecular supercomplexes at the plasma membrane consisting of Wnt-Frizzled-LRP5/6-DVL-AXIN. This promotes the disassembly of the β-catenin destruction machinery, β-catenin accumulation and consequent activation of Wnt signaling. Therefore, DVLs are considered to be key regulators that rescue cytoplasmic β-catenin from degradation. The overexpression of DVL has been shown to potentiate the activation of Wnt signaling and it is now apparent that upregulation of DVLs is involved in several types of cancer [13,16]. Our present investigations are showing that the dishevelleds, too, are targeted in glioblastoma. We observed gross deletions (loss of heterozygosity, LOH) as well as microsatellite instability (MSI) of dishevelled family of genes in glioblastoma samples. LOH of *DVL1* was found in 11% of glioblastoma samples, of *DVL2* in 6.25% and *DVL3* gene in 39.3%. MSI was detected in 22% cases for *DVL1*, 25% for *DVL2* gene and 6% for *DVL3* gene. Protein expressions in glioblastoma were moderate or strong in 65, 73.5 and 67% of samples, respectively, for DVL1, DVL2 and DVL3.

Although brain tumors rarely metastize to distant organs, probably because of blood–brain barrier, their local invasion is the reflection of their motility and invasive behavior. The formation of mobile cells with invasive potential and the delamination of cells from primary tumors are performed through the process of epithelial-to-mesenchymal transition (EMT). Being a very complex event, EMT requires the specific spatiotemporal expression of molecules, their interaction and modification of a range of cellular and extracellular factors to allow cellular motility and invasion to proceed. The classical Wnt signaling pathway has a particularly tight link with EMT. The nuclear translocation of β-catenin can induce EMT [17]. Besides being the signaling molecule, β-catenin is involved in cellular architecture too, bound to E-cadherin it is an essential component of adherens junctions. Let me accentuate that the most prominent feature of EMT is the loss of expression of adhesion molecule E-cadherin. Moreover, the stabilization and nuclear accumulation of β-catenin can activate the transcriptional repressors Snail and Slug that suppress E-cadherin expression and thus induce EMT [17].

Reflecting on the beginning of this paper and at the battle fought against this deadly tumor, we must with regret say that at present we are losing the battle. Clinically effective treatments remain elusive and individuals with malignant astrocytomas experience significant morbidity and mortality. Tumor progression and resistance to therapy have been associated with tumor heterogeneity both at the genetic and the phenotypic level. Thus, the success of any therapeutic approach will need to include the consideration of astrocytoma heterogeneity. It has repeatedly been shown that the activity of Wnt signaling correlated with patients' outcomes in different types of cancer [18]. For example, high nuclear and cytoplasmic β-catenin levels, as well as the increased staining of WNT1, were associated with poor survival in glioblastoma [19]. Therefore, in our opinion, it is important to mention the research oriented in finding possible molecular targets for the therapeutic intervention in the Wnt signaling. Multiple strategies – ranging from blocking antibodies and small inhibitor molecules to peptide agonists and antagonists – are now in development, and number of clinical trials is under way hopefully bringing effective therapeutics [15,18]. One strategy has focused on suppressing β-catenin expression by promoting its degradation [20], but the disadvantage of this approach is that it is not tumor cell specific and eliminates β-catenin's cellular pool everywhere. There are also studies dealing with restoration of functional APC and those trying to prevent the transcriptional activity of β-catenin/TCF complexes in order to restrain expression of target genes. A number of small molecules inhibitors that disrupt β-catenin/TCF binding have been studied [18]. There is also a group of Wnt signaling's own inhibitors that could be employed as therapeutics. Several sFRPs

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(secreted Frizzled-related proteins) competitively bind to Wnt ligands and prevent the interaction between Wnt and its receptors. Another class of Wnt inhibitors, the DKK (Dickkopf) family of proteins, potently inhibits signaling by binding to the Wnt coreceptors LRP5/6.

In conclusion, as the old proverb teaches us – *vivere est militare*. Therefore, we would like to emphasize that better understanding of human astrocytic brain tumor molecular profile is an imperative in future research on effective treatments. Elucidating the role of Wnt signalling in astrocytoma etiology is important for the characterization of molecular biomarkers that will help us in diagnostics and the design of therapeutic weapons.

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