Angiogenesis and angiogenic tyrosine-kinase receptor and claudin-5 expression in pediatric brain tumors

Doctoral thesis

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INTRODUCTION

The second most common disease among pediatric malignancies is brain tumor. We can differentiate three main types, namely ependymoma, astrocytoma and medulloblastoma. Major progress has been made in oncology as the development of molecular biology has provided new and more efficient therapy options in the past few years. Various tumors in adults are now treated with several targeted and efficient therapies in clinical practice for several years (bevacizumab, cetuximab, sunitinib, sorafenib, etc.), however, their use in pediatric oncology is so far limited.

Among pediatric tumors, astrocytomas are of glial origin and are often well differentiated and are generally respond well to treatment. Glioblastoma is characterized by poor prognosis and is indeed a rarity among children. Medulloblastoma originates from germ cells, most commonly located in the cerebellum and usually reacts well to chemotherapy. Ependymoma originates from the ependymal cells of the ventricle and is often poorly differentiated and has poor prognosis. Multimodal treatments play the most important role in the therapy of pediatric tumors. The timely removal of the tumor can result in full recovery. Chemotherapy is mainly used in malignant astrocytoma and medulloblastoma; modern radiotherapy methods are also important elements of the complex therapy. In the past few years the development of molecular oncology has created some new opportunities in the treatment of tumors. Several examinations have been conducted to identify the new molecular targets in pediatric tumors and to apply a targeted therapy which could provide a more effective treatment with lower toxicity.

Angiogenesis plays an important role in the progression of tumors by providing blood flow to the

constantly growing tumor. The inhibition of angiogenesis has been already introduced to clinical practice in several cases (coclorectal cancer, kidney cancer, lung cancer, glioblastoma). Several distinct mechanisms can contribute to the development of the blood flow in the tumor tissue including neovascularization, vessel incorporation, intussusceptive angiogenesis or vascular mimicry. The so called glomeruloid formations are often present in certain brain tumors. All vascularization mechanisms are characterized by specific angiogenic tyrosine kinase receptor expression patterns.

Tyrosine kinase receptors (TKR) are integral membrane proteins and play an essential role in the formation and progression of most malignant tumors. TKR genes can be divided into 17 families and code 58 proteins. They form complexes in the cell membrane and by virtue of their kinase activity control several cell signaling processes. The protein VEGFR1 does not have kinase activity and primarily functions as a decoy-receptor, while the activation of VEGFR2 by the VEGF-A ligand is a critical regulator of angiogenesis. The VEGFR3 mainly binds VEGF-C and -D proteins and is one of the most important molecular regulators of lymphangiogenesis. Tumor cells expressing VEGFR2 can directly support their growth via autocrine signaling and a synergistic interaction between VEGFR and EGFR has been described in the progression of certain malignancies. The monomer proteins of the PDGF family exerts its effect as dimers. These ligands can be bound by two PDGF receptors, the PDGFRα and the PDGFRβ. The PDGF/PDGFR system is one of the defining regulators of the differentiation processes of the kidney, the lung, the circulation and hematopoiesis throughout the embryonic development and it regulates the differentiation of the neuroglial progenitors in the nervous system. The developmental/stem cell receptor c-Kit is also an angiogenic tyrosine kinase receptor which is expressed at the endothelial progenitor cells too. Among the different

angiogenic mechanisms, neovascularization is supported by VEGFR2, vascular mimicry is supported by EphA and FAK and glomeruloid angiogenesis is supported by VEGFR2 and PDGFR proteins. Several studies have examined the presence of these proteins in pediatric brain tumors and the hypothesis has been conceived that the regression of the tumor could be induced through inhibiting these tyrosine kinases by reducing the extent of vascularization. Several other proteins are involved in the process of angiogenesis, including the ligands of receptors, the matrix metalloproteinases or different cell adhesion molecules. PDGF proteins and their receptors play an important role in regulating the perivascular elements of more mature vessels. The PDGF-BB produced by endothelial cells is able to stimulate the motility and proliferation of the pericytes and vascular smooth muscle cells covering the vessels.

Through the specific inhibition of the tyrosine kinase receptors of tumor cells, there is a possibility that the tumor growth can be inhibited by the disruption of the given signaling processes. These specific inhibitors have provided a new paradigm in molecular therapies. The inhibition of the signaling network can lead to the inhibition of the tumor cell's development and induction of apoptosis in certain cases. Antibodies like cetuximab with high molecular mass can also selectively inhibit tyrosine kinase receptors by binding their extracellular domains. Inhibitors with low molecular mass enter the cell membrane and bind the intracellular domains inhibiting their enzymatic function.

Besides angiogenesis, proteins responsible for cell-cell adhesion also play an important role in the progression of tumors. Among these proteins, members of the claudin family are the main components of the complexes creating the tight junctions between cells. 24 different claudin genes have been identified in the human body so far. They play a role in the adhesion between cells and the development of organs.

Claudin proteins can be found in all tissues and their expression is to a great extent tissue and organ specific. Claudins -1, -2, -3, -5, -7, -11, -12 have been described in the central nervous system. Claudin-2 can be found in the choroid plexus; claudin-3, -5 and -12 in the cerebral capillary endothelium and claudin-11 in the Schwann-cells. Claudin-5 and claudin-12 characterize the cerebral endothelial cells that constitute the blood-brain barrier. Several claudins can play a role in the formation of nervous system tumors, including claudin-1 and claudin-3 proteins. The elevated expression of claudin-2, -5 and -7 has also been detected in ependymoma so far.

OBJECTIVES

1. ***Are there major differences in pediatric brain tumors with regards to microvascular density and mechanisms of angiogenesis?***
   In order to answer this question, altogether 44 cases of the three most common types of pediatric brain tumors (astrocytoma, ependymoma, medulloblastoma) have been collected. After CD34 and smooth muscle actin immunohistochemistry we determined microvascular density, frequency of glomeruloid vascular structures and the ratio of vessels with pericyte coverage.

2. ***Which angiogenic tyrosine kinase receptors are present in the blood vessels and tumor cells of pediatric brain tumors?***
   We determined the expression pattern of VEGFR, PDGFR and c-Kit tyrosine kinase receptors in the tumor vasculature and in the tumor cells of the aforementioned 44 pediatric astrocytoma, medulloblastoma and ependymoma specimens. Furthermore, we examined the correlation of expression pattern and the angiogenic mechanisms. These studies can contribute to determining which tyrosine kinase receptors can be potential targets in the therapy of pediatric brain tumors.

3. ***What is the clinical significance of the claudin-5 expression of ependymoma cells?***
   During the claudin-5 labeling of the vessels of pediatric brain tumors we noticed that the protein can also be found in the plasma membrane of the tumor cells in certain ependymomas. Hence we described the claudin-5 expression of the normal ependyma and choroid

plexus and compared the ultrastructural structure of cell-cell adhesions. Moreover, through an international collaboration we collected 54 intracranial pediatric ependymoma cases and described the correlation of claudin-5 expression with clinicopathological parameters and examined its potential prognostic significance.

METHODS

Patient groups

We examined 14 astrocytoma, 16 ependymoma and 14 medulloblastoma cases for the angiogenetic mechanisms and tyrosine kinase receptor patterns and 54 ependymoma cases for the claudin-5 expression.

Human autopsy ependyma samples

Ependyma tissue samples (from ventricules and plexus choroideus) were collected during routine autopsy of newborns deceased between the 35th and the 40th gestational week.

Method of determining vessel density

An immunohistochemical method was applied to detect vascular density with the help of anti-SMA and anti-CD34 antibodies.

Determining the expression of tyrosine kinase receptors

VEGFR1, VEGFR2, PDGFRα, PDGFRβ, c-Kit specific antibodies were used for immunohistochemical labeling to detect tyrosine kinase receptors on blood vessels and tumor cells.

Tests on the expression of cell junction structures

In order to detect cell-cell adhesion proteins and identify ependymal cells through immunohistochemical reaction, we used the following antibodies: claudin-1, claudin-2, claudin-5, claudin-7, E-cadherin, N-cadherin, occludin and vimentin, respectively.

Quantitative real-time PCR

We isolated the RNA from eleven FFPE ependymoma tumor samples and after a reverse transcription we measured the transcription level of with a quantitative real-time PCR method.

Electron microscopy imaging

Transmission electron microscope was used for the ultrastructural analysis of cell-cell adhesions in the ependyma and choroid plexus epithelium.

Statistical analysis

The Mann-Whitney and $\chi 2$ methods were used for the statistical comparison of the patient groups. The percentage of positive tumor cells was calculated with the Kruskal-Wallis method and then Dunn's multiple comparison tests. The overall survival analysis was performed with the Kaplan-Meier method. The different survival curves were compared with log-rank statistics. The program GraphPad Prism 5 was used for all the statistical analysis. Differences with $P<0.05$ significance were regarded significant.

RESULTS

Microvascular density in pediatric brain tumors

Vessels in all tumor types evidently stained with the anti-CD34 antibody. A significantly higher amount of CD34 and SMA positive vessels can be found in ependymoma when compared to astrocytoma or medulloblastoma. The number of vessels stained with anti-CD34 antibody was higher than that of SMA positive structures in all three tumor types. It is worth mentioning that SMA positive vessels are also more significantly common in ependymoma than in astrocytoma or medulloblastoma. The highest SMA/CD34 ratio was detected in medulloblastoma which was significantly higher than the ratio detected in astrocytoma. However, we have not found increased vascularization in the 4 pediatric glioblastoma cases when compared to 10 pilocytic astrocytoma specimens.

Glomeruloid vascular elements in pediatric brain tumors

CD34 positive glomeruloid vessels were found in all medulloblastomas, while significantly less astrocytoma cases had glomeruloid structures and they were almost completely missing from ependymoma cases. All pediatric glioblastoma cases had this type of vessels. In contrast, the SMA-marked glomeruloid structures were less frequent than CD34 postive ones, but they were also more abundant in most of the medulloblastoma cases.

Expression of tyrosine kinase receptors in tumor associated blood vessels

Almost all (93%) medulloblastomas showed strongly PDGFRα positive vessels, but PDGFRβ only showed feeble and low expression in each tumor type. Vessel specific PDGFRα expression was significantly higher in astrocytoma than in the case of ependymoma. The highest density of c-Kit

labeled vessels were found in ependymoma cases and the labeling was much higher when compared to either medulloblastoma or astrocytoma which hardly showed positivity. In the case of certain ependymomas, strong VEGFR2 and c-Kit expression was detected on the tumpr associated vessels. VEGFR2 receptor positive vessels were not detected in medulloblastoma cases. Interestingly, the vessels showed VEGFR1 labeling in most cases regardless of the tumor type.

Expression of tyrosine kinase receptors on tumor cells

The intensive staining of the PDGFRα was typical of all three tumor types. The most PDGFRβ receptors were found in medulloblastoma cases (71%) and in less than half of the ependymoma and astrocytoma cases. Three medulloblastoma samples showed strong c-Kit staining in the plasma membrane of tumor cells. Low intensity tumor cell specific c-Kit expression was only present in 2 astrocytoma and 5 ependymoma cases. Strong VEGFR1 labeling was present in pediatric brain tumor cells regardless of the tumor type (66% of all cases), while VEGFR2 expression was not detected in either tumor on the tumor cells.

Non-endothelial claudin-5 expression in pediatric ependymoma

Claudin-5 labeling limited to the cell membrane was detected in certain ependymoma tumors on the tumor cells. Tumor cell specific claudin-5 expression occured only in certain supratentorial tumors and was not present in infratentorial or spinal tumors. Among 20 supratentorial ependymomas, the plasma membrane of tumor cells was stained with anti-claudin-5 antibody in 9 cases (45%). In contrast, claudin-5 was limited to endothelial cells in infratentorial tumors. Claudin-5 transcription was significantly higher in 6 claudin-5 positive ependymomas when compared to the 5 claudin-5 negative

samples. It is worth mentioning that as a result of the high claudin-5 expression in the endothelial cells, even the tumor cell specific claudin-5 negative ependymoma samples showed relatively high transcription.

Prognostic factors in pediatric ependymoma

There were 20 supratentorial (37%) and 34 infratentorial (63%) among the 54 examined ependymoma cases. The average and median age of patients at the time of the operation was 6,3 and 6,4, the youngest patient at the time of the diagnosis was 2 months old and the oldest was 17 years old. Average age in the supratentorial patient group was higher. Among the 54 patients, 33 (61,1%) had macroscopically total tumor resection and in 21 cases (38,9%) only partial resection was possible. A strong tendency was seen towards increased survival rate in the group with full tumor resection. 27 cases (50%) had grade 2 tumor differentiation and the other 27 cases (50%) had grade 3 differentiation. There was no significant difference in survival in regard of the differentiation of the tumor. As for the location of the tumor, supratentorial localization was clearly associated with higher median overall survival.

Non-endothelial claudin-5 expression in the developing central nervous system

The ependyma and choroid plexus epithelium were identified with vimentin labeling in the neonatal ventricular system. The endothelial cells of subependymal layer were claudin-5 positive, unlike the ependyma cells. However, the choroid plexus epithelium showed intensive claudin-5 staining connected to the plasma membrane.

Expression of other cell-cell adhesion proteins

Intensive claudin-1, -2 and occludin staining was detected in the plasma membrane and the cytoplasma in the neonatal

plexus choroideus. E-cadherin labeling was clearly limited to the lateral plasma membrane.

Ultrastructure of cell-cell adhesion in the ependyma and choroid plexus epithelium

In the neonatal ependyma the lack of claudin-5 expressions associated with the lack of tight junctions between ependyma cells, however, adherent junctions were detected. In the choroid plexus epithelium, tight junctions were found between cells. Both cell types had microvillus on the apical surface. In contrast, only ependyma cells carried cilias.

Clinical significance of the claudin-5 expression of ependymoma cells

The overall survival of the intracranial claudin-5 expressing ependymoma group was significantly longer than the negative cases. The survival of the claudin-5 negative supratentorial patients resembled that of the infratentorial ones.

CONCLUSIONS

1. Comparing the vascularization of the three most common pediatric brain tumor types, we found the highest vessel density in ependymoma and the most glomeruloid structures in medulloblastoma, while the highest rate of vessels without pericyte coverage was present in astrocytomas.
2. Angiogenic tyrosine kinase receptor patterns specific to each tumor type were found in the blood vessels of the brain tumors; most VEGFR2 and c-Kit positive vessels were found in ependymoma and most PDGFRα labeled vessels were present in medulloblastoma.
3. Regardless of the tumor type, significant PDGFRα and VEGFR1 labeling was found on the tumor cells. PDGFRβ and c-Kit proteins on tumor cells were primarily identified in medulloblastoma. These results suggest that in certain pediatric brain tumors the specific inhibitors of the angiogenic tyrosine kinase receptors might play a role in therapy both due to their antiangiogenic and direct antitumor effect.
4. The cerebral endothelial cell marker claudin-5 protein was found to be specifically expressed on the tumor cell in certain supratentorial but not in infratentorial or spinal cases. This claudin-5 expression, similarly to complete resection and supratentorial localization had a positive prognostic value in this pediatric patient cohort.
5. Reflecting this regional distribution, claudin-5 expression was only identified in the choroid plexus epithelium but not in the ependyma cells of the ventricular system or in the spinal cord. Accordingly, tight junctions were only found in the epithelial cells of choroid plexus as demonstrated by ultrastructural studies by transmission electron microscopy.

PUBLICATIONS

1. Virág J, Kenessey I, Haberler C, Piurkó V, Bálint K, Döme B, Tímár J, Garami M, Hegedűs B: Angiogenesis and angiogenic tyrosine kinase receptor expression in pediatric brain tumors. *Pathol Oncol Res. 2014:20(2):417-26* (impact factor: 1.855)
2. Virág J, Haberler C, Baksa G, Piurkó V, Hegedüs Z, Reiniger L, Bálint K, Chocholous M, Kiss A, Lotz G, Glasz T, Schaff Z, Garami M, Hegedűs B: Region specific differences of claudin-5 expression in pediatric intracranial ependymomas: potential prognostic role in supratentorial cases. Accepted in *Pathol Oncol Res. 2016* (impact factor: 1.94)

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