The pilocytic astrocytoma
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 9

SUMMARY
CHAPTER 1

INTRODUCTION AND PURPOSE OF THIS THESIS

The tumors, studied in this thesis, were named “pilocytic astrocytomas” in the WHO classification of 1979, before that time they had been described under different names, such as gliocytoma embryonale and spongioblastoma. Pilocytic astrocytomas account for 6% of all brain tumors and occur mainly in the pediatric age group. Two-third of these tumors are located in the cerebellum and the remainder can be localized anywhere in the central nervous system. Well known other localizations are the brain stem, diencephalon and optic pathways. In the latter situation the patient often suffers from neurofibromatosis type 1 (von Recklinghausen’s disease). The presenting symptoms are most often caused by increased intracranial pressure. The majority of pilocytic astrocytomas contain cysts. When there is a solitary cyst the tumor mass occurs as a mural nodule. The microscopic appearance is dominated by a biphasic pattern, in which areas containing stellate or protoplasmic astrocytes with microcysts and eosinophilic granular bodies alternate with areas containing the typical elongated (piloid) astrocytes with Rosenthal fibers. Previous efforts to subdivide these tumors in groups with favorable and unfavorable prognosis, based upon histological characteristics, have failed.

Surgical resection is the treatment of first choice. When this can be achieved the tumor will not recur and the prognosis of the patient is excellent. Residual tumors after incomplete resection have an unpredictable behavior. These residual tumors can remain stable for many years, can even regress, but may also show rapid progression. Radiation therapy has no proven beneficial effect on cerebellar pilocytic astrocytomas and the side-effects on the brain of young patients are devastating. Therefore, it is generally accepted that conventional radiation therapy has no place in the treatment of cerebellar pilocytic astrocytomas. However, a favorable response to radiation therapy has been reported in the treatment of optic pilocytic astrocytomas. Results of chemotherapy and stereotactic radiation therapy are scarce but promising.

Since pilocytic astrocytomas and their residual tumors may remain “quiescent” for many years, treatment options for patients with tumors on localizations which are difficult to access surgically, are very controversial, varying from riskily surgery to a conservative expectative attitude. This controversy forms the basis for the efforts made in this thesis to acquire more insight in the biological behavior of pilocytic astrocytomas.

CHAPTER 2

THE CEREBELLAR PILOCYTIC ASTROCYTOMA: A TREATMENT PROTOCOL BASED UPON ANALYSIS OF 73 CASES AND A REVIEW OF THE LITERATURE

In a retrospective study of 73 patients operated on for cerebellar pilocytic astrocytomas, results of treatment, outcome and biological behavior of residual tumor were analyzed. Complete tumor resection proven by CT or MRI-scans within one year after surgery was achieved only in 69% of
cases. In 31% of cases the surgeons opinion and the result of post-operative neuro-imaging regarding the extent of surgical resection did not correlate. Progression of residual tumor or tumor recurrence appeared in 19% of patients, 1 patient showed metastatic spread along the craniospinal axis and in 1 patient malignant degeneration appeared during follow-up. Stable residual tumor or regression of residual tumor was seen in 14% of patients. Outcome after surgical treatment, which was combined with irradiation in 10 patients (14%), was favorable in 80% and unfavorable in 20% of patients. This outcome of treatment was not influenced by a second operation for progression of residual tumor or recurrent tumor. Characteristics of patients with tumor progression after the first operation did not differ from those of the whole group. Ten out of 17 re-operations for residual or recurring tumor took place within 4 years after initial surgical treatment. Surgery-related morbidity was 15% and mortality 4%. Irradiation of residual tumor in 8 patients was followed by complete regression in 1 patient, progression in 4 patients and no changes in 1 patient. For the remaining 2 patients the effect of irradiation on the residual tumor is unknown. Factors that determine the prognosis of the patient are discussed on the basis of this retrospective analysis and the data from the literature.

It is concluded that optimal treatment for a cerebellar pilocytic astrocytoma does not consist solely in surgery with the aim of total tumor removal. Careful tumor handling needs to be performed, in order to avoid spread of tumor cells and subsequent metastases. Additional radiation therapy can be considered in strictly selected cases. Posttreatment follow-up, based on direct postoperative neuroimaging, preferably by MRI, is of utmost importance. An algorithm for postoperative follow-up management is presented.

CHAPTER 3

INTRODUCTION TO THE METHODS USED FOR STUDYING THE BIOLOGICAL BEHAVIOR OF PILOCYTIC ASTROCYTOMAS

AgNOR staining

Nucleolar organizer regions (NORs) are portions of DNA that are involved in the transcription of DNA to RNA. RNA is responsible for protein synthesis in the cell, including the proteins needed for proliferation. NORs are argyrophilic and therefore, by using silver staining, resulting in the precipitation of AgNOR dots, easily made visible. The technique of silver staining and the interpretation of the results show a large variation among the numerous previous studies in which AgNOR staining was tested as a proliferation marker. Of 109 reviewed articles, 73% showed correlation between AgNOR score and tumor grade and 60% showed a correlation between AgNOR score and patient outcome. AgNOR staining is positive in all cells that show protein synthesis. AgNOR expression reflects the rapidity of the cell cycle and is related with tumor doubling time.

KI-67 and MIB-1 labeling
KI-67 is a protein of unknown function, only present in the active phases of the cell cycle (G1, M, G2 and S phase). The protein can be detected with the monoclonal KI-67 antibody in fresh tissue and with the MIB-1 antibody in formalin fixed tissue. Since proliferating cells are in one of the active phases of the cell cycle, the number of cells showing presence of KI-67 protein, the KI-67 or MIB-1 labeling index (LI), reflects the number of proliferating cells, or the growth fraction. A majority of previous studies that have tested MIB-1 LI as a proliferation marker found a correlation between MIB-1 LI and patient outcome or tumor grade.

The TP53 gene and p53 protein

One of the functions of the TP53 tumor suppressor gene is to prevent carcinogenic events: the p53 protein is involved in inducing G1-arrest, DNA-repair and apoptosis. In high grade astrocytomas TP53 mutation is considered to be an early event in tumorigenesis. The gene is involved in the progression of low grade astrocytomas to a higher grade. The function of the gene can be studied either by analyzing the structure of the gene itself (LOH studies, mutation screening), or immunohistochemically by using antibodies to the p53 protein. Only 3 mutations among 107 studied pilocytic astrocytomas have been described. However, in those previous studies mostly not the entire coding sequence of the TP53 gene was investigated. Results of immunohistochemical p53 analyses are controversial. Therefore, it is believed that the TP53 gene is not involved in pilocytic astrocytoma formation.

Some studies reported a correlation between p53 function and tumor grade or patient outcome. This relation has not been studied in pilocytic astrocytomas.

The NF1 gene

Pilocytic astrocytomas, especially of the optic nerve, are closely related to neurofibromatosis 1 (NF1). Patients with NF1 develop pilocytic astrocytomas in 15% of cases. Structural changes in the NF1 gene may cause NF1, a disease with very variable expression, autosomal dominant inheriting and one of the most frequent occurring genetic disorders worldwide. One of the functions of neurofibromin, the product of the NF1 gene, is to inhibit the transduction of growth stimulating signals that are mediated by p21ras. Therefore, the NF1 gene has a tumor suppressor function, as has been proven in several malignant neoplasms. However, the tumor suppressor function for NF1 in pilocytic astrocytomas has not been established.

CHAPTER 4

AgNOR STAINING MAY REFLECT THE GROWTH POTENTIAL OF PILOCYTIC ASTROCYTOMAS

The value of AgNOR staining as a tumor biological marker was tested in 26 children with pilocytic astrocytomas (20) and fibrillary astrocytomas (6). All patients were surgically treated and followed
by periodical MRI- or CT scans. Follow-up ranged from 8 to 84 months, with a mean of 44 months. AgNOR expression was determined by using semi-automated computer assisted surface area measurement. AgNOR values ranged from 1.4 to 81.4 square micrometer per cell, with a mean of 26.6 and a median of 15.2.

The median value was taken as a "cut-off" score, separating 2 groups of patients with low and high AgNOR scores. In the group with low scoring tumors there were 9 total resections, of which 1 showed a recurrence after 6 years, this tumor was a fibrillary astrocytoma of the cerebral hemisphere. The 4 remaining patients had partial resections, the residual tumor remained stable in 3 and regressed in 1 patient. In the group with high scoring tumors only 2 patients had complete resection without recurrence, whereas in 11 patients resection was incomplete. Among the 11 residual tumors 5 remained stable and 6 showed progression within 1 year. These progressive tumor remnants were of optic pilocytic astrocytomas in 3 cases, of cerebellar pilocytic astrocytomas in 2 cases and of a fibrillary cerebral astrocytoma in 1 case.

In the small group of 7 patients with optic/hypothalamic pilocytic astrocytomas 4 patients do well, having low AgNOR scores (8 to 33), whereas 3 show progression of residual tumor having high AgNOR scores (39 to 64).

In the group of pilocytic astrocytomas with low AgNOR expression, i.e. below the median value of the whole group, no progression of residual tumors or recurrences appeared. Possibly the AgNOR score is related to tumor resectability; a high AgNOR score might correspond to infiltrating tumor growth with hence a more difficult surgical resectability. The determination of AgNOR expression might be of help in selecting patients, when there is residual tumor after surgery, for additional chemo- or (stereotactic) radiation therapy.

CHAPTER 5

THE PROLIFERATIVE POTENTIAL OF THE PILOCYTIC ASTROCYTOMA: THE RELATION BETWEEN MIB-1 LABELING AND CLINICAL AND NEURO-RADIOLOGICAL FOLLOW-UP

The proliferative potential of 39 pilocytic and 5 low grade astrocytomas was studied in relation to the KI-67 activity as measured by the MIB-1 Labeling Index. The results were correlated to the biological behavior of the tumor as measured by clinical and neuro-radiological (CT- or MRI-scans) follow-up of the patient. This study was undertaken to answer the question wether MIB-1 expression reflects differences in biological behavior of these tumors, such as rapid progression of residual tumor or stable remaining tumor.

MIB-1 LI values ranged from 0 to 19% in the group of pilocytic astrocytomas (mean 4,2%) and from 0 to 15% in the 5 low grade astrocytomas ( mean 4,2%).

All patients were operated and 23 of them had incomplete tumor resection as proven on postoperative neuro-imaging studies. Those 23 patients could be subdivided into two groups; one without progression of residual tumor during follow-up (n=12) and the other with tumor progression (n=11). Mean MIB-1 LI in the group with "quiescent" tumor tended to be lower than in the group
Residual tumors which were negative for MIB-1 staining showed fewer progressions of residual tumor compared to those being positive for MIB-1 staining, however this difference was not significant (p=0.15, Fisher exact test).

Tumor samples of a second operation of the same patient had lower MIB-1 LI values than those of the samples taken at first operation. The proliferating potential seemed to be decreased after part of the tumor was resected.

Pilocytic astrocytomas with a negative MIB-1 LI are unlikely to show progression of residual tumor after partial resection. MIB-1 staining might be an additional tool in determining the frequency and duration of follow-up and in making decisions regarding further treatment of a patient operated for a pilocytic astrocytoma with residual tumor.

CHAPTER 6

TP53 IS INVOLVED IN PILOCYTIC ASTROCYTOMAS BUT HAS NO RELATION WITH TUMOR BEHAVIOR

The TP53 gene is the most frequent mutated gene in human neoplasms. The mutation frequency in high grade astrocytomas is 30%-90% and in grade II astrocytomas 5%-60%. In pilocytic astrocytomas TP53 mutations occur in less than 3%. Results of immunohistochemical p53 screening in previous studies are conflicting.

The role of the TP53 gene in 43 pilocytic and 5 low grade astrocytomas was investigated by immunohistochemical and mutation analysis, the relation between the results of these 2 techniques and a possible relation to tumor behavior were studied.

All tumor specimens were stained with the monoclonal antibody BP53-12 and the entire coding region of TP53 in 12 tumors was screened for mutations using nested PCR, DGGE and sequencing. Patients were categorized in two groups based on behavior of residual tumor after surgery during follow-up: one group with regressive or stable residual tumors (n=13) and one group with progression of residual tumor (n=11). Among pilocytic astrocytomas 58% were positive and 42% were negative for p53 immunolabeling. These results did not differ among the 2 groups, therefore, a clear relation between immunolabeling results and tumor behavior could not be found.

Among 10 pilocytic astrocytomas genetically screened 8 TP53 mutations were found in 5 tumors. One of the 2 low grade astrocytomas also showed a mutation. Five mutations have not been reported to occur in any tumor or tissue previously, which makes them novel. Three mutations are described for the first time in pilocytic astrocytomas, and one has been described previously in a pilocytic astrocytoma but not in any other tumor.

Of all mutations 3 are very likely, and another 3 are possibly causative for the production of a dysfunctional p53 protein. TP53 mutation frequency among pilocytic astrocytomas is estimated 30%-60%.

A clear relation between the presence or absence of TP53 mutations and tumor behavior could not be found.

Results of p53 immunohistochemistry and TP53 mutation analysis were not concordant.
It is concluded that the *TP53* gene is involved in tumorigenesis of pilocytic astrocytomas but has no relation with tumor behavior.

**CHAPTER 7**

**UP-REGULATION OF SPECIFIC *NF1* GENE TRANSCRIPTS IN SPORADIC PILOCYTIC ASTROCYTOMAS**

Due to the frequent occurrence of optic pilocytic astrocytomas in Neurofibromatosis 1 and the presence of chromosomal alterations in the region of the *NF1* gene in sporadic pilocytic astrocytomas, a causative relation between the *NF1* gene and tumor formation is suggested. The existence of this relation is further supported by the fact that the gene product, being neurofibromin, contains a region that is homologous to GTPase-activating proteins (GAP). GAPs play a significant role in the p21*ras* mediated signal transduction pathway that is involved in tumorigenesis. Results of previous studies support the hypothesis that *NF1* acts as a tumor suppressor gene. According to this hypothesis one expects to find in pilocytic astrocytomas a dysfunctional *NF1* gene or gene product, which can be reflected in either alterations at the level of the *NF1* gene, such as mutations, or a reduced or absent level of neurofibromin.

To test this hypothesis we analyzed 6 pilocytic astrocytomas of non-NF1 patients. Since the *NF1* gene is very large and difficult to screen for mutations entirely, and the protein neurofibromin is expressed in several isoforms, each possibly having different functions, this analysis was predominantly focused on quantitative RNA-transcript measurements. However, part of the *NF1* DNA was directly screened for mutations and qualitative immunohistochemical analysis with a polyclonal antibody to full length neurofibromin was also performed.

The small part of the gene, containing the GAP related domain, that was analyzed by SSCP technique, did not disclose any mutations. However, RNA analysis showed an increased neurofibromin expression compared to normal brain, with levels ranging from 1.4 to 4.2 times the level of normal brain. Also 3 glioblastomas multiforme were analyzed, all had increased levels but less high than in the pilocytic astrocytomas. Furthermore, the 5 pilocytic astrocytomas that were immunohistochemically screened all showed strong cytoplasmic neurofibromin positiveness. The isoforms of the protein were differently expressed in normal brain and tumors, the former showing predominance of the type 1 isoform, and the latter of type 2.

The unexpected finding of an increased level of neurofibromin, very likely reflects an accumulation of wild type, based on the fact that the antibody reaction is quite specific for wild type neurofibromin.

Since normal rat glial cells mainly express type 2 neurofibromin and the neurons type 1, our finding of type 2 predominance in the tumors can be explained by the fact that the tumors contain more glial cells than neurons. A more tempting explanation for the overexpression of neurofibromin would be that this is a normal physiological response to reduce the p21*ras* mediated proliferative signals. This is supported by the finding of elevated neurofibromin levels together with elevated levels of activated p21*ras* in high grade astrocytic tumors.
These data illustrate that pilocytic astrocytomas overexpress specific \textit{NF1} gene transcripts, perhaps as a regulatory response to growth stimuli. The role of the \textit{NF1} gene as a tumor suppressor in pilocytic astrocytomas, however, remains to be proven.

**CHAPTER 8**

**GENERAL DISCUSSION AND CONCLUSIONS**

On theoretical grounds, the results of AgNOR staining and MIB-1 labeling combined in a mathematical multiplication, may reflect the proliferative potential more accurate than each of these proliferation markers by itself. When this is applied to the tumors that are studied, this theory is supported.

The absence of mutations in the \textit{NF1} gene suggests that this gene is not involved in pilocytic astrocytoma formation. The fact that tumors with \textit{TP53} mutations do not behave more malignant than those lacking mutations, makes it unlikely that \textit{TP53} gene mutations are an early event in tumorigenesis of pilocytic astrocytomas. Furthermore, it can be questioned whether the \textit{TP53} gene is involved in the formation of pilocytic astrocytomas. It is more likely that in pilocytic astrocytomas the \textit{TP53} and \textit{NF1} genes are both upregulated as a physiological response to abnormal cell proliferation, but they appear to be unable to correct the carcinogenic events that already have occurred in these tumors.

The algorithm for treatment and follow-up of patients with a pilocytic astrocytoma, as given in chapter 2, is adjusted. When the directly postoperative MRI-scan is negative for residual tumor, and after 1 year the control MRI-scan remains negative, the patient needs no further control. In case of postoperative residual tumor, MIB-1 LI and AgNOR staining values direct the further follow-up strategy. Patients with stable residual tumor and low AgNOR score in combination with negative MIB-1 labeling can be discharged from further control after 4 years. Residual tumors which are positive for MIB-1 labeling need yearly MRI surveillance life-long. Progressive residual tumors need to be re-operated. In case of progressive residual tumor at an inoperable site additional chemotherapy or (stereotactic) radiation therapy can be considered.