THE PILOCYTIC ASTROCYTOMA

IMMUNOHISTOCHEMICAL AND GENETIC STUDIES
IN RELATION TO TUMOR BEHAVIOR
The pilocytic astrocytoma, immunohistochemical and genetic studies in relation to tumor behavior.

Thesis Rijksuniversiteit Groningen - With a summary in Dutch.

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THE PILOCYTIC ASTROCYTOMA

immunohistochemical and genetic studies
in relation to tumor behavior

Proefschrift

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PROF. DR. W.M. MOLENAAR

REFERENT: DR. J. KOUDSTAAL
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CHAPTER 1

INTRODUCTION AND PURPOSE OF THIS THESIS
HISTORY

Harvey Cushing in 1931 described his experience with 76 treated cerebellar astrocytoma-like tumors and was the first to separate this group of tumors from other gliomas on the basis of 4 specific properties he had observed (1). The properties of these tumors were a cerebellar localization, frequently a cystic appearance, occurrence at a young age, and most important, a very good prognosis for the patient. Most of the cystic lesions of the cerebellum were previously referred to as "gliomatous cysts". It was Bergstrand in 1932 who first described the specific histologic appearance of these prognostic favorable lesions (2). He found that many of the cells in these tumors were uni- or bipolar spongioblasts, reminiscent of cells found during the late embryonal stage of development. Therefore, he proposed the term "gliocytoma embryonale" for these tumors. He also suggested that these lesions were congenital malformations and considered them to be hamartomas. Bucy and Gustafson could not support this theory and stated that the cerebellar astrocytoma is a neoplastic entity (3). Furthermore, they drew attention to the typical hyaline bodies so often found in these lesions and called them "Rosenthal fibers". Zülch stressed the fundamental difference in the cellular picture between cerebellar astrocytomas and cerebral astrocytomas, the former being a type of "spongioblastoma" (4). Ringertz and Nordenstam described their experience with 140 cerebellar astrocytomas, operated by Olivecrona between the years 1924 and 1948 (5). They also proposed the term spongioblastoma and noted that these tumors not only occur in the cerebellum but also in the cerebral hemispheres and in the brainstem. In 1977 the term "juvenile pilocytic astrocytoma" was introduced by Russell and Rubinstein (6). First in 1979 and later in 1993, the name "pilocytic astrocytoma" was given to these tumors by the World Health Organization in their classification of tumors of the central nervous system (7).

INCIDENCE

Pilocytic astrocytomas account for 6% of all brain tumors in humans, but in childhood for approximately 15% (8). After leukemia, brain tumors are the second most common malignancy of childhood. More than 50% of all pediatric brain tumors are located in the posterior fossa and one-third of these are pilocytic astrocytomas. Most frequently the cerebellar pilocytic astrocytoma occurs between the age of 5 and 10 years, equally distributed among both sexes (9). Two-third of the pilocytic astrocytomas are located in the cerebellum, the remainder in the optic pathways, in the hypothalamic area, in the third ventricle, in the cerebral hemispheres, in the pons, in the medulla oblongata and in the spinal cord. This means that the tumor can actually arise at any location in the central nervous system. The tumor occurs predominantly in the pediatric population. However in a recent series of 131 pilocytic astrocytomas, 28% occurred in patients above the age of 18 (10). Pilocytic astrocytomas are strongly associated with neurofibromatosis 1 (von Recklinghausen’s disease): 15% of patients suffering from neurofibromatosis 1 will develop a pilocytic astrocytoma, mostly in the optic pathways and 30% of patients presenting with an optic glioma will appear to have neurofibromatosis 1 (11). A case of a cerebellar astrocytoma has been described occurring in a child with a deletion of a part
of the long arm of chromosome 18 (12). A "cystic astrocytoma" with pilocytic areas and Rosenthal fibers has been described in association with Fahr's disease, which is synonymous to idiopathic nonarteriosclerotic cerebral calcification (13). The occurrence of a cerebellar juvenile pilocytic astrocytoma in a patient with alcaptonuria, later followed by the occurrence of a pituitary adenoma, has been reported (14).

**SYMPTOMS AND SIGNS**

Presenting symptoms and signs can be divided in those caused by increased intracranial pressure, and those which are the result of local brain dysfunction at the site of the tumor. Headache, vomiting and papilledema are the result of increased intracranial pressure, mostly caused by hydrocephalus due to obstruction of cerebrospinal fluid pathways. In children with a cerebellar tumor in general, gait abnormality, a wide-based gait, gait-ataxia and later on repeatedly falling may occur. Less common features are dysmetria and nystagmus. Sometimes stiffness of the neck or head tilt due to extension of the tumor or the cerebellar tonsils in the foramen magnum may be seen. Seldom there is strabismus, which is then caused by sixth nerve paresis due to increased intracranial pressure. In babies the increased head size may be the first symptom of a cerebellar tumor, furthermore the raised intracranial pressure may cause split sutures and a bulging fontanelle. Later the child becomes irritable, lethargic and has impaired attention. Occasionally a child may present with extreme drowsiness, bradycardia and slowed respiration. In the past, when these patients were presented late in the course of their disease, there were also the "cerebellar fits", short periods of coma accompanied by opisthotonic posture. Pilocytic astrocytomas on other locations give focal symptoms, such as visual disturbances, hypothalamic dysfunction and brainstem symptoms.

**MACROSCOPIC APPEARANCE**

Pilocytic astrocytomas of the cerebellum show a pink-grey color in most cases. The tumors are in 60%-80% of cases cystic, the remainder is solid (5,15-18). Most often they are located in the vermis of the cerebellum, expanding asymmetrically to one or both cerebellar hemispheres. Sometimes they are confined to one cerebellar hemisphere, showing a lateral position; then they mostly consist of a single large cyst containing a mural tumor nodule. They seldom grow infiltrating, about 8% of cerebellar pilocytic astrocytomas were assumed to infiltrate into the brainstem (19). However, in a recent study 30% of tumors showed brainstem infiltration during surgery (20). On computed tomography 80% of the cerebellar astrocytomas show contrast enhancement (21). This is probably due to vascular proliferation inside the tumor. In supratentorial low grade astrocytomas contrast enhancement is associated with a poor prognosis, whereas in pilocytic astrocytomas it has no prognostic value. On magnetic resonance imaging half the number of childhood cerebellar astrocytomas enhanced after gadolinium administration (22). However, in another study the T1-weighted post-gadolinium
MR images of 30 pilocytic astrocytomas at various localizations, showed enhancement in 93% of cases (23). In this study the T1 spin-echo signal of pilocytic astrocytomas was generally decreased and the T2 signal increased. Furthermore, 63% of cases had no associated edema in the surrounding brain and among 4 cases there was evidence of previous hemorrhage.

HISTOLOGY

The histologic appearance of the pilocytic astrocytoma is very distinct. Most tumors have a biphasic pattern, in which areas of loose texture with microcysts and stellate or protoplasmic-type astrocytes alternate with more compact areas containing the typical elongated (piloid) astrocytes with cytoplasmatic fibrillation (10). These two types of areas are present in highly variable proportions among different tumors. Some tumors even may show only one of these two areas. In the microcystic areas eosinophilic granular bodies are often seen. In the more compact areas Rosenthal fibers are almost invariably present. The tumors that only show the more loose textured area with microcysts and lacking the typical piloid areas were formerly referred to as the "diffuse type" of pilocytic astrocytoma. They make up for 15% of the pilocytic astrocytomas (10). Their behavior and prognosis is equal to the biphasic pilocytic astrocytomas.

Mitoses are infrequent, however they do occur in 7 to 20% of the cases but then they are very sparse (24). Necrosis is also rare and can be found in approximately the same frequency (24). Microcalcification is quite frequently found. Vascular proliferation is commonly seen, in such a degree that the picture resembles a vascular malformation, whereas endothelial proliferation is rare. Focal oligodendroglial features can be found in small amounts in the tumor.

Based on the variations in different histologic characteristics many authors have tried to distinguish subgroups among these tumors. The most well known subdivision has been made in the "juvenile pilocytic astrocytoma" and the "diffuse" type (6), the first one showing the typical picture of alternating areas of loose microcystic structure and of solid and compact structure. In the second type there is a more even distribution of glial fibrils and there are small and uniform cells. The diffuse type is supposed to be far less frequent than the juvenile type, it appears in older children and grows more infiltrating. The 25 year survival for the juvenile type is 94% and for the diffuse type 38%. Winston et al. scored all the single microscopical characteristics of 132 cerebellar gliomas and related these to survival (25). This led them to the conclusion that the so called type A cerebellar glioma, consisting of microcysts, Rosenthal fibers, leptomeningeal deposit and focal oligodendroglial components, had a 10 year survival rate of 94%, whereas the type B, consisting of high cellular density, mitosis, necrosis and calcification had a 10 year survival rate of 29%. Another study regarded the uneven distribution of fibrils in connection with lack of high cell density, necrosis and mitosis as the most important favorable factor (26).

In later studies the existence of these subclassifications was not supported and especially the differences in survival could not be confirmed. The features necrosis, mitosis and endothelial proliferation were not related to survival in these studies (10,27-30). Consequently, the conventional Kernohan or St. Anne-Mayo grading systems for astrocytomas do not apply for pilocytic astrocytomas as far as a relation to survival is concerned (31). Also in the WHO-classification the pilocytic astrocytoma is a distinct entity among astrocytic tumors.

The above information about the histology is mainly derived from studies that investigated cerebellar...
pilocytic astrocytomas, however it applies also for other pilocytic astrocytomas, on every possible localization.

Most optic pathway and hypothalamic gliomas are thought to be pilocytic. However, it is difficult to establish to which extent, since most studies only speak of "optic glioma" or "low grade glioma", 60% seems to be pilocytic and 40% fibrillary (32). Most optic gliomas occur in early childhood. When they occur in adults they may exhibit characteristics of malignant gliomas and show an aggressive behavior (33). In the brainstem and in the cerebral hemispheres the diffuse (grade 2) astrocytoma far outnumbers the pilocytic astrocytoma (10).

Also malignant forms of pilocytic astrocytomas, with aggressive behavior, exist. They are infrequent and most are reported to occur on the site of a "benign" pilocytic astrocytoma treated many years previously (34,35). Almost all of these patients had undergone radiation therapy many years before and possibly the malignant recurrence was radiation induced. Also primary forms of malignant pilocytic astrocytomas have been described, these tumors showed mitotic activity of more than one per high power field (x250), endothelial proliferation and necrosis (36). The prognosis of these tumors seems to be much less favorable than for the “benign” pilocytic astrocytomas, but still much better in comparison to diffuse fibrillary astrocytomas showing the same histological features.

TREATMENT AND PROGNOSIS

The treatment of first choice is surgical resection; a statement already made by Harvey Cushing and upheld until now. The surgeon must aim for total resection. This is an achievable task for most cerebellar and cerebral hemispheric lesions. However, for deep seated lesions, such as the optic pathway, hypothalamic and brainstem localizations this will often be impossible.

There is some discussion about the cyst-wall, when there is a single cyst with a mural tumor nodule. If this wall shows contrast-enhancement on computed tomography or magnetic resonance imaging, it very likely contains tumor cells, and should be resected (29). In non-enhancing cases resection of the nodule only will be sufficient. When the inner wall of the cyst is smooth and glossy, the presence of tumor cells is very unlikely. On the other hand, when the cyst-wall is thick, not shiny and shows a gelatinous aspect it is probably infiltrated by tumor and should be resected (37). Others advise to biopsy the cyst-wall in all cases and to resect it when tumor cells are seen on frozen sections (30). Recently it was stated that cyst-wall excision did not influence outcome or risk of recurrence (20).

Survival after total tumor removal is excellent: Cushing in his well known series of 76 patients established 28 total resections of cerebellar lesions, the 20 year survival among this subgroup was 100% (1). True tumor recurrence, which is recurring tumor after complete resection, is extremely rare for pilocytic astrocytomas (38). Therefore, the prognosis for pilocytic astrocytomas, when resected totally, is excellent.

The problem in treatment for these tumors lies in those patients where total tumor resection is not feasible. In cerebellar tumors this problem occurs when the tumor has infiltrated the brainstem; than total resection is not always possible. Pilocytic astrocytomas of the optic pathways, hypothalamus or basal ganglia are hardly ever totally resectable. Therefore, twenty year survival for chiasmal gliomas in one study of 28 patients was only 43% (39). Nevertheless, even after incomplete resection the prognosis can be very favorable since the tumor remnant may remain "quiescent" for many years (16,18,27,40). It has been reported that pilocytic astrocytomas of the brainstem or the
optic-hypothalamic area behave more benign when associated with neurofibromatosis 1 (41,42). Formerly, radiation therapy was often used to treat the tumor remnant and a beneficial effect was reported several times (9,43-45). Others stated that radiotherapy has no influence on the tumor (1,30,46). Because of the deleterious side effects of irradiation on the developing brain, the use of it is contra-indicated below the age of 4 years and after macroscopically total resection. In the present time it is generally believed that conventional radiation therapy has no place in the treatment of cerebellar pilocytic astrocytomas; exceptions are a different histologic appearance or re-recurring tumor at an inoperable site (8,46). However, radiation therapy is still frequently used in the treatment of optic, diencephalic and brainstem gliomas (47-50). Probably such treated patient series contain pilocytic astrocytomas next to other type of gliomas.

Results of chemotherapy are only reported for the treatment of optic and brainstem gliomas. In two studies a decrease of tumor size was reported (51,52). The best treatment for chiasmatic-hypothalamic gliomas is uncertain. Patient series that were treated with radical surgical (incomplete) resections had no better outcome than those where the tumors were only biopsied and tumor growth was controlled by either chemotherapy in very young infants or radiation therapy in older patients, with 10-year survival rates ranging from 57% to 90% (47-50,53). Experience with stereotactic radiosurgery is very limited. However, Grabb et al demonstrated a possible beneficial effect among 8 patients with residual pilocytic astrocytomas after surgery: 3 tumors disappeared, 4 decreased in size and 1 remained unchanged after stereotactic radiosurgery (54).

The successful treatment of a patient with a pilocytic astrocytoma depends primarily on the extent or possibility of surgical resection. It is known from older studies that tumors and tumor remnants may remain quiescent for many years (16,18,27,40). Therefore, the neurosurgeon has to make a difficult decision: taking the risk of surgically induced neurological damage when aiming for total tumor resection on one hand or refraining from risky surgery and accepting residual tumor on the other. This problem, as has been stated before, is especially encountered in the treatment of tumors on deep seated localizations of the brain, such as in the brainstem, the hypothalamus and the optic chiasm.

Different neurosurgical clinics uphold controversial opinions on the best treatment for these patients. These range from very aggressive surgical to strictly conservative expectative attitudes. Advocates of aggressive treatment use as motivation the fact that residual tumors may progress, sometimes even rapidly (55,56). Supporters of the opposite opinion are motivated by the frequently seen benign and quiescent behavior of the residual tumor (39,57).

The use of other treatment modalities such as radiation therapy and chemotherapy, are subject to similar diverging opinions, since it is not known how effective they are.

The existence of these dualistic views is mainly caused by the fact that we do not know what the behavior of the residual tumor will be. Therefore, it would be very rewarding to find new characteristics of the tumor that can predict behavior, and can be of help in determining prognosis and treatment for patients with residual pilocytic astrocytoma.
THE PURPOSE OF THIS THESIS

1. Determining the frequency and behavior of residual tumor after surgical treatment of pilocytic astrocytomas localized in an operable site: An extensive literature review is performed on previous patient studies. Medical files of 73 patients with cerebellar pilocytic astrocytomas are retrospectively investigated and follow-up neuroimaging studies are analyzed in order to draw up an inventory of possible manifestations of biological tumor behavior. Patient outcome is listed and a treatment protocol drafted (chapter 2).

2. Finding methods and techniques to study cell kinetics and proliferative potential, which have been applied to other tumors and not yet to pilocytic astrocytomas: Two relatively simple tissue staining techniques, AgNOR staining and Ki-67 labeling, are described, including their physiological background and technical properties. The reliability of these techniques is discussed in relation to predictive value on tumor behavior as derived from previous studies (Chapter 3, first two paragraphs).

3. Exploring recent knowledge and insights in molecular genetics of oncogenesis to understand the paradoxical neoplastic character of pilocytic astrocytomas: Implications of two genes and the products these genes code for in carcinogenesis are reviewed. The TP53 gene for being the most frequent mutated gene in human cancers and for being involved in the formation of astrocytomas grade II-IV. The Neurofibromatosis 1 gene for the existing relation between von Recklinghausen’s disease and (optic) pilocytic astrocytomas. (Chapter 3, last two paragraphs).

4. Application of cell kinetic studies to tumor material and relating the results to true tumor behavior as observed on follow-up neuroimaging studies of the patient: AgNOR staining and Ki-67 labeling (MIB1-labeling) are performed on tumor tissues and the results are related to behavior of the residual tumor (Chapters 4 and 5).

5. Application of molecular genetic studies on tumor tissue to study the role of known tumor suppressor genes: Immunohistochemical p53 labeling and screening for TP53 mutations are performed in order to establish the role of this gene in pilocytic astrocytomas. The product of the NF1 gene, neurofibromin, is qualitatively and quantitatively studied to test the hypothesis that the NF1 gene functions as a tumor suppressor gene also in pilocytic astrocytomas. (Chapters 6 and 7).

6. Establishing new parameters that can predict the behavior of the tumor in order to set a rationale for the treatment and follow-up of patients with residual or inoperable pilocytic astrocytomas: An attempt is made to combine results of the performed studies in order to find a combination of tests that gives a high predictive value on tumor behavior. The role of TP53 and NF1 is discussed. An adjusted treatment and follow-up algorithm is presented. (Chapter 8).
REFERENCES

CHAPTER 2

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

Dirven CMF, Mooij JJA, Molenaar WM
INTRODUCTION

The pilocytic astrocytoma was first described as a separate entity by Cushing in 1931 (8). Other authors gave the same tumor different names: gliocytopma embryonale (Bergstrand in 1932), spongioblastoma (Zulch in 1940), classic juvenile astrocytoma (Rubinstein and Russel in 1977) and astrocytoma grade I according to the WHO classification from 1979. Controversial opinions exist concerning the origin of these tumor cells, whether this tumor arises from true astrocytes or from subependymal cells called tanycyes remains unclear. However, strong agreement exists on the very good prognosis, since this tumor has a twenty-five year survival rate after surgical resection varying from 50% to 94 %. This tumor accounts for 6% of all primary brain tumors, for 20% of all primary brain tumours in children under 15 years of age, and for 30% of all posterior fossa tumors in these children. Of all pilocytic astrocytomas 80% have a cerebellar localization and 20% are located in the hypothalamic region, in the cerebral hemispheres, third ventricle, optic pathways or brainstem. Treatment consists primarily in surgical resection and in the past sometimes radiotherapy after incomplete removal.

Despite the very good prognosis, the outcome is not always favorable, mainly because of surgical morbidity and tumor recurrence. The behavior of this tumor after complete or incomplete resection is unpredictable. The use of additional radiation therapy in the treatment remains controversial. The question as to wether all patients or only patients in whom resection has been incomplete need follow-up and how long such follow-up should be, remains unanswered.

This retrospective study was undertaken to assess characteristics of the patient, the treatment-modality and the tumor, specifically its behavior during follow-up, that might be related to the outcome of the patient. Therefore, all data in the records of 73 patients treated for cerebellar pilocytic astrocytoma in three major neurosurgical units in the Netherlands were analyzed. Based on these data and previously reported material, we established a protocol for treatment and follow-up of these patients.
MATERIAL AND METHODS

Medical files of 73 patients, treated for a cerebellar pilocytic astrocytoma between 1969 and 1990 at three University Hospitals in the Netherlands, were analyzed. They were screened for patient age, sex, treatment modality (surgery or surgery and radiation therapy), and duration of follow-up. The radicalness of resection was estimated by analyzing the surgeons’ surgical reports and by the results of postoperative neuroimaging. All neuroimaging results during the period of follow-up, either by MRI or CT scan, were obtained and reviewed. They were judged on the presence or absence of residual or recurring tumor. Recurring tumor is defined as the appearance of a new tumor after neuro-radiologically proven total resection. The clinical status of the patient at the end of the follow-up period was used as a basis for classification in a five grade outcome scale. Grade 1: no neurological deficit, grade 2: minor neurological deficit but leading normal life, grade 3: severe neurological deficit requiring special therapy or schooling, grade 4: disabled with permanent institutional stay, grade 5: death. Grade 1 and 2 are regarded as favorable, grade 3, 4 and 5 as unfavorable. Complications of treatment were listed and regarded directly related to surgery when they appeared within one month after the surgical procedure. Number and causes of deaths were listed. Archival tumor tissue was reviewed (W.M.M.).

RESULTS

The mean age at first operation was 10.8 years. All patients were under 25 years of age, except 3 who were 27, 33 and 39 years. Male to female ratio was 1.1:1. Neuro-radiological follow-up either by CT- or MRI-scans was established in 62 patients and the duration ranged up to 20 years after the operation, with a mean of 5.3 years. The follow-up period of most of the patients was extended by interviewing the family doctor or the pediatric doctor, and is referred to as "clinical follow-up" in these cases. The duration of clinical follow-up ranged from 4 months to 26 years with a mean of 8.2 years for all 72 patients, 1 patient died within one month of the operation. Surgical complications can be divided into those occurring early, i.e. within 1 month after operation, and those occurring late. The number and nature of the surgery-related complications in the whole group are listed in table 1.
**TABLE 1.** Early (within one month after surgery) and late complications related to surgery, number of patients and percentages in parentheses.

<table>
<thead>
<tr>
<th>Surgical complications:</th>
<th>Morbidity</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early (10; 13%)</td>
<td>-postoperative blindness (4)</td>
<td>-postoperative hematoma causing death (1)</td>
</tr>
<tr>
<td></td>
<td>-outcome grade 3 or 4 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-subdural hygroma (1)</td>
<td></td>
</tr>
<tr>
<td>Late (4; 6%)</td>
<td>-cervical kyphosis, internal fixation (1)</td>
<td>-Draindysfunction, elevated ICP leading to death (2)</td>
</tr>
<tr>
<td></td>
<td>-Intraventricular hemorrhage after shuntrevision (1)</td>
<td></td>
</tr>
<tr>
<td>Total (14; 19%)</td>
<td>(11; 15%)</td>
<td>(3; 4%)</td>
</tr>
</tbody>
</table>

Postoperative irradiation was administered to 10 patients (14%). Two of them had had total tumor removal according to the surgeon and 8 had residual tumor. Irradiation doses given were 5000-6000 rad in 6 cases, 4450 rad in one case, 3180 rad in one case and are unknown in two cases. Postirradiation follow-up of the patients irradiated for residual tumor showed progression of tumor in 4 patients, 2 of whom developed intratumoral hemorrhage requiring surgical decompression. In 1 patient the residual tumor remained unchanged and in 1 patient it regressed until it was disappeared on MRI-scan 4 years after irradiation. Two patients had no follow-up imaging but are alive 2 years and 12 years after surgery.

Table 2 lists overall outcome at the end of follow-up for the whole group. This grossly results in 80% favorable outcomes (grade 1 and 2) and 20% unfavorable outcomes (grade 3, 4 and 5).

**TABLE 2.** Outcome at end of follow-up for whole group.

<table>
<thead>
<tr>
<th>Grade</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1: No neurological deficit</td>
<td>56%</td>
</tr>
<tr>
<td>Grade 2: Minor neurological deficit, leading normal life</td>
<td>23%</td>
</tr>
<tr>
<td>Grade 3: Severe neurological deficit, requiring special therapy or schooling</td>
<td>11%</td>
</tr>
<tr>
<td>Grade 4: Severely disabled, permanent institutional stay</td>
<td>3%</td>
</tr>
<tr>
<td>Grade 5: Death</td>
<td>7%</td>
</tr>
</tbody>
</table>
Five patients died; 1 patient in the postoperative period because of hematoma, and 4 some months or years later; 1 after malignant transformation and brainstem invasion of the tumor, and 2 because of transtentorial herniation owing to elevated intracranial pressure resulting from dysfunction of a ventriculoperitoneal shunt. One patient died from progression of optic astrocytoma since this patient suffered from neurofibromatosis type 1. Table 3 shows a comparison of the results noted in surgical reports and the results of postoperative neuroimaging concerning extent of surgical resection. Postoperative CT or MRI scans performed within 1 year after surgery were available for 48 patients: 33 (69%) showed no tumor and 15 (31%) showed residual tumor. In 15 out of 48 cases (31%) the surgeons’ opinion did not correlate with the result of neuroimaging.

**TABLE 3.** Comparison of the neurosurgeons opinion and the result of postoperative neuro-imaging, CT- or MRI- scans within one year after surgery, concerning the extent of surgical resection in 48 cases.

<table>
<thead>
<tr>
<th>Resection according to neurosurgeon:</th>
<th>Result of postoperative neuroimaging:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete: 32 cases (67%)</td>
<td>No tumor: 25 cases</td>
</tr>
<tr>
<td></td>
<td>Residual tumor: 7 cases</td>
</tr>
<tr>
<td>Incomplete: 16 cases (33%)</td>
<td>No tumor : 8 cases</td>
</tr>
<tr>
<td></td>
<td>Residual tumor: 8 cases</td>
</tr>
</tbody>
</table>

In total 25 patients had residual or recurring tumor. There were 15 in whom tumor progression was detected during further follow-up, whereas in 8 patients the residual tumor remained "silent" in a follow up period from 1-11 years (mean 4.5 years). In 2 patients the residual tumor showed regression; in 1 this was spontaneous after 10 years and in 1 it followed radiation therapy. Thus during continued follow-up different patterns of biological behavior of the residual tumor were seen. These patterns are listed in table 4.
TABLE 4. Different patterns of biological behavior as seen among 25 patients with residual or recurring tumor.

<table>
<thead>
<tr>
<th>Biological behavior following surgical treatment</th>
<th>nr.of cases:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progression of residual tumor:</td>
<td>11</td>
</tr>
<tr>
<td>Recurrence after &quot;total&quot; neurosurgical tumor removal:</td>
<td>1</td>
</tr>
<tr>
<td>Recurrence and metastatic spread along craniospinal axis:</td>
<td>1</td>
</tr>
<tr>
<td>Progression of residual tumor with malignant transformation:</td>
<td>1</td>
</tr>
<tr>
<td>Hemorrhage and progression of residual tumor after irradiation:</td>
<td>1</td>
</tr>
<tr>
<td>Residual tumor without progression on follow-up imaging:</td>
<td>8</td>
</tr>
<tr>
<td>Spontaneous regression of residual tumor:</td>
<td>1</td>
</tr>
<tr>
<td>Regression of residual tumor after irradiation:</td>
<td>1</td>
</tr>
</tbody>
</table>

Neuroradiological follow-up demonstrated progression of residual tumor in 13 cases and true recurrence of tumor in only 2 cases. Characteristics of these 15 patients are listed in table 5. The final results of neuro-imaging at the end of follow-up in the whole group, including the re-operated patients, show that of 68 living patients 43 (63%) are free of tumor, and 15 (22%) still have residual tumor. For the remaining 10 patients (15%) no postoperative CT or MRI scans were available.

TABLE 5. Characteristics of 15 patients with tumor-progression after surgical treatment compared to results in whole group.

<table>
<thead>
<tr>
<th></th>
<th>15 patients with tumor-progression:</th>
<th>whole group:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at first operation:</td>
<td>8.7 years</td>
<td>10.8 years</td>
</tr>
<tr>
<td>Male to female ratio:</td>
<td>1:2</td>
<td>1:1:1</td>
</tr>
<tr>
<td>Outcome: favorable (grade 1 and 2):</td>
<td>80 %</td>
<td>80 %</td>
</tr>
<tr>
<td>unfavorable (grade 3,4 and 5):</td>
<td>20 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Resection complete:</td>
<td>30 %</td>
<td>69 %</td>
</tr>
<tr>
<td>resection incomplete:</td>
<td>70 %</td>
<td>31 %</td>
</tr>
<tr>
<td>Interval between first and second operation</td>
<td>0.5-4 years:10 6</td>
<td></td>
</tr>
<tr>
<td>(2 patients were operated three times; n=17):</td>
<td>7 years: 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9-11 years: 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 years: 1</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Several factors might have an influence on patient-outcome after treatment for a cerebellar pilocytic astrocytoma. In this group of patients these factors can be categorized in three groups: patient-related, treatment-related and tumor-related factors.

Age and sex are patient-related factors that had no predictive value for the outcome of the patients in previous studies (9,14). Treatment-related factors are the extent of surgical tumor-removal, surgical mortality and morbidity, the effect of radiation therapy on the tumor and the side effects of irradiation. As can be seen from table 3 the surgeons estimation of extent of tumor removal did not correspond with postoperative neuroimaging results in 31% of cases. This makes a postoperative scan, preferably an MRI-scan obligatory to assess the patient’s needs for follow-up screening. The development of better neurosurgical and anaesthesiological techniques in recent decades have led to a fall in the mortality and the morbidity of the surgical procedure. Mortality rates recorded in several studies are given in Table 6.

<table>
<thead>
<tr>
<th>Author</th>
<th>Operated between</th>
<th>Number of patients</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garcia et al (12)</td>
<td>1928-1980</td>
<td>84</td>
<td>10%</td>
</tr>
<tr>
<td>Laws et al (24)</td>
<td>1916-1976</td>
<td>190</td>
<td>15%</td>
</tr>
<tr>
<td>Ferbert, Gulotta (11)</td>
<td>1950-1972</td>
<td>89</td>
<td>50%</td>
</tr>
<tr>
<td>Szenazy, Slowik (32)</td>
<td>1954-1975</td>
<td>128</td>
<td>10%</td>
</tr>
<tr>
<td>Lapras et al (23)</td>
<td>? - 1987</td>
<td>63</td>
<td>5%</td>
</tr>
<tr>
<td>Undjian et al (34)</td>
<td>1954-1984</td>
<td>100</td>
<td>29%</td>
</tr>
<tr>
<td>Cushing (8)</td>
<td>? - 1931</td>
<td>76</td>
<td>18%</td>
</tr>
<tr>
<td>Kehler et al (18)</td>
<td>1955-1980</td>
<td>99</td>
<td>12.1%</td>
</tr>
<tr>
<td>Hojer et al (16)</td>
<td>1978-1993</td>
<td>33</td>
<td>6%</td>
</tr>
<tr>
<td>This study</td>
<td>1969-1990</td>
<td>73</td>
<td>4%</td>
</tr>
</tbody>
</table>
The effect of radiation therapy on residual tumor after surgery is disputed in the literature, the long-term prognosis of patients treated with surgery and radiation therapy apparently being not different from that of those treated with surgery alone (11,12,23,24). However, the numbers in these studies are small. On the other hand the use of radiotherapy seems to delay or stop the progression of residual tumor (7,9,15,31) but may also play a part in the late recurrence with malignant transformation and in the induction of new tumors in the irradiated field such as meningiomas and sarcomas and tumors of the parotid and thyroid glands. Some authors advocate irradiation after incomplete removal of the tumor when histological "malignant" features are present, regardless the extent of resection (34,36). Irradiation is contraindicated in children under the age of 3 years because of the devastating effect it has on the developing brain, in particular intellectual impairment and dysfunction of the endocrine system.

Earlier studies have tried to identify tumor-related factors that had a predictive value on outcome (5,13). The lateral localized tumors and the cystic types had a better prognosis than the medial localized and solid ones. Later these results were attributed to the fact that in those days such tumors were more easily completely resected (6,9,12,14,34).

Another distinction was made between a diffuse and a classic juvenile type (14,29) on the basis of histological characteristics; the 25-year survival rate was 94% for the classic juvenile type and 38% for the diffuse type. Later studies confirmed neither the existence of these subtypes, nor the difference in prognosis (23,28). Winston et al (37) classified cerebellar astrocytomas as subtypes A and B, based upon clinical characteristics retrospectively correlated to outcome. Subtype A had any of the following histologic features: microcysts, leptomeningeal deposits, Rosenthal fibers and focus of oligodendroglia. Subtype B had perivascular pseudorosettes, high cell density, necrosis, mitosis, calcification and a less uniform histologic pattern as subtype A. Type A had a 10-year survival rate of 94% and type B, one of 29%.

From the different possible manifestations of the biological behavior, the progression of residual tumor, often in previous studies referred to as "recurrence", is most frequently encountered (table 4). The incidence of "recurrences" varied in 14 larger studies from 7% to 35%, with a mean of 24% (Table 7). Whether these "recurrences" were new tumors after complete surgical resection, or residual tumors that show progressive growth, remains unclear. In our material we found progression of tumor after operation in 21% (15 patients). However in only two of those patients was there true recurring tumor, while in 13 there was progression of residual tumor. We could not identify any factors related to patients who developed progression of residual tumor or recurring tumor that were different from those related to the whole group (table 5). The male-to-female ratio in the group with progressive tumor was 1:2, and that in the whole group 1.1:1. Age at first operation for the tumor was lower in the group with tumor-progression than in the whole group; 8.7 vs. 10.8 years. Most tumor progressions were detected within 4 years after operation (10 out of 17).
<table>
<thead>
<tr>
<th>Study</th>
<th>nr. of cases</th>
<th>corrected (*1)</th>
<th>nr. recurrences</th>
<th>% recurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cushing 1931 (8)</td>
<td>76</td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Ringertz, Nordenstam 1951 (19)</td>
<td>140</td>
<td>93</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Geissinger, Bucy 1971 (13)</td>
<td>38</td>
<td>34</td>
<td>12</td>
<td>35</td>
</tr>
<tr>
<td>Gjerris, Klinken 1978 (14)</td>
<td>44</td>
<td>44</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Griffin et al 1979 (15)</td>
<td>39</td>
<td>38</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>Davis, Joglekar 1981 (9)</td>
<td>43</td>
<td></td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>Szenasy, Slowik 1983 (32)</td>
<td>128</td>
<td>125</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Palma et al 1984 (28)</td>
<td>49</td>
<td>49</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td>Ferbert, Gullotta 1985 (11)</td>
<td>89</td>
<td>44</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Ilgren, Stiller 1986 (17)</td>
<td>112</td>
<td>112</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>Lapras et al 1986 (22)</td>
<td>63</td>
<td>59</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Austin, Alvord 1988 (3)</td>
<td>41</td>
<td>41</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>Undjian et al 1989 (34)</td>
<td>100</td>
<td>71</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td>Garcia et al 1989 (12)</td>
<td>84</td>
<td>66</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Present study</td>
<td>73</td>
<td>72</td>
<td>15(*2)</td>
<td>21</td>
</tr>
</tbody>
</table>

*1 = Number of cases corrected for post-operative deaths and patients lost to follow-up.

*2 = In this study 13 cases with progression of residual tumor and 2 cases of recurring tumor.

The outcome in the whole group is regarded as favorable (grade 1 and 2) in 79% of patients. The outcome in the group treated for postoperative tumor progression was also favorable in approximately 80% of patients. However, complete resection during a subsequent operation was only possible in 30% of patients, whereas complete resection of the primary tumor at first operation was achieved in 69% of cases. This means that re-operation for progressing tumor does not influence final outcome, but patients with residual tumor do need control neuroimaging studies and will very likely need one or more re-operations.

It is important to stress that not every patient with residual tumor after operation develops tumor progression. In this group 8 patients have residual tumor that has not shown any progression during 1 to 11 years (mean 4.5 years) follow-up by neuroimaging. Another two patients experienced regression during follow-up; 1 spontaneously and 1 after irradiation. These phenomena, stabilization of residual tumor many years after operation, even after only biopsy of a large tumor, and
spontaneous regression, are well known from the literature (3,6,9,11,13,20,23). Metastatic spread is mentioned in the literature in only a few cases: Eade and Urich (10) describe 5 young patients, 4 with a spinal and 1 with a thalamic glioma spreading via the cerebrospinal fluid. McLaughlin described four thalamic gliomas in children with cerebrospinal fluid seeding (26), Shapiro and Shulman reported 3 cerebellar astrocytomas that seeded to the spinal cord (30), Auer et al described 1 case of benign cerebellar astrocytoma with massive craniospinal leptomeningeal spread prior to surgery (2), and Wallner et al report 1 case of diffuse leptomeningeal seeding after four local recurrences of a cerebellar pilocytic astrocytoma over a 23-year period (36). In another reported case leptomeningeal seeding occurred 6 years after surgery for a cerebellar pilocytic astrocytoma located in the vermis (27). The multiple nodular metastatic lesions remained stable during a two year observation period. In a recent study 11 out of 90 patients with pilocytic astrocytomas at different localizations had metastatic spread along the craniospinal axis, proven by MRI studies (25). Only 1 of these 11 patients had a primary cerebellar pilocytic astrocytoma, and it was concluded that the hypothalamic pilocytic astrocytoma was 23 times more likely to show metastatic spread than the cerebellar one. Garcia et al report 1 patient with a spinal recurrence of a cerebellar pilocytic astrocytoma (12). In our study 1 patient had metastatic lesions in the third ventricle and the spinal canal, detected 1 year after surgery for a cerebellar lesion together with a local recurrence, the local recurrence was extirpated and showed no different histology than the benign pilocytic astrocytoma extirpated one year before. In the 2-year follow-up after re-operation the metastatic lesions remained stable and were not treated since the patient had no complaints. The easier detection of spinal lesions by the more frequent use of MRI studies might show that metastatic spread of the pilocytic cerebellar astrocytoma is not as rare as formerly thought. In this respect careful tumor handling and intraoperative closure of the spinal canal at the foramen magnum or C1 level may be of importance to prevent the spread of tumor cells.

Malignant or anaplastic transformation is also a rare phenomenon of the cerebellar pilocytic astrocytoma. Wallner et al (36) describe 3 patients who showed anaplastic transformation of recurring tumor. In one of these the primary tumor had a cerebellar localization and this patient had undergone radiation therapy 21 years before. Kleinman et al (21) report 1 case of malignant degeneration 48 years after partial removal and irradiation. Their literature review lists 4 more cases of malignant transformation 20-39 years after surgery, 3 of them in patients who had had radiation therapy. Five other cases have previously been described with time intervals between surgery and malignant recurrences of 50, 21, 10 and 5 years (1,33,35,38). In all cases radiation therapy had been given in dosages of 12, 50 and 60 Gray.

In the patient in our study, who was operated on twice for local recurrence of a benign pilocytic astrocytoma in a period of 2.5 years, 16 months after the last operation the tumor recurred again; then subtotal resection of a malignant ependymoma invading the brainstem was performed, 3 weeks later the patient died. This patient had not previously been treated with radiation therapy. In the aforementioned 12 cases of malignant degeneration 10 of the patients had been treated with radiation therapy many years before. For 1 patient, in whom the malignant recurrence appeared after 20 years, the case report does not mention the use of irradiation (4; case 1). The patient from our study, who was not treated with irradiation, showed malignant recurrence after 2.5 years. The initial histologic appearance of our patient was of a mixed type, the pilocytic astrocytoma also having areas reminiscent of subependymoma. The recurrence showed characteristics of a malignant ependymoma.

Spontaneous arising or de novo malignant transformation in pilocytic astrocytomas of the cerebellum
is described in 6 cases (33). It remains questionable whether very late occurring malignant recurrences are the result of spontaneous tumor degeneration or of induction by the radiation therapy applied many years previously. The literature records only 1 case of local invasive growth from a cerebellar pilocytic astrocytoma: this tumor had infiltrated the dura mater, the bone of the skull and the soft tissues of the neck and is the same tumor as described earlier, which showed metastatic spread (19).

CONCLUSION

In the study group of 73 patients treated for cerebellar pilocytic astrocytoma, 15 patients (21%) had tumor progression after the initial surgical treatment. The majority of these patients had postoperative residual tumor. Only in 2 patients was a true recurrence present and 1 of these also had metastatic spread of the tumor. The surgeons’ opinion of the extent of tumor resection is not very reliable, whether it is thought to have been complete or incomplete. Therefore, early postoperative neuroimaging, preferably by MRI, is indicated to establish the duration and frequency of follow-up consultations with or without imaging studies. Tumor progression mostly appeared within 4 years after surgery. Despite its well-known benign nature, this tumor is capable of metastatic spread and malignant transformation. Steps must be taken to prevent craniospinal spread of tumor cells during the operation. Re-operation for progression of residual tumor or recurring tumor had no influence on outcome; however, total removal was only possible in 30% of cases. This means that the majority of patients with residual tumor after operation need to be subjected to the burden of periodic medical controls and neuroimaging and, possibly reoperations for the rest of their lives. Since residual tumor may remain silent for many years, not every residual tumor needs to be operated on. In this respect a “wait-and-see” policy can be advocated. Neuro-radiologically proven progression of residual tumor in the absence of clinical symptoms is, however, an indication for re-operation. The importance of aiming for total tumor removal at first operation can not be overstressed. Patients at risk of an unfavorable outcome or recurring tumor could not be determined by this study. Adequate postoperative follow-up is of the utmost importance; we suggest a follow-up flow-chart such as that seen in figure 1. In case of total tumor removal confirmed by postoperative MRI scan, the follow-up period can be short. Such a patient can be discharged as needing no further controls after 4 years when a third MRI scan remains negative for residual or recurring tumor. When residual tumor is seen on the first postoperative MRI scan follow-up needs to be continued with MRI scans on a yearly base. In general, radiation therapy has no place in the treatment of this tumor. However, earlier reports do suggest a beneficial response of the tumor to this therapy in certain cases. The use of radiotherapy can be considered in children over 3 years of age in the case of a radiological or symptomatic recurrence, which means residual or recurring tumor after a second operation, in the case of a recurrence with "malignant" histological characteristics and in the case of a progressive or recurring lesion at an inoperable site, such as the brain stem. More research is needed to determine the biological behavior of the pilocytic astrocytoma, to find optimal treatment and to decide whether or not particular patients might be helped by radiotherapy.
FIGURE 1. Algorithm for the treatment of patients with a cerebellar pilocytic astrocytoma:

Operation for cerebellar pilocytic astrocytoma

Direct post-operative MRI
(within 48 hours)

Residual tumor

RESIDENTIAL ON

Repeat MRI after 1 year

Repeat MRI after 1 and 4 years

Residual tumor

Progression or recurrence

No residual tumor

No further control

Re-operation

Re-occurring tumor
- at inoperable site (brainstem)
- "malignant" histology
- over 3 years of age

Consider radiation therapy

Progression
or Spontaneous regression
REFERENCES

CHAPTER 3

INTRODUCTION TO THE METHODS USED FOR STUDYING THE BIOLOGICAL BEHAVIOR OF PILOCYTIC ASTROCYTOMAS
1. AgNOR-STAINING

Nucleolar organizer regions (NORs) are specific portions of DNA, called rDNA, that, by using the enzyme RNA-polymerase-1, code for the transcription of ribosomal RNA (rRNA). This rRNA inside the ribosomes is responsible for protein synthesis of the cell. Protein synthesis is a necessary step in the process of cell proliferation. Therefore a relation between NORs and cell proliferation is suggested.

The existence of NORs has been well-known to cytogeneticists for many years, this NOR-DNA was visualized by in situ hybridization making use of radio-labeled rRNA, a reliable but time-consuming method. With the use of silver colloid impregnation, described by Goodpasture and Bloom in 1975 (1) and modified by Ploton et al. in 1986 (2), NORs can be identified much easier. By using the silver nucleolar organizer region (AgNOR) impregnation technique the number, size and shape of NORs can be studied in a fast and simple way, not only in fresh frozen tissue specimens but also in formalin fixed paraffin embedded material. The amount of silver deposit in a cell, reflecting the amount of NORs that are involved in protein-synthesis, is thought to be related to the proliferative capacity of that cell. The exact relationship between proliferation, protein-synthesis and expression of AgNORs is, however, not yet well understood. The expression of AgNOR is either causally or indirectly coupled to DNA-synthesis and thus AgNOR can be considered as a cell proliferation marker (3). Therefore, in recent years the AgNOR staining method has become a new tool among the diagnostic possibilities for histopathologists.

NORs reside on the short arms of the acrocentric human chromosomes 13, 14, 15, 21 and 22, these areas are the sites which hybridize with rRNA and are of importance with respect to the ultimate synthesis of protein. NOR-DNA, the associated proteins and rRNA are located in the nucleolus of the cell, where also the nucleolar chromosomes rest in interphase (3). In simple terms: NORs are parts of DNA in the nucleolus that encode for rRNA, this rRNA forms the ribosomes, the "protein-factories" of the cell.

The NOR-associated proteins bind very well to silver. This argyrophilia is associated to the step of phosphorylation of the protein nucleolin (protein C23), by which this protein is activated, and the transcription of rDNA is made possible. Nucleolin is the major silver staining protein in this process. Nucleolin is a 92 kd nucleolar protein, which is thought to control rDNA transcription. The phosphorylation of the protein nucleolin is probably performed by p34cdc2 kinase, which is a subunit of M phase kinase, an enzyme involved in bringing cells into mitosis (4). Another important NOR-associated protein beside nucleolin is "protein B23". There exists a good correlation between mean AgNOR area count and amounts of nucleolin and protein B23 inside the same cell (5). However, stimulation of rRNA synthesis, and thus of proteins associated to rRNA, does not necessarily give a quantitative increase in amounts of nucleolin and protein B23. This means that increased rRNA synthesis may occur without increase in NORs (6).

The numerous previous reports on studies of AgNOR staining in human tumors and tissues could not unequivocally support a direct positive relation between number or size of AgNORs and biological behavior of the tumor studied. The different and sometimes conflicting results of these studies may be partially attributed to differences in the used staining technique and to differences in interpreting the results of the silver-staining.

As for the technique used for silver staining, many variations and improvements are reported. Wet-autoclaving pretreatment gives better staining results (7). A staining period of longer duration has
been made possible by using polyethylene glycol-thiosulfate (PEG-Th), this improved the contrast between silver deposit and background (8). The use of microwave-irradiation, giving a shorter processing time, results in less unspecific silver deposits and increases the contrast of the deposit. Gelatin as a protective colloid and Farmer's solution to optimize the specificity, are recommended (9). Triton-X-100 staining prior to the usual AgNOR staining has been reported to give better contrast and improves the background, without influencing the total AgNOR count (10). By using the blue-toning technique, which consists of a mixture of FeCl3, hexacyanoferrate and oxalic-acid, the resolution and the number of counted AgNORs increases two- to threefold (11). Important factors during the staining procedure appeared to be the oxidation-reduction level and the type of gelatin used (12). In another study, many types of fixatives were tested; acetone, ethanol, methanol, Bouin's solution, 4% gluteraldehyde, 10% formalin and 10% formol-salin. It appeared that none of these were a limitation in acquiring reliable AgNOR counts (13).

For scoring the amount of silver deposit after staining of a tissue slice, many different methods are being used. In older studies the number of intranuclear "silver-dots" were hand-counted, making use of a light-microscope. After counting at least 100 cells a mean number of AgNOR dots per cell could be calculated. In most studies this method is still used and AgNOR staining is expressed as mean number of silverdots or AgNORs per cell or per nucleus or per nucleolus. A major problem is caused by the clustering of small deposits to one larger dot, which makes it difficult and sometimes impossible to establish the total number of small dots. To overcome this problem the total surface area of the silver deposit can be measured, which has the advantage that an automated computer-assisted analysis can be performed. The disadvantage of the latter technique is that also extranuclear precipitated silver particles will be counted.

Counting the number of AgNORs is subject to intra- and interobserver variability, which is often regarded as a limitation to the reliability of the results. However, when the interobserver variation was tested, it appeared that there was a statistical significant correlation between the results found by two observers (14,15), but the correlation coefficient was higher in counting AgNOR-areas than in AgNOR numbers (16,17). To make the counting more specific, the silver-deposit only inside the nucleolus instead of the whole nucleus can be regarded. In one study this led to the conclusion that measurement of the whole nucleolar size has the same relevance as AgNOR scoring in these nucleoli (18). Another study found a positive correlation between AgNOR number and nuclear size (19) but both had no value in predicting survival of the patient. These results were contradicted by Tosi et al. who found that the number of AgNORs had a significant predictive value in patient outcome, but form, shape and size of the nucleus had not (20).

The mean number of AgNOR dots per cell, or per nucleus, is often referred to as mAgNOR. The mAgNOR scores in cells with slow proliferation may be in the range between 0.5 and 1.5, however there are only very few reports giving "normal-values" of mAgNOR for different types of human tissues. The mAgNOR scores are higher in fast proliferating tissues. Most AgNOR studies focus on the difference in AgNOR counts among tumors of different pathological grades and tissues in different stages of neoplasia, i.e. dysplasia, in situ carcinoma or invasive carcinoma.

The pAgNOR refers to the percentage of cells in a tumor or tissue slice that harbors more than a certain number of AgNORs per cell (mostly more than 5), this is also called the AgNOR distribution score and sometimes the AgNOR proliferation index. Some studies, correlating the results of
flowcytometry and BrdU-labeling with AgNOR staining, demonstrated that pAgNOR correlates with percentage of cells in S-phase of the cell cycle, or with proliferative activity, whereas mAgNOR correlates with ploidy (21,22,23).

Many investigators have regarded the size and some of them also the shape and the localization of the AgNOR-dots. An inverse relationship between number of AgNORs and AgNOR size is often reported (24,25,26). However, in contradiction to this also an increase in AgNOR size together with increasing malignancy is reported (27,28,29). In general it is assumed that malignant cells, showing more AgNORs per nucleus, have smaller AgNOR dots than benign cells, showing less and larger AgNORs. While individual AgNOR dots become smaller as their number increases with increasing malignancy, total AgNOR area per cell increases together with increasing malignancy (30,31). AgNOR expression is also related to cell maturation; aging cells from the anterior lobe of rat-hypophysis show that the number of AgNORs and the total AgNOR area decrease, whereas the size of the individual AgNOR particles increase (32,33,34). Furthermore, AgNOR expression depends on the level of cell-differentiation, resulting in a decrease of AgNOR number and size with increasing differentiation (35). This decrease of AgNOR number and size during aging, maturation and cell-differentiation may reflect suppression of rDNA transcription.

AgNOR scores are modified in different ways in various reports. These modifications mostly try to incorporate not only the AgNOR number, but also the AgNOR size and the nuclear size. This has led to AgNOR scores being expressed as the ratio of mean AgNOR-number and mean AgNOR-size (26), the ratio of AgNOR-area and nuclear-area (36) or the ratio of number of small AgNORs (< 3 microns) and number of large AgNORs (> 3 microns) (20). Also the localization of the AgNOR inside the nucleus might be related to cell behavior; with increasing malignancy in astrocytic tumors AgNORs moved from central to peripheral inside the nucleus (29,37).

With the improvement of the AgNOR-staining technique over the years and the use of automated computer-assisted surface-area measurement of the precipitated silver-colloid inside the nucleus, the AgNOR staining has become much more standardized and reliable. However it should be realized that the number of detectable NORs depends on several factors: the level of transcriptional activity, the number of NOR-bearing chromosomes in the karyotype and the stage of the cell-cycle in which they are sought (38).

In an attempt to estimate the value of AgNOR staining in tumor pathology we reviewed all reported studies on AgNOR staining during the years 1992, 1993, 1994 and 1995. In a "med-line" search, under the keyword "AgNOR", 285 articles were produced. These were all screened for conclusions on the presence or absence of a statistical significant relation between AgNOR score and tumor type, or between AgNOR score and patient outcome. Furthermore, the type of AgNOR score used and a possible relation to other proliferation markers were noted. These criteria were met in 109 articles. Table 1 presents the results of these studies.

In the 109 reviewed reports on AgNOR expression in tumors, 118 times a conclusion was drawn concerning the relation to tumor type (histopathological grade, benign versus malignant) or to patient outcome (survival and prognosis). Of the 75 conclusions relating AgNOR score to tumor type, 55 (73%) showed a positive statistical significant correlation and 20 (27%) showed no relation. The other 43 conclusions concerned the relation between AgNOR score and patient outcome; 26
(60%) showed a statistical significant positive correlation and 17 (40%) showed no relation (table 2).

Almost two-thirds of the reviewed papers studied the relation of AgNOR score to tumor type. As mentioned before, the determination of AgNOR expression is a subjective method regarding the variations in staining methods and in counting and interpretation of the results. Also the determination of the tumor grade, using various histopathological parameters, is based on subjective variables. Thus, it can be argued that the majority of the previously published reports (64%) seek to draw conclusions on the results of two subjective "measurements". Most of these reports, even after having shown a positive correlation between AgNOR expression and tumor-type, doubt therefore, the clinical relevance of their conclusions.

A good parameter for testing the relation between AgNOR score and tumor proliferation would be the assessment of tumor growth during follow-up of the patient. One single study was found in our review, done on adenocarcinomas of the lung, where tumor size was radiologically determined during patient follow-up (39). Interestingly the results of this study showed lack of correlation between AgNOR score and degree of histological differentiation or pathologic staging, but a high inverse relation between AgNOR count and doubling time of the tumor.

Still an indirect, but a clinical more useful parameter for determining the value of AgNOR staining is the patient-outcome as expressed in survival percentage or prognosis, which is done in one-third (36%) of the reviewed studies. In a small majority of these studies (60%) a positive correlation between AgNOR expression and patient outcome was found. However among the 26 studies that reported this positive correlation, in 9 this relation could only be established when separating the patients into two groups, with high and low AgNOR scores, using a "cut-off" score. The level of this cut-off score was rather high in most studies; 5 or 6 AgNOR dots per nucleus and in one study even 11. Furthermore, the number of AgNOR dots in the tumors showed such variation that often a great overlap existed between groups with favorable and unfavorable outcome, preventing clinical usefulness in the individual case.

Almost all reports express the result of AgNOR staining in number of AgNORs per nucleus. Only 29 (25%) of 109 reports express the result of AgNOR staining in area-measurement and often not only the AgNOR area is measured but also the nuclear area. Considering these 29 reviewed studies separately, the same conclusions regarding AgNOR score in relation to tumor type or patient outcome are drawn as in the whole group of reviewed articles. In 69% there is a positive relation between AgNOR score and tumor type or patient outcome, and in 31% this relation could not be proven. Thus in this respect AgNOR area measurement leads to the same conclusions as AgNOR number measurement.

The relation of AgNOR scores to other proliferation markers is as controversial as to the aforementioned parameters. Among the 109 reviewed studies, in 27 the AgNOR score was correlated to one or more other proliferation markers. These results are listed in table 3. Only in 1 of 4 studies dating from the period 1992-1995 a positive relation to Ki-67 labeling could be found (40). Another nucleolar antigen is PCNA; from the 15 studies comparing PCNA labeling index to AgNOR expression only 60% showed a clear positive relation between the two. The same was found for the mitotic index, but in only 5 studies this relation was studied. S-phase fraction and
ploidy did correlate with AgNOR score in 6 out of 8 studies. Possibly the lack of a positive relation between the aforementioned markers and AgNOR score lies in the fact that AgNOR is expressed as well in proliferating as in resting cells, whereas for example PCNA and Ki-67 expression are absent in resting cells. Furthermore, AgNOR staining reflects only the process of protein synthesis in the cell and not necessarily cell proliferation. Since AgNOR expression is not an indicator for number of growing cells or growth fraction but reflects the rapidity of the cell cycle and is related with tumor doubling time, and the technique is simple, fast and reliable when using the computer assisted area measurement, it is felt that this technique might be an adjunct to the diagnostic possibilities for determining tumor behavior (5,39).

In order to test the clinical usefulness of AgNOR staining as a proliferation marker in pilocytic astrocytomas, AgNOR expression is studied in relation to behavior of residual tumor, as determined by follow-up neuroimaging studies of the patient (chapter 4).

**TABLE 1.** Results of 109 reviewed studies relating AgNOR score to histopathological type or grade of tumor, or to patient outcome.

<table>
<thead>
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<td>urin bl</td>
<td></td>
<td>hyperpl vs ca</td>
<td>-</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>urin bl</td>
<td>170</td>
<td>all tissues</td>
<td>+</td>
<td>mAg</td>
<td>ratio mAg area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>urin bl</td>
<td>50</td>
<td>carcinomas</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>prost.</td>
<td></td>
<td>var tumors</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116</td>
<td>prost</td>
<td>50</td>
<td>carcinomas</td>
<td>-</td>
<td>Agarea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>prost</td>
<td>74</td>
<td>carcinomas</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>118</td>
<td>prost</td>
<td>20</td>
<td>all tissues</td>
<td>-</td>
<td>mAg</td>
<td>-Spf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>prost</td>
<td>78</td>
<td>adenoca</td>
<td>-</td>
<td>mAg</td>
<td>Nuarea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>prost</td>
<td>28</td>
<td>adenoca</td>
<td>+</td>
<td>mAg</td>
<td>-DNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>blood/lymf</td>
<td>58</td>
<td>NHL</td>
<td>+</td>
<td>+</td>
<td>mAg&gt;2,9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>blood/lymf</td>
<td>101</td>
<td>NHL vs hyperplasia</td>
<td>-</td>
<td>Agarea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>blood/lymf</td>
<td>200</td>
<td>NHL</td>
<td>+</td>
<td>mAg</td>
<td>+PCNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>blood/lymf</td>
<td></td>
<td>NHL</td>
<td></td>
<td>mAg</td>
<td>-PCNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>blood/lymf</td>
<td>35</td>
<td>NHL</td>
<td>+</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ref nr.</td>
<td>tumor loc.</td>
<td>nr.</td>
<td>tumor-type</td>
<td>histol grade</td>
<td>outcome</td>
<td>Ag-NOR score</td>
<td>other prol. markers</td>
<td>remarks</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-----</td>
<td>------------</td>
<td>--------------</td>
<td>----------</td>
<td>--------------</td>
<td>-------------------</td>
<td>--------</td>
</tr>
<tr>
<td>126</td>
<td>muscle/soft ti</td>
<td>151</td>
<td>Soft tissue sarcoma</td>
<td>+</td>
<td>mAg</td>
<td>+Spf +DNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>muscle/soft ti</td>
<td>194</td>
<td>Soft tissue sarcoma</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>muscle</td>
<td>leiomyoma vs leiomyosar</td>
<td>+</td>
<td>mAg</td>
<td>+MI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>blood</td>
<td>50</td>
<td>leukemia</td>
<td>+</td>
<td>mAg</td>
<td>pAg&gt;5 +DNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>kidney</td>
<td>95</td>
<td>renal cell ca</td>
<td>+</td>
<td>mAg</td>
<td>-PCNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>kidney</td>
<td>59</td>
<td>renal cell ca</td>
<td>+</td>
<td>Agind.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>kidney</td>
<td>173</td>
<td>renal cell ca</td>
<td>+</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>kidney</td>
<td>44</td>
<td>rcc vs sarcomatoid rcc</td>
<td>+</td>
<td>mAg</td>
<td>+PCNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>liver</td>
<td>43</td>
<td>all tissues</td>
<td>-</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>134</td>
<td>liver</td>
<td>48</td>
<td>Cholangiocel-lular ca</td>
<td>+</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>liver</td>
<td>89</td>
<td>hepatocel-lular ca</td>
<td>+</td>
<td>+</td>
<td>mAg&gt;3,04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>galbl</td>
<td>53</td>
<td>normal vs ca</td>
<td>+</td>
<td>+</td>
<td>mAg Agarea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>testis</td>
<td>45</td>
<td>var tumors</td>
<td>-</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>bone</td>
<td>54</td>
<td>osteosarcoma</td>
<td>-</td>
<td>mAg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>skull</td>
<td>36</td>
<td>chordomas</td>
<td>-</td>
<td>mAg Agarea</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend table 1:
- Ref nr. = reference number of study cited.
- Tumor loc. = location of tumor, pancr= pancreatic gland, thyroid= thyroid gland, colorec= colorectal, gastint= gastrointestinal, esophag= esophagus, saliv= salivary gland, maxil= maxillary, sin= sinus, urin bl= urinary bladder, prost= prostate, galbl= galbladder
- Nr. = number of tumors studied with AgNOR staining;
- type tumor= type of tumor or tissue studied, var. = various, ca= carcinoma, vs. = versus
(when two types of tumors or tissues are compared to each other regarding AgNOR score),
spor. = sporadic, ben. = benign, sweatgl. = sweat gland, sq. cell ca = squamous cell carcinoma,
astro. = astrocytoma, hyperpl = hyperplasia, NHL = Non Hodgkin Lymphoma, leiomyosar =
leimyosarcoma
ma, rcc = renal cell carcinoma.
- histol/grade = significant relation of AgNOR score to histological type or grade of tumor:
  + = positive correlation, - = no correlation.
- Outcome = significant relation of AgNOR score to prognosis, survival or outcome of the
  patient:
  + = positive correlation, - = no correlation.
- AgNOR score = type of score used, being mAg = mean number of AgNOR per cell or per
  nucleus,
pAg = percentage of cells showing AgNOR score above a certain number; Agarea = mean
  area of
  AgNOR per cell or per nucleus, Nuarea = mean total nuclear area, Agind. = ratio of mAg in
tumor
  cell to mAg in normal cell.
- Other prol. markers = relation of AgNOR score to other tested proliferation markers
  (PCNA =
  proliferating cell nuclear antigen, BrdU LI = BromodeoxyUridine labeling index, 3HdT LI =
  3H thymidine labeling index, MI = mitotic index, Spf = S-phase fraction, DNAc = DNA content
  by
  flowcytometry) or relation to other tumor characteristics (metas = to metastases): + =
  positive
correlation, - = no correlation.
- Remarks: overl = great overlap between AgNOR scores in subsets of groups, so that there
  is no
diagnostic value to an individual AgNOR score.
*1 Negative relation of AgNOR number to histological grade, but positive relation to tumor
  proliferation rate as measured by tumor size on chest-radiographs.
*2 Only prognostic value of mAgNOR in combination with DNA-content as measured by
  flowcytometry.

TABLE 2. Results of 109 reviewed papers on the relation of AgNOR score to tumor type and to
patient-outcome.

<table>
<thead>
<tr>
<th>AgNOR SCORE RELATED TO:</th>
<th>TUMOR TYPE</th>
<th>PATIENT OUTCOME</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive relation</td>
<td>47%</td>
<td>22%</td>
<td>69%</td>
</tr>
<tr>
<td>no relation</td>
<td>17%</td>
<td>14%</td>
<td>31%</td>
</tr>
<tr>
<td>total</td>
<td>64%</td>
<td>36%</td>
<td>100%</td>
</tr>
</tbody>
</table>
### TABLE 3. Relation of AgNOR score to several other tumor proliferation markers in 109 reviewed papers.

<table>
<thead>
<tr>
<th>Proliferation marker</th>
<th>Number of studies</th>
<th>Positive relation</th>
<th>No relation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>15</td>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>Mitotic index</td>
<td>5</td>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>S-phase fraction</td>
<td>4</td>
<td>75%</td>
<td>25%</td>
</tr>
<tr>
<td>DNA-content</td>
<td>4</td>
<td>75%</td>
<td>25%</td>
</tr>
<tr>
<td>Ki-67 LI</td>
<td>4</td>
<td>25%</td>
<td>75%</td>
</tr>
<tr>
<td>p53 LI</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>BrdU LI</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>3HdT LI</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

**Legend table 3.**

*Number of studies:* Among the 109 reviewed papers, the number of times the specific proliferation marker was studied in relation to AgNOR score.

PCNA: Proliferating Cell Nuclear Antigen.

S-phase fraction: percentage of cells in S-phase determined by flowcytometry.

DNA-content: ploidy, as measured by flowcytometry.

Ki-67 LI: Ki-67 or MIB-1 labeling index.


BrdU LI: Bromodeoxy-Uridine labeling index.

3HdT LI: radiolabeled Hydrogen-Thymidin labeling index.

### 2. KI-67 AND MIB-1 LABELING

In the search for antibodies against nuclear antigens specific to Hodgkin and Reed-Sternberg cells, Gerdes et al. in 1983, discovered a mouse-monoclonal antibody, named Ki-67, that recognized a nuclear antigen present only in proliferating cells (140). The Ki-67 reactive nuclear antigen is only expressed in the G1, G2, M and S-phase of the cell cycle, and not in the resting or Go-phase. Schonk et al. concluded that the gene involved in the expression of the Ki-67 antigen is localized on chromosome 10 (141). The immunoreaction between the Ki-67 antibody and the reactive antigen could only take place in fresh frozen tissues. Apparently, the routinely used formalin fixation process destroyed the antigenic activity to the Ki-67 antibody. However, in 1991, by the same group as in 1983, part of the gene, encoding for the Ki-67 protein was cloned and sequenced (142).
immunizing mice with recombinant Ki-67 gene product a new monoclonal antibody was developed, named MIB-1. It appeared that this antibody reacted with the Ki-67 antigen in formalin fixed tissues after antigen retrieval. The deduced aminoacid sequence of the gene encoding for the Ki-67 protein did not reveal homology to any known cell-cycle protein, thus the function of the Ki-67 protein remains unknown (143).

Many studies have been undertaken to use the Ki-67 antibody, or the later developed MIB-1 antibody, for assessment of the amount of proliferating cells in all kinds of normal human tissues, tumors and cell lines. Especially in neoplastic diseases the quantifying of the fraction of proliferating cells can be an adjunct to conventional histologic diagnosis for predicting the biological behavior of the tumor and the prognosis of the patient. The Ki-67 labeling index (LI), being the number of positive staining cells divided by all counted cells, expressed as a percentage and also called the proliferating cell index (PCI) or proliferation index (PI), correlates well with the behavior of many malignant tumors, such as lymphomas and breast carcinomas (144).

The reliability of the Ki-67 immunostaining technique in gliomas was confirmed when comparing it to other labeling techniques, such as incorporation of radiolabeled 3H-Thymidine in the tumor cell DNA or measurement of the uptake of the thymidine-analogue Bromodeoxyuridine (BrdU) (145,146,147). These older techniques have proven their usefulness in determining the number of proliferating cells or the growth fraction, but have the great disadvantage of being invasive, since they make it necessary to administer, by intravenous injection, a tracer into the patient.

Table 4 lists the results of 32 previous studies of Ki-67 labeling in gliomas. The earliest studies merely tried to establish "normal-values" of Ki-67 LI in different types and grades of gliomas. Others compared Ki-67 labeling to results of other methods of growth fraction assessment in these tumors, such as Proliferating Cell Nuclear Antigen (PCNA) labeling, Bromodeoxyuridine (BrdU) labeling, p53 immunostaining, determination of S-phase fraction by flow-cytometry and counting number of mitoses. More recent studies investigated a possible relation between MIB-1 labeling and patient survival or outcome.

Values for Ki-67 LI are in general lower than for MIB-1 LI. In one study these 2 different antibodies were compared using the same tumors to test both (148). It appeared that MIB-1 LI is approximately 1.6 times higher than Ki-67 LI. Probably the antigenic activity is better preserved in paraffin embedded tissues than in fresh frozen tissues. Furthermore, cells in early G1 phase contain very little antigenic activity, possibly these cells are detected with MIB-1 but not with Ki-67.

In 17 of the 32 cited previous studies Ki-67 has a clear correlation with astrocytoma grade; LI values being higher in tumors of higher grade. Mean Ki-67 and MIB-1 LI values of 25 grade I astrocytomas were 0.25%-6%, of 159 grade II astrocytomas 0.5%-10.7%, of 230 grade III astrocytomas 3.0%-18.4% and from 424 glioblastomas (grade IV) 5.2%-31.6%. Despite the good correlation between tumor grade and Ki-67 expression, the clinical usefulness for individual tumors is very limited, since the values overlap significantly. Furthermore, the Ki-67 LI values in pilocytic astrocytomas are not different from grade II astrocytomas; the first even have slightly higher values in some studies (149,150,151,152). MIB-1 labeling studies on pilocytic astrocytomas have not been performed before 1997. Ki-67 LI values of 23 pilocytic astrocytomas were found to range from 0.7%-6% (145,149-154,162). Among 92 grade II astrocytomas Ki-67 LI values ranged
from 0.5%-2%, whereas MIB-1 LI values of 65 grade II astrocytomas ranged from 2.03%-10.7%. In one study dating from 1997 the mean MIB-1 LI of 2 pilocytic astrocytomas was 0.25% (155). The paradox of equal Ki-67 antigenic expression among grade I and grade II astrocytomas and different prognosis, being much better in grade I tumors than in grade II, has not been studied yet.

The 7 studies that showed a good correlation between MIB-1 LI and survival or prognosis of the patient were all referring to astrocytomas grade II-IV (155-161). In 5 studies this correlation was achieved by using a statistical method that sets a cut-off score for the labeling index at a level where patients scoring lower have a better prognosis than patients scoring higher. The level of the LI-cut-off score varied considerably, from 1.5%-15.3% (156,157,159,162,163).

In the 3 studies that compared Ki-67 labeling to BrdU labeling or S-phase fraction, a good correlation was found (146,148,164). A relation with PCNA labeling, performed in one study, could not be proven (161). Comparing Ki-67 labeling to p53 immunostaining, mitotic index and AgNOR staining, as done in several previous studies, gives equivocal results.

Summarizing the information as given above, leads to the following conclusions:

- Ki-67 labeling and MIB-1 labeling are methods used to determine the fraction of proliferating cells in tumors.
- MIB-1 LI values have a statistically significant relation to tumor grade among gliomas grade II, III and IV.
- When using a cut-off score, that separates two groups, one with lower and one with higher MIB-1 LI's in high grade gliomas, the MIB-1 labeling index has a predictive value concerning patient-outcome.
- MIB-1 LI values for pilocytic astrocytomas have hardly been assessed.
- Ki-67 LI values of pilocytic astrocytomas are in the same range as for astrocytomas grade II, despite their totally different prognoses.

The following questions remain:

- What are "normal-values" of MIB-1 LI in pilocytic astrocytomas ?
- Are MIB-1 LI values of pilocytic astrocytomas and grade II astrocytomas in the same range?
- Has MIB-1 labeling a predictive value towards outcome or tumor behavior in the relatively homogenous group of pilocytic astrocytomas ?

In an attempt to answer these questions a MIB-1 labeling study was performed among 39 pilocytic and 5 low grade astrocytomas in children and the results were analyzed in relation to neuroradiological follow-up of the patients (chapter 5).
TABLE 4. Results of previous Ki-67 and MIB-1 labeling studies on gliomas, in chronological order.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3 grade</th>
<th>4 grade</th>
<th>5 grade</th>
<th>6 grade</th>
<th>7 relation to survival(G) or tumor grade(G)</th>
<th>8 remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>study</td>
<td>anti-body</td>
<td>I nr. LI</td>
<td>II nr. LI</td>
<td>III nr. LI</td>
<td>IV nr. LI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>Ki-67</td>
<td>1 1%</td>
<td>7 10-40%</td>
<td>G+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>Ki-67</td>
<td>13 0.9%</td>
<td>12 1.8%</td>
<td>6 4.3%</td>
<td>53 9.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>Ki-67</td>
<td>1 4.5%</td>
<td>15 0.7%</td>
<td>8 3.5%</td>
<td>27 11.1%</td>
<td>G+ S- &quot;grade II piloc. astr.&quot; in a 53 y. old patient</td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>Ki-67</td>
<td>28 1-8.5%</td>
<td>13 8.6-14.2%</td>
<td>18 1-22.1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>166</td>
<td>Ki-67</td>
<td>5 0.5%</td>
<td>5 3%</td>
<td>3 5.2%</td>
<td>G+ AgNOR: +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>Ki-67</td>
<td>1 0.7%</td>
<td>6 1.5%</td>
<td>4 5.1%</td>
<td>18 9.9%</td>
<td>AgNOR: -</td>
<td></td>
</tr>
<tr>
<td>167</td>
<td>Ki-67</td>
<td>45 gliomas, various grades</td>
<td>AgNOR: +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>Ki-67</td>
<td>26 0.5%</td>
<td>26 4.1%</td>
<td>38 6.4%</td>
<td>G+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>Ki-67</td>
<td>1 6%</td>
<td>4 &lt;1%</td>
<td>8 4.4%</td>
<td>7 21%</td>
<td>G+ Ki-67 superior to PCNA</td>
<td></td>
</tr>
<tr>
<td>169</td>
<td>Ki-67</td>
<td>21</td>
<td>20</td>
<td>32</td>
<td>G+ MI: +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>Ki-67</td>
<td>1 5.6%</td>
<td>&lt;1%</td>
<td>8 10.1%</td>
<td>G+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>Ki-67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S- only GBMs</td>
<td></td>
</tr>
<tr>
<td>162</td>
<td>Ki-67</td>
<td>3 0.9%</td>
<td>7 1.2%</td>
<td>13 7.5%</td>
<td>20 11.1%</td>
<td>G+ S+(5%)</td>
<td>p53: -</td>
</tr>
<tr>
<td>163</td>
<td>Ki-67</td>
<td></td>
<td>20 5.2%</td>
<td>S+ (1.5%)</td>
<td>MI: -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>Ki-67</td>
<td>7 0.8%</td>
<td>9 7.2%</td>
<td>G+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 146 | Ki-67 | 200 brain tumors, various grades | G+ | BrdU (S-phase): +
| 152 | Ki67 | 2 | 2.1% | 10 | 2% | 5 | 6.1% | 20 | 18.5% | G+
| 147 | MIB-1 | 24 | 2.03% | 26 | 12.8% | 9 | 14.6% |
| 148 | MIB-1 | 22 | 7.3% | 30 | 23.9% |
| 156 | MIB-1 | 50 astrocytomas, various grades | G+ | S+ (15.3%) |
| 157 | Ki-67 | 24 | 31 | 68 | S+(2%) | p53: + |
| 172 | MIB-1 | 137 brain tumors | G+ |
| 173 | MIB-1 | 11 | <10% | 2 | <10% | 8 | 0-15% | 8 | 0-15% |
| 164 | MIB-1 | 31 glial tumors | G+ | S-phase: + |
| 174 | MIB-1 | 48 astrocytomas, various gr. | G+ |
| 158 | MIB-1 | 19 intramedullary spinal cord tumors | S+ |
| 159 | MIB-1 | 10 | 11.9% | 19 | 27.3% | S+ (12%) |
| 160 | MIB-1 | 19 | 3.8% | 25 | 18.4% | 28 | 31.6% | S+ |
| 161 | MIB-1 | 15 | 9.0% | 19 | 17.9% | 86 | 23.3% | S+ | G+ | p53: - | PCNA: - |
| 175 | MIB-1 | 75 astrocytomas, various gr. | G+ |
| 155 | MIB-1 | 2 | 0.25% | 7 | 10.7% | 7 | 6.3% | 37 | 15.8% | S+ |
| 176 | MIB-1 | 46 astrocytomas, various gr. | telomerase RNA: + (associated with tumorigenesis) |

Legend Table 4:
1= reference number of study. 2= Type of antibody used. 3= Astrocytoma grade I, or pilocytic astrocytoma, number of tumors studied and mean Ki-67 LI. 4= Astrocytoma grade II. 5= Astrocytoma grade III. 6= Astrocytoma grade IV or glioblastoma multiforme; various gr.= various grades. 7= Relation between Ki-67 LI and tumor grade(G) or survival (S), if present: + , the value of the "cut-off" Ki-67 LI score that discriminates between good and poor outcome is...
The TP53 gene and p53 protein

The TP53 gene is known as a tumor suppressor gene located on the short arm of chromosome 17. The p53 protein was discovered in 1979, and in the mid-1980's the gene was cloned. It appeared that parts of the gene remained identical during evolution among species and this suggests that these highly conserved regions of the gene play a vital role for protein function (177). The gene spans 20 kilobases of genomic DNA and consists of 11 exons (178).

The TP53 gene and its product, being the wildtype p53-protein, have a key function in the regulation of the cell cycle. Overwhelming evidence exists for a pivotal role of p53 in regulating cell proliferation, differentiation, DNA-repair and apoptosis (177,180-184) (figure 1). Numerous mutations in the p53 encoding gene have been described that strongly associate with carcinogenesis. The TP53 gene is the most frequently mutated gene in human tumors.

In it's normal function p53 is up regulated in case of anoxia, DNA damage or disturbances in the replicative process of the cell. This leads to several possible p53 mediated actions in the cell, all directed towards "guarding" the genome (183). The p53 protein induces p21 (WAF1, Wildtype Activating Factor), which inhibits the cdk (cyclin dependent kinase) mediated signal transduction pathway resulting in a block of the cell-cycle into a G1-arrest. By stimulating the GADD (Growth Arrest and DNA Damage)-gene, DNA-repair mechanisms become active. When these mechanisms are insufficient, p53 can accelerate the transcription of the bax-gene and inhibit bcl-2, which both lead to increased apoptosis, or programmed cell death (185).

The function of the TP53 gene can be studied in different ways, either by analyzing the structure of the short arm of chromosome 17 and the TP53 gene itself by molecular techniques, or by studying the presence or absence of the gene product, being the p53 protein by immunohistochemical methods. Currently, molecular techniques like Single Strand Conformation Polymorphism (SSCP) determination and Denaturing Gradient Gel Electrophoresis (DGGE) in combination with subsequent DNA sequencing, allow rapid mutation screening. It appears that tumor related mutations in TP53 are predominantly present in exons 5,7 and 8, which are the evolutionary highly conserved regions of the gene. Mutations outside these "hot spots" have also been reported though. The p53 protein in it's normal appearance is called "wildtype". The half life of this wildtype protein is very short, in the range of 15 to 30 minutes and present only in very low concentrations in normal cells (177). Therefore it is thought to be undetectable by monoclonal antibodies used in even the most recent immunostaining techniques. Positive immunostaining with these monoclonal antibodies therefore, reflects the presence of an altered p53 protein, with a longer half life, or an abundant presence of the wildtype, which is normally not the case. The existence of such an altered p53 protein is thought to be the result of a structurally changed TP53 gene, for example because of a mutation. Depending on the severity of damage that is caused in the encoding process to the protein, which is dependant on the type of mutation, the function of the p53 protein may be impaired.
Mutation of the TP53 gene is an early event in the development of malignant gliomas of the brain (186,187). High grade astrocytic tumors of adulthood show over expression of the p53 protein or mutations in the TP53 gene in 32%-90% of cases (188-192). In adult low-grade astrocytomas this frequency varies from 5%- 60 % (186,187,188,189,191,193,194,195). Mutations of TP53 are strongly associated with progression of low grade astrocytomas to high grade astrocytomas. Weber et al. studied 5 astrocytomas grade II that recurred as an anaplastic astrocytoma, all had a mutation of the TP53 gene (196). Different pathways have been distinguished in the development of a grade IV astrocytoma (glioblastoma) (197). Some astrocytomas grade IV are the result of malignant progression of a lower grade astrocytoma, these tumors showed disturbed function of the TP53 gene, either immunohistochemically or genetically. Other grade IV astrocytomas arise “de novo”, those are p53-immunonegative and have no mutations in the TP53 gene.

In malignant gliomas of adulthood TP53 mutations are more often found in younger patients than in the older (198). However, the reported incidence of these mutations in pediatric astrocytomas is very variable. Litofsky et al. found no mutation among 35 pediatric astrocytomas of all grades (199), Rasheed et al. found only 1 mutation among 48 astrocytomas of all grades in patients under eighteen years of age (200). Neither did Felix et al. find a mutation among 17 low grade astrocytomas of childhood, but 2 of 3 glioblastomas of childhood appeared to have a TP53 mutation, of which one also had a germline TP53 mutation (201). Among 29 malignant brainstem gliomas of childhood 18 were p53-immunopositive and 11 showed a TP53 mutation (202). The prognosis of the children with TP53 mutations was less favorable. It was concluded that TP53 mutation frequency among high grade gliomas of childhood is in the same range as for adults, however the frequency among children under 4 years of age was lower, and the prognosis of these very young children was better. In another study among 21 malignant gliomas in childhood almost 50 % was p53 immunopositive (203). However, in this study the p53 immunostatus had no relation with survival (203).

In pilocytic astrocytomas p53 dysfunction is very rare. In the 19 previously published papers a total of 218 pilocytic astrocytomas were studied for p53 abnormalities, either immunohistochemically, or by molecular analysis techniques. Results of these studies are listed in table 5. From the 77 tumors immunohistochemically studied 19 showed p53 protein over expression (25%). Chromosomal analysis, performed in 75 tumors, showed in 8 structural changes (LOH, Loss of Heterozygosity) on the short arm of chromosome 17 (11%), but in 4 cases these changes occurred just outside the region of the TP53 gene. Furthermore, the one tumor with LOH of chromosome 17p in the study of Wu et al. is probably an adult low grade astrocytoma, which leaves only 3 pilocytic astrocytomas with LOH of 17p on the site of the TP53 gene (204). From the 107 tumors studied by genetic analysis techniques, mainly being SSCP and sequencing, only 3 showed a mutation in the gene. These 3 mutations were found on 3 different localizations: At codon 47 in exon 4, codon 248 in exon 7 and codon 324 in exon 9. Most of these genetic analyses have screened the gene incompletely, they were restricted to the known frequent mutation hot-spots of other tumors, which are exons 5 to 8. Willert et al. demonstrated one of these mutations in a study of 20 juvenile pilocytic astrocytomas (JPA's), exons 4 to 9 were analyzed, after PCR, denaturing gradient gel
electrophoresis and sequencing (205). The mutation was found in exon 7 at codon 248, which is a known hot-spot for TP53 mutations in many other malignancies. However, they found other places on the short arm of chromosome 17 where loss of heterozygosity was detected, i.e. in two of 20 JPA's telomeric to TP53 and in 6 of 20 JPA's centromeric to the TP53 region. Of those 6 JPA's with centromeric loss, 4 tumors (66%) behaved rather aggressively, whereas only 5 of the remaining 14 tumors (36%) showed this behavior. One of the conclusions of the authors is that the TP53 gene may have a role in the formation of JPA's.

Immunohistochemical analysis of the p53 protein may show immunopositiveness without mutation of the gene (186). The frequency of p53-immunopositiveness among astrocytic tumors is much higher than of TP53 mutations. In the study of Lang et al. p53 immunopositiveness was found in 71% of pilocytic astrocytomas (n=7), 63% of grade II astrocytomas (n=8) and in 63% of grade III astrocytomas (n=16) (186). However, DNA sequencing after SSCP revealed among the same tumors TP53 mutations in only 14%, 25% and 19% respectively. Another study found similar high incidences of p53 immunopositiveness, i.e. 54% in grade II, 75% in grade III and 90% in grade IV astrocytomas (189). TP53 mutation frequency determined by SSCP and DNA sequencing in the study of Patt et al. (187) was again low: in pilocytic astrocytomas 14% (1 out of 7), in grade II astrocytomas 5.6%, in grade III astrocytomas 25% and none in glioblastomas. Although it was believed that immunostaining identified only mutant forms of the p53 protein, since the wildtype protein has a half-life too short to be detected by the monoclonal antibody, these results led to the assumption that the antibody also reacts with the wildtype p53 protein but only when present in an excessive amount, which means an over expression. Such an over expression of wildtype p53 can be caused by cell-cycle specific variations, amplification of the MDM2 gene resulting in binding of the gene product to the p53 protein and thus accumulation, DNA damage and anoxia leading to stress-induced increases of wildtype p53 expression (202,206).

Rubio et al. demonstrated that the often used monoclonal antibody PAb 1801 binds to mutant as well as to wildtype p53 protein, whereas the antibody PAb 240 only binds to the mutant form (207). In this study it was proved that over expression of wildtype p53 protein can occur without a TP53 mutation. Another study showed the same discrepancy between results of immunostaining and DNA analysis in gliomas grade II-IV (190). However, in this study there was a correlation between the results of the two techniques among tumors that showed more than 5% of positive p53 immunostaining cells. Most of the highly immunopositive tumors showed indeed a high mutation frequency, whereas in many of the tumors that were immunopositive for less than 5% of the total amount of cells a DNA mutation could not be detected. This was attributed to the low sensitivity of the DNA analysis technique used, being SSCP followed by direct sequencing of DNA. The conclusion of the authors was that the immunostaining technique might be more useful to detect p53 alterations than the DNA analysis technique. Furthermore, it was stated that genetic analysis techniques using the PCR method, only screen a very small sample of tumor DNA, mostly from a very small area of the whole tumor. Knowing that astrocytomas are heterogenous tumors, this may lead to sample errors giving rise to false-negative results.
In summary, immunohistochemical p53 positiveness can occur in the following situations:

1. There is over expression of wildtype p53 protein: the p53 protein is present in an increased amount but has a normal function. This reaction occurs as a physiological response to DNA-damage (185). An example of this are the elevated levels of wildtype p53 found in skin-cells after a sunburn (208).

2. There is accumulation of p53 protein: the p53 protein is bound in the cell to other proteins which cause an increased stability of the protein and a prolonged half-life. The function of the p53 protein is impaired in these circumstances. Proteins that are able to bind to p53 are the product of the MDM-2 gene, viral oncoproteins such as those from SV-40, HPV E6 and adenovirus E1B. Also sequestration of the p53 protein in the cytoplasm, away from the target DNA in the nucleus of the cell may render the p53 protein to become dysfunctional (178).

3. There is mutant p53 protein: the TP53 gene is altered and depending on the type of mutation the protein produced is full-length with an amino acid change, truncated or the protein is absent. The first form of protein leads to impaired function and a prolonged half-life, which makes the protein detectable for the used antibody, in the remaining two situations the protein will not be detectable by an antibody (178).

In the situations as described above under 1. and 2. p53 immunopositiveness may occur without the presence of a TP53 gene mutation.

The involvement of the TP53 gene in the development of high grade astrocytic tumors is not debated, but the majority of studies trying to establish a relation between p53 function and patient survival fail to prove this relation (Table 6). The reason for a lack of correlation between p53 status and survival among high grade glioma patients might be that in these gliomas so many other carcinogenic genetic events have taken place that p53 dysfunction by itself only plays a minor role. Assuming a relatively more important role for the TP53 gene in low grade astrocytomas, where not so many other carcinogenic genetic events have occurred yet, p53 status in these tumors may have a predictive value. This is supported by the study of Chozick et al. who did find a relation between p53 function and survival among 24 patients with low grade astrocytomias. However, possibly due to the low number of patients, this relation was not significant (p=0.08) (198). Another study among 52 grade II astrocytomas showed a difference in survival after 5 years of follow-up between p53 immunopositive and immunonegative patients (193). The group of Kraus et al. could not establish a relation between p53 mutations and survival among grade II astrocytoma patients (209). From the 38 grade II astrocytomas studied, 10 had a malignant recurrence. In 6 out of those 10 a TP53 mutation was found, however in 5 this mutation was already present in the precursory grade II astrocytoma. In 2 cases the TP53 mutation of the grade II astrocytoma could not be found in the corresponding malignant recurrence. These authors conclude that TP53 plays no role in the malignant progression of grade II astrocytomas and has no prognostic significance. In contrast, Bodey et al. have found 100% immunopositiveness among 5 studied JPA’s (210). These authors state that p53 immunopositiveness without TP53 gene mutation is one of the primary steps in astrocytoma formation. Furthermore, they assume that this p53 accumulation may prohibit the normal function of the p53 protein. When the primary step of p53 protein dysfunction has occurred, which might be the case in those immunopositive pilocytic astrocytomas, and the genome becomes
altered, subsequently a cascade of further disturbances in DNA replication may be initiated, since the DNA-repairing and protective role of p53 is then missing. In this way dedifferentiated cell-clones may develop and cause the malignant degeneration of grade I to grade II, and of grade II to grade III or IV astrocytomas. The observation that increased levels of wildtype p53 in normal fibroblasts enhance the tumorigenic potential of these cells is in line with this hypothesis (211). Also the observation that transfection of the human wildtype p53 in a pilocytic astrocytoma-derived cell line resulted in a growth suppressor effect on the cell line and the induction of important morphological changes in the cells, resembling those of differentiated astrocytes, suggests a role for p53 in the formation of pilocytic astrocytomas (195).

In summary, the above mentioned information leads to the following conclusions:

- **The TP53 gene**: The most frequently mutated gene in human neoplasms. p53 is a cell cycle regulator and "guards the genome". In case of DNA damage or disturbances in DNA replication, p53 induces G1-arrest with DNA-repair or triggers apoptosis. Transfer of TP53 gene products into p53-mutant glioma-cells (including a cell line derived from pilocytic astrocytoma) results in a growth suppressor effect and rapid cell death via apoptosis.

- **Malignant gliomas**: TP53 mutation frequency varying from 30%-90%, in adults as well as in children. The TP53 gene is certainly involved in the formation of these tumors, probably when arising from malignant progression of low grade astrocytomas. But, p53 status, immunohistochemically established, has no predictive value for patient survival.

- **Low grade gliomas**: TP53 mutation frequency varying from 5%-60% in adults. In children probably a much lower frequency. The role of the TP53 gene in the formation of these tumors is unclear. This role is accepted in malignant progression of low to high grade astrocytomas. Two studies reported a positive relation between p53 status and survival, whereas one rejected this relation. The TP53 gene seems to play no role in pediatric low grade gliomas.

- **Pilocytic astrocytomas**: TP53 mutation frequency is very rare (<3%). But in most studies only the well known "hot-spots" of the gene (exons 5-8) were examined. Of the 3 mutations reported, 1 is on exon 7, while the other 2 are on exons 4 and 9. Results of p53-immunostaining are very conflicting: in 4 studies negative, but strongly positive in another 3 studies. In 2 of these 3 studies p53 dysfunction is supposed to be a major initiating event in astrocytoma formation and subsequent dedifferentiation to higher grades. The relation between p53 status and patient survival or prognosis has not been studied before.
Figure 1. Tumor suppressor functions of p53.

DNA-damage
Disturbed replication
Ischemia

→

P53 activation

Activation

P21waf
GADD
CDK inhibition
Gl-arrest

DNA Repair

Apoptosis

Inhibition

Bax

Bcl-2

Cyclin A

Apoptosis

blocking S-phase entry
**TABLE 5.** Results of previous p53 studies in pilocytic astrocytomas.

<table>
<thead>
<tr>
<th>Study</th>
<th>Antibody</th>
<th>Immunohistochemical nr. pos/tot</th>
<th>Structural (LOH) study</th>
<th>Structural (LOH) nr. pos/tot</th>
<th>Genetic analysis (SSCP and sequencing) study</th>
<th>Genetic analysis (SSCP and sequencing) exons studied</th>
<th>Genetic analysis (SSCP and sequencing) nr. pos/tot</th>
<th>Genetic analysis (SSCP and sequencing) mutation</th>
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<tr>
<td>210</td>
<td>PAb-1801</td>
<td>5/5</td>
<td>204</td>
<td>1/2 (*1)</td>
<td>218</td>
<td>5,7,8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>212</td>
<td>PAb-1801</td>
<td>8/21</td>
<td>205</td>
<td>6/20</td>
<td>219</td>
<td>5-8</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>192</td>
<td>DO-7</td>
<td>0/12</td>
<td>200</td>
<td>0/28 (*2)</td>
<td>199</td>
<td>5-8</td>
<td>0/17</td>
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</tr>
<tr>
<td></td>
<td>CM-1</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>191</td>
<td>DO-7</td>
<td>0/8</td>
<td>216</td>
<td>1/20</td>
<td>187</td>
<td>5-9</td>
<td>1/7</td>
<td>exon 9 co.324</td>
</tr>
<tr>
<td>213</td>
<td>PAb-1801</td>
<td>0/11</td>
<td>217</td>
<td>0/5</td>
<td>213</td>
<td>5-8</td>
<td>0/11</td>
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</tr>
<tr>
<td></td>
<td>DO-1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>214</td>
<td>PAb-1801</td>
<td>0/4</td>
<td>201</td>
<td>4-8</td>
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<td>186</td>
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<td>205</td>
<td>4-9</td>
<td>205</td>
<td>1/20</td>
<td>exon 7 co.248</td>
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<tr>
<td>215</td>
<td>DO-1</td>
<td>1/9</td>
<td>220</td>
<td>0/3</td>
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<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL 19/77 (25%)</td>
<td>TOT 8/75 (11%)</td>
<td>TOTAL 3/107 (3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*1: the positive tumor is a grade I astrocytoma in an adult, but the authors use a grading system of I to III, in which grade III refers to a glioblastoma multiforme, so probably this tumor is actually a low grade astrocytoma.

*2: The 28 tumors investigated are "low grade astrocytomas of childhood".
<table>
<thead>
<tr>
<th>study ref.nr.</th>
<th>type of astrocytoma</th>
<th>nr. of tumors</th>
<th>method of p53 analysis</th>
<th>relation p53 to survival</th>
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</thead>
<tbody>
<tr>
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<td>grade 4</td>
<td>?</td>
<td>immuno CM-1</td>
<td>yes</td>
</tr>
<tr>
<td>222</td>
<td>all grades</td>
<td>53</td>
<td>immuno DO-1</td>
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<tr>
<td>202</td>
<td>pediatric malign.brain stem</td>
<td>29</td>
<td>immuno DO-1, SSCP, seq.</td>
<td>yes</td>
</tr>
<tr>
<td>198</td>
<td>grade 2</td>
<td>24</td>
<td>immuno PAb1801</td>
<td>yes (p=0.08)</td>
</tr>
<tr>
<td>200</td>
<td>all grades</td>
<td>126</td>
<td>immuno</td>
<td>no</td>
</tr>
<tr>
<td>193</td>
<td>grade 2</td>
<td>52</td>
<td>immuno DO-7</td>
<td>no</td>
</tr>
<tr>
<td>223</td>
<td>all grades</td>
<td>105</td>
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<td>grades 2,3,4</td>
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<tr>
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<td>grade 3,4</td>
<td>63</td>
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<td>no</td>
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<td>191</td>
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<td>56</td>
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<td>no</td>
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<tr>
<td>224</td>
<td>grades 2,3,4</td>
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</tr>
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<td>206</td>
<td>grade 3</td>
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<tr>
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<td>grade 3,4</td>
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<td>209</td>
<td>grade 2</td>
<td>38</td>
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<td>225</td>
<td>grade 2,3 childhood</td>
<td>21</td>
<td>immuno PAb1801, PAb240, DO1, DO7 BP53-12, CM-1</td>
<td>no</td>
</tr>
</tbody>
</table>
Neurofibromatosis 1 (NF1), the von Recklinghausen or peripheral form of neurofibromatosis, is closely associated with pilocytic astrocytomas. NF1 patients develop in 15% of cases pilocytic astrocytomas and one third of all patients with pilocytic astrocytomas of the optic nerve have NF1 (226).

NF1 is one of the most frequently occurring genetic disorders as it affects 1 in 3500 individuals worldwide. It is inherited in an autosomal dominant fashion, but half of the cases are new mutations in the germline without prior family history of the disease. The disease is characterized by multiple cafe-au-lait spots, neurofibromas, Lisch noduli, axillary freckling, optic nerve gliomas, distinct osseous lesions, and sometimes development of malignant tumors (227). However, the disease shows a very variable expression, meaning that patients from the same family and presumably carrying the same mutation, may exhibit a wide range of symptoms.

The gene locus was mapped in 1987 within band q11.2 on the long arm of chromosome 17 (228,229). It is a large gene, spanning approximately 300,000 nucleotides, but with an open reading frame of 8454 nucleotides (230). The gene product is a 250 kDa protein called neurofibromin; a small part of this protein is homologous to human GTPase-activating proteins (GAPs). The part of the NF1 gene that encodes for the GAP is called the GAP-related domain (GRD). GAPs are involved in the regulation of the p21ras proto-oncogene, in such a way that GAPs inactivate p21ras. P21ras is involved in cell proliferation and differentiation via the tyrosine kinase signal transduction pathway, in which the epidermal, nerve and platelet derived growth factors (EGF, NGF and PDGF) play a role. In normal resting cells p21ras is in its inactive state. Activation of p21ras leads to the development of tumors. It is suggested that GAPs regulate the p21ras mediated growth and differentiation pathways by keeping p21ras in its inactive state. Assuming that neurofibromin has a function homologous to GAP, by analogy, a mutation in the NF1-GRD would cause a dysfunction of neurofibromin and subsequently dysregulation of p21ras leading to unlimited cell proliferation and the formation of tumors. In this way the NF1 gene can be regarded as a tumor-suppressor gene.

To test the hypothesis that NF1 acts as a tumor suppressor gene, molecular genetic analyses have been performed on tumors occurring in NF1 patients. The "two-hit" model, established by Knudson after studying the sporadic and familiar occurrence of retinoblastomas and the retinoblastoma (Rb)-gene, can also be applied to NF1 patients (231). In case of inherited loss of one NF1-allele in the germline DNA, the most frequently occurring symptoms would arise, including the formation of neurofibromas. The formation of other tumors in these patients, such as the optic glioma or neurofibrosarcomas, would be caused by loss of the remaining NF1-allele, due to a somatic mutation, being the "second hit". Therefore, studying these tumors, one would expect to find loss of heterozygosity near or at the NF1-gene locus or mutation of the gene itself.

Some studies have found results supporting this hypothesis: Genetic analysis of neuro-fibrosarcomas from NF1 patients showed a deletion of chromosome 17, including the NF1 gene (232,233). Homozygous deletions of chromosome 17 were found in a malignant melanoma cell line and in neuroblastoma cell line (234,235). Li et al. have found mutations in at least one allele of the NF1 gene in 1 anaplastic astrocytoma, in a colon carcinoma and in myelodysplastic syndrome (236).
NF1 mutations were found in 1 recurrent low grade astrocytoma, 1 ependymoma, 2 glioblastomas multiforme and 1 primitive neuroectodermal tumor (PNET) out of a group of 31 gliomas and 3 PNETs from non-NF1 patients (237). In the study of von Deimling et al. 4 of 20 pilocytic astrocytomas showed LOH of chromosome 17, in 3 of the long arm and in 1 of the whole chromosome 17 (226). One of these pilocytic astrocytomas occurred in a NF1 patient; in this tumor the LOH included the NF1 locus. It is tempting to assume that in all these 4 tumors the responsible gene for pilocytic astrocytoma development would be the NF1 gene, however, evidence for this was not established, since the gene itself was not analyzed.

Other findings however, oppose the theory of NF1 being a tumor suppressor gene: In neurofibromas, the hallmark tumor of NF1 patients, LOH on or near 17q11.2 could not be found (238). Among the 31 gliomas in the study of Thiel et al. there were 3 pilocytic astrocytomas including 1 from a NF1 patient, however, none of those 3 showed DNA abnormalities in the region of the NF1 gene. In 51 pediatric brain tumors, including 16 pilocytic astrocytomas, the part of the NF1 gene that encodes for the part of the protein with the GAP-like function, which is called the FLR exon of the NF1 gene, was screened for mutations by SSCP, but these were not found (239). In this study only the FLR exon of the NF1 gene was screened, which forms less than 2% of the whole gene. Neither were mutations found in the FLR exon among 18 glioblastomas in another study (240). This leaves however the possibility that mutations are present in other parts of the gene. These data, especially the lack of LOH 17q in neurofibromas of NF1 patients and the enormous size of the NF1 gene, which makes it very difficult to screen for mutations, cause that the status of the NF1 gene as a tumor suppressor gene remains to be proven. Moreover, the NF1 gene carries a dominant inheritance, whereas the typical tumor suppressor genes are recessive.

Other studies were performed to look more specifically at the function of the gene product, neurofibromin. Structural studies of this protein disclosed that it has 3 isoforms due to alternative splicing of the gene product before transcription. The levels of GAP activity of these isoforms are not identical and the expression of the 3 isoforms differs among cells of certain tissues and in various stages of development and differentiation of these tissues (241). From this it is suggested that the various isoforms of neurofibromin have specific functions, mainly related to the stage of embryonic development of the certain tissues. Nevertheless, in relation to the supposed tumor suppressor function of NF1, neurofibromin expression can be studied by using a specific antibody to this protein, or by studying the amount of RNA transcript. In the case of DNA damage at the level of the NF1 gene one would expect a lowered or absent expression of neurofibromin. In many of the tumors that showed NF1 mutations, being melanomas, neuroblastomas, pheochromocytomas and neurofibrosarcomas the levels of neurofibromin were indeed absent or reduced (234,235,242).

To evaluate the possible tumor-suppressor function of the NF1 gene neurofibromin expression in 6 pilocytic astrocytomas was analyzed by studying the amount of RNA-transcript and by antibody, directed against neurofibromin, detection. (Chapter 7).
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CHAPTER 4

AgNOR STAINING MAY REFLECT THE GROWTH POTENTIAL OF PILOCYTIC ASTROCYTOMAS

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submitted for publication.
INTRODUCTION

Nucleolar Organizer Regions (NORs) are portions of DNA that code for rRNA. Some argyrophilic proteins, the most important being nucleolin, are involved in the transcription of NOR-DNA to rRNA. By using a silver colloid staining technique the transcriptionally active NORs and the associated argyrophilic proteins can be easily visualized in fixed and paraffin embedded tissue samples. These silver binding DNA-protein complexes are called AgNORs. AgNOR expression of a cell is causally or indirectly related to protein synthesis (1). Since protein synthesis is obligatory for cell proliferation, a relation between the extent of AgNOR expression and proliferative activity is suggested (2).

Patients with pilocytic astrocytomas have a very good prognosis and survival after total surgical resection. The clinical problem lies in those patients who have undergone a partial resection of their pilocytic astrocytoma. The residual tumor has an unpredictable behavior, it may remain stable, grow or even spontaneously regress (3). Therefore controversy exists about the usefulness and necessity of additional therapy, such as (stereotactic) radiation therapy or chemotherapy, on the residual tumor. The behavior of the residual tumor can not be predicted by conventional histological classification. Proliferation markers have gained much attention as possible predictors for biological behavior of tumors. We recently studied Ki-67 expression in pilocytic astrocytomas in relation to biological behavior, but a statistically significant correlation could not be found (4). Since the quantity of AgNOR-proteins is related to the rapidity of cell proliferation (5), in this study we related AgNOR expression to different patterns of biological behavior of pilocytic astrocytomas, as observed by neuro-radiological follow-up.
MATERIAL AND METHODS

Twenty-six tumor specimens of 26 patients were studied. The age of the patients ranged from 5 months to 16 years, with a mean of 6.4 years. All patients were surgically treated between 1985 and 1995 in the department of neurosurgery at the University Hospital Groningen, The Netherlands. All patients were post-operatively followed by CT- or MRI-scans. Duration of neuro-radiological follow-up lasted from 8 to 84 months, with a mean of 44 months. Thirteen tumor samples were from classic cerebellar pilocytic astrocytomas, 7 from optic nerve/hypothalamic gliomas of which 6 were of the pilocytic type and 1 (patientnr. 8) showed a rather diffuse gliomatous swelling of the optic nerve, 1 from a fibrillary astrocytoma of the spinal cord and 5 were from the cerebral hemispheres including one pilocytic astrocytoma (patientnr. 24), 3 fibrillary astrocytomas and one having the histologic pattern of a superficial cerebral astrocytoma with dural attachment (patientnr. 12) as described by Taratuto et al (6). Formalin fixed and paraffin embedded tumor material was used for this study. All histologic material was reviewed and classified according to the WHO-classification. AgNOR staining was performed essentially according to Howell and Black (7). The silver solution consisted of: 1 volume gelatin in 1% formic acid (12.5 ml) and 2 volumes of 20% silver nitrate solution (5 gr/ 25 ml). Incubation was performed at 37 degrees Celsius for 30 minutes, without preheating. Sections were dehydrated through a graded series of alcohol, cleared in xylene and mounted with synthetic resin mounting medium. The area of AgNORs was quantitatively assessed using an interactive image analysis system. A total magnification of x4840 was realized. After digitizing, a threshold was interactively set as to measure only AgNORs. As proposed by Crocker, we decided to use only a quantitative measurement, in which total AgNOR, both intra- and extra-nucleolar, is counted (8). For each tumor at least 20 randomly selected fields were quantified by meandering through the section. At the same time the total number of cells was hand-counted. The total amount of silver deposit was divided by the total number of cells, the obtained value is the mean amount of silver deposit per cell (square micrometer per cell). The number of counted cells per tumor-slice ranged from 81 to 294, with a mean of 176 cells per tumor.
RESULTS

AgNOR area measurements per cell among the 26 tumor samples ranged from 1.4 to 81.4 square micrometer per cell (sqmcm/c) with a mean of 26.6 and a median of 15.2 (table 1). The value of the median score was taken as a cut-off point for discriminating two groups; i.e. one group of patients with an AgNOR-score below the median and one group scoring above this value. All tumors in the group with scores below the median showed a benign behavior; 8 tumors were totally resected and did not recur, 3 residual tumors, two of which were irradiated, remained stable during further follow-up and 1 residual tumor regressed after radiation therapy. The remaining patient had a fibrillary astrocytoma which recurred 6 years after surgery and shows very slow progression.

In contrast, in the group of patients with AgNOR scores above the median value, there were only 2 total resections without recurrences, and 5 patients had residual tumor without evidence of progression, whereas 6 patients had postoperative residual tumor that progressed during further follow-up, all within the first year.

The above mentioned differences between the groups with low and high AgNOR scores appeared to be independent of tumor localization (table 2). Thus in the group of 13 cerebellar pilocytic astrocytomas only two cases showed progression of residual tumor during follow-up, both had AgNOR scores above the median, i.e. 18.2 and 48.0. In the small group of 7 optic nerve/hypothalamic gliomas the 3 patients who do very well have low AgNOR scores of 8.2, 9.5 and 15.9. This is in striking contrast to the 3 other patients with an optic pathway pilocytic astrocytoma, two of which, with AgNOR scores of 39.1 and 64.0, have rapidly progressing tumor, whereas the third with an AgNOR score of 57.8 died. The one patient with a pilocytic astrocytoma in the hypothalamic/third ventricle region had an AgNOR score of 32.9 but remained stable after radiation therapy. Among the five patients with tumors in the cerebral hemispheres 3 were completely resected without recurrence and 1 developed a slowly progressive recurrence 5 years after surgery (AgNOR score 12.1), the remaining patient died of rapid tumor progression, having a high AgNOR score of 44.1.

In total 15 patients had postoperative residual tumor. Of these residual tumors 1 regressed after radiation therapy, 4 remained stable during follow-up ranging from 12 to 81 months, 4 remained stable after being irradiated and 6 progressed, all within one year after surgery. All 6 progressive tumors had AgNOR scores above the median value. None of the pilocytic astrocytomas with AgNOR scores below the median value showed recurrence or progression of residual tumor. The only recurrence in the group of low AgNOR scores appeared in a fibrillary astrocytoma.
**TABLE 1.** Listing of all patients with AgNOR scores and results of follow-up.

<table>
<thead>
<tr>
<th>pat. nr.</th>
<th>age/sex</th>
<th>tumor type</th>
<th>tumor site</th>
<th>Ag-NOR score</th>
<th>resid. tumor</th>
<th>CT/MRI fu mo.</th>
<th>add. ther</th>
<th>follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11/F</td>
<td>pil</td>
<td>Cb.</td>
<td>1.4</td>
<td>no</td>
<td>M 33</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>2</td>
<td>3/F</td>
<td>pil</td>
<td>Cb.</td>
<td>2.5</td>
<td>no</td>
<td>M 77</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>3</td>
<td>7/M</td>
<td>pil</td>
<td>Cb.</td>
<td>2.8</td>
<td>yes</td>
<td>M 84</td>
<td>RT</td>
<td>regression</td>
</tr>
<tr>
<td>4</td>
<td>5/M</td>
<td>pil</td>
<td>Cb.</td>
<td>4.1</td>
<td>no</td>
<td>C 33</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>5</td>
<td>1/M</td>
<td>pil</td>
<td>Cb.</td>
<td>4.7</td>
<td>yes</td>
<td>M 57</td>
<td></td>
<td>stable</td>
</tr>
<tr>
<td>6</td>
<td>1/M</td>
<td>pil</td>
<td>Cb.</td>
<td>4.8</td>
<td>no</td>
<td>C 15</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>7</td>
<td>12/M</td>
<td>pil</td>
<td>Spin.</td>
<td>8.2</td>
<td>yes</td>
<td>M 33</td>
<td>RT</td>
<td>stable</td>
</tr>
<tr>
<td>8</td>
<td>4/M</td>
<td>fibr?</td>
<td>Opt.</td>
<td>8.2</td>
<td>no</td>
<td>M 37</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>9</td>
<td>5/F</td>
<td>pil</td>
<td>Cb.</td>
<td>8.8</td>
<td>no</td>
<td>C 52</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>10</td>
<td>10/F</td>
<td>pil</td>
<td>Opt.</td>
<td>9.5</td>
<td>yes</td>
<td>M 71</td>
<td>RT</td>
<td>stable</td>
</tr>
<tr>
<td>11</td>
<td>10/F</td>
<td>fibr</td>
<td>Cer.</td>
<td>12.1</td>
<td>no</td>
<td>M 76</td>
<td></td>
<td>recurrence</td>
</tr>
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<td>12</td>
<td>0.8/F</td>
<td>SCA</td>
<td>Cer.</td>
<td>13.9</td>
<td>no</td>
<td>M 63</td>
<td></td>
<td>NED</td>
</tr>
<tr>
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<td>fibr</td>
<td>Cer.</td>
<td>14.4</td>
<td>no</td>
<td>M 50</td>
<td>ChT</td>
<td>NED</td>
</tr>
<tr>
<td>14</td>
<td>1/F</td>
<td>pil</td>
<td>Opt.</td>
<td>15.9</td>
<td>no</td>
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<td></td>
<td>stable</td>
</tr>
<tr>
<td>15</td>
<td>5/M</td>
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<td>Cb.</td>
<td>18.2</td>
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<td>M 49</td>
<td></td>
<td>progression</td>
</tr>
<tr>
<td>16</td>
<td>8/M</td>
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<td>M 19</td>
<td></td>
<td>stable</td>
</tr>
<tr>
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<td>4/F</td>
<td>pil</td>
<td>Hypot</td>
<td>32.9</td>
<td>yes</td>
<td>M 64</td>
<td>RT</td>
<td>stable</td>
</tr>
<tr>
<td>18</td>
<td>13/F</td>
<td>pil</td>
<td>Cb.</td>
<td>33.5</td>
<td>no</td>
<td>M 24</td>
<td></td>
<td>NED</td>
</tr>
<tr>
<td>19</td>
<td>4/F</td>
<td>pil</td>
<td>Opt.</td>
<td>39.1</td>
<td>yes</td>
<td>M 19</td>
<td></td>
<td>progression</td>
</tr>
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<td>12/M</td>
<td>fibr</td>
<td>Cer.</td>
<td>44.1</td>
<td>yes</td>
<td>M 24</td>
<td></td>
<td>DOD</td>
</tr>
<tr>
<td>21</td>
<td>11/F</td>
<td>pil</td>
<td>Cb.</td>
<td>48.0</td>
<td>yes</td>
<td>C 1.5</td>
<td></td>
<td>progression</td>
</tr>
<tr>
<td>22</td>
<td>0.4/M</td>
<td>pil</td>
<td>Opt.</td>
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<td>yes</td>
<td>M 8</td>
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<td>DOD</td>
</tr>
<tr>
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<td></td>
<td>progression</td>
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<td>Cer.</td>
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<td>M 47</td>
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<td>NED</td>
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<tr>
<td>25</td>
<td>16/M</td>
<td>pil</td>
<td>Cb.</td>
<td>65.6</td>
<td>yes</td>
<td>M 27</td>
<td></td>
<td>stable</td>
</tr>
<tr>
<td>1 pat. nr.</td>
<td>2 age/sex</td>
<td>3 tumor type</td>
<td>4 tumor site</td>
<td>5 Ag-NOR score</td>
<td>6 resid. tumor</td>
<td>7 CT/MRI fu mo.</td>
<td>8 add. ther</td>
<td>7 follow-up</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>--------------</td>
<td>----------------</td>
<td>--------------</td>
<td>----------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
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<td>pil</td>
<td>Cb.</td>
<td>81.2</td>
<td>yes</td>
<td>M 12</td>
<td></td>
<td>stable</td>
</tr>
</tbody>
</table>

Legend table 1.

Columns: 1 = patient number. 2 = age of the patient at operation and sex, M: male, F: female. 3 = tumor type, pil: pilocytic astrocytoma, fibr: fibrillary astrocytoma, SCA: superficial cerebral astrocytoma with dural attachment. 4 = site of tumor, Cb.: cerebellar, Opt.: optic nerve, Spind.: spinal, Cer.: cerebral hemisphere, Hypot: hypothalamic region. 5 = AgNOR expression in squaremicrometer per cell. 6 = presence of postoperative residual tumor determined by CT- or MRI-scan. 7 = duration of follow-up in months by CT- (C) or MRI-scan (M). 8 = additional therapy, RT: radiation therapy, Cht.: chemotherapy. 9 = follow-up results, NED: No evidence of disease, DOD: Dead of disease, stable: residual tumor without progression.

**TABLE 2**. Relation between AgNOR score and clinical behavior by tumor localization.

<table>
<thead>
<tr>
<th></th>
<th>low AgNOR</th>
<th>high AgNOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>complete resection(6)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>stable/regression (5)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>progression (2)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Optic nerve/ hypothalamic (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>complete resection (1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>stable (3)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>progression (3)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Cerebral hemisphere (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>complete resection (3)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>progression (2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Spinal (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stable (1)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
DISCUSSION

Several studies among gliomas of all grades have found a correlation between AgNOR score and tumor grade (9,10,11,12) or patient survival (13), such that tumors with high AgNOR counts were histologically high grade and clinically aggressive. Louis et al. (14) further demonstrated the usefulness of AgNOR staining in the differentiation between gliosis and low grade astrocytoma by establishing a significantly lower AgNOR score in the former than in the latter. In most of the glioma studies AgNOR score could discriminate between low grade and high grade gliomas. The question arises whether AgNOR expression is sensitive enough to discriminate between tumors with an indolent behavior and those with a more aggressive course within a group of tumors whose histopathological grades are not far apart. The findings in the current study suggest that this may indeed be the case. In a group of low grade pediatric astrocytic tumors it was found that tumors with AgNOR values below the median for the group, follow an indolent clinical course, whereas those with AgNOR scores above the median can behave rather aggressively.

Results of studies relating AgNOR expression to established proliferation markers are unequivocal. Some showed a linear correlation of AgNOR score to S-phase fraction and to Ki-67 Labeling Index (9,10,15). Others failed to demonstrate a relation between AgNOR score on one hand and Ki-67 LI, BrdUrd LI or protein synthesis rate as assessed by 11C-Tyrosine Positron Emission Tomography on the other (16,17,18). The AgNOR scores of the present study showed no relation with MIB-1 LI, as determined in a previous study, of the same tumors (4). The above studies suggest that though AgNOR activity and proliferation may parallel each other, they presumably reflect different tumor biological phenomena. This notion is supported by the observations in atrophic and regenerating rat calf muscles, where AgNOR counts appeared to parallel protein synthesis not accompanied by proliferation (19) and with the findings in rat experimental pancreatitis, where AgNORs appeared to be a marker of regulatory cellular processes independent of the total intracellular protein synthesis rate (20). Furthermore Carbajo et al. showed that in the developing cells of rat anterior lobe of the hypophysis variations in AgNOR expression are more related to phenomena of cellular maturation than to proliferative activity (21).

Two different factors may cause the different and sometimes conflicting results of studies assessing the value of AgNOR staining as a proliferation marker. First, the method and interpretation of AgNOR scoring and secondly, the choice of the parameter that is used to relate AgNOR score to. The available data on how to score AgNOR expression are numerous and conflicting. Counting the individual silverdots per tumor cell nucleus has the problem of misinterpreting because of clustering of individual dots to one single larger dot (8). Surface area measurement of individual dots, in previous glioma studies has lead to different conclusions; to smaller dots with increasing malignancy (12), to larger dots with increasing malignancy (11), or to a stable dot-size independent of tumor grade (22). An inverse relation between mean AgNOR number per cell and mean AgNOR area has been reported (12).

Only 5 previous studies among astrocytomas of all grades reported the AgNOR score of pilocytic astrocytomas, all expressed this score in mean number of AgNOR dots per nucleus (11,12,16,22,23). In all, except the study of Maier et al. (16), the AgNOR score increased with tumor grade, but conclusions on the value of AgNOR staining within the group of pilocytic
astrocytomas were not drawn. Two previous studies (24,25) have investigated AgNOR expression within the homogenous group of optic gliomas. In both studies the results of counting AgNOR dots per nucleus did not correlate with biological behavior of the tumor during follow-up. Concerning the second factor, it appears that most studies relate the AgNOR score to histological degree of differentiation or to tumor grade. The determination of the tumor grade is based on various histopathological parameters and does not always directly correlate to proliferative activity of the tumor. Therefore the significance of AgNOR score as a tumor biological marker can not be assessed by studying it exclusively in relation to histopathological characteristics. Among 109 studies we reviewed on AgNOR expression in various kinds of human tumors, published between 1992 and 1995 (complete list of references available from the author), almost two-thirds related AgNOR score to histological parameters. The majority did find a statistically significant positive relation between the two parameters, but in 25% no relation could be established. The remainder of these studies related AgNOR score to patient outcome, prognosis or survival and a positive relation was found in 60%. This is clinically a more useful parameter but still an indirect way to test AgNOR expression as a marker for tumor growth. In only one study, involving adenocarcinomas of the lung, AgNOR score was directly related to tumor growth, assessed by periodical radiological follow-up of the patient (26). Interestingly, this study showed no relation between AgNOR score and pathological tumor grade, but a strong statistically significant relation between high AgNOR score and short tumor doubling time.

The clear result of the study of Derenzini et al. proving that quantitative changes of the AgNOR proteins, as determined by area measurements, are related to cell doubling time in human cancer cell-lines (5) and the lack of correlations in previous AgNOR enumeration studies among pilocytic astrocytomas and optic gliomas have led us to the decision to use only AgNOR area measurements in the present study. Also our results clearly demonstrate a positive relation between AgNOR area measurement and tumor growth as assessed by follow-up imaging studies.

The extent of surgical resection might influence our results. In the low AgNOR scoring group 8 patients had complete surgical resections compared to only 2 in the high scoring group. Although this may bias our results, it may also reflect a difference in growth pattern, such that tumors with high AgNOR scores grow more infiltrating and are therefore more difficult to resect completely.

In the clinical setting there is debate on how to treat the patient with a residual pilocytic astrocytoma after incomplete surgical resection. Additional chemotherapy or (stereotactic) radiation therapy might be used, but are not always necessary since some of the residual tumors remain "quiescent".

In our group of 26 patients, 15 had postoperative residual tumor. The 6 of those that showed progression, and might benefit from additional therapy, all had high AgNOR scores. Therefore, we conclude that AgNOR staining may play a role in the prediction of the behavior of residual tumor after incomplete surgical resection. For patients with residual tumor having a low AgNOR score a "wait and see" follow-up policy can be advocated. Patients with high scoring residual tumors could be selected for additional chemo- or radiation therapy.
REFERENCES

16. Maier H, Morimura T, Ofner D, Hallbrucker C, Kitz K, Budka H: Argyrophilic nucleolar organizer region proteins (Ag-NORs) in human brain tumors: relations with grade of malignancy and


CHAPTER 5

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

Dirven CMF, Koudstaal J, Mooij JJA, Molenaar WM
INTRODUCTION

In routine neuropathology the grading of gliomas is done on the basis of morphological criteria such as nuclear morphology, presence or absence of necrosis, vascular proliferation and mitoses. For the group of pilocytic astrocytomas this grading system lacks the possibility to give specific information on the growth potential and biological behavior of these tumors. There is increasing evidence that the amount of proliferating cells in a tumor correlates with the individual prognosis of the patient with a glioma.

Several techniques to investigate cell kinetics have been developed. Some of them are based upon the detection of antigens specifically expressed by proliferating cells. One of these techniques is Ki-67 immunolabeling. Ki-67 is a monoclonal antibody, that reacts to the Ki-67 antigen in fresh frozen tissue, which is expressed in the G1, S, G2, and M phase of the cell cycle, but not in the G0 phase (3). The commercially available monoclonal antibody MIB-1 reacts to the Ki-67 antigen in formalin fixed tissue (4). The amount of proliferating tumor cells that is recognized and stained by the antibody can be counted as a fraction of the total amount of tumor cells. This number of cells expressed as a percentage is called the labeling index (LI) or proliferating cell index (PCI). So, the LI or PCI reflects the amount of cells in a tumor that shows proliferative activity.

Ki-67 labeling has only sporadically been performed on pilocytic astrocytomas. The previous studies in which the proliferative potential of gliomas was observed focused mainly on the malignant glioma types in adults. Furthermore, only a few of those studies correlated the results of Ki-67 labeling to prognosis and outcome of the patient. Although the pilocytic astrocytoma is a benign tumor, the biological behavior is unpredictable; metastatic spread, malignant degeneration and spontaneous tumor regression are well known features of this tumor (2).

The present study was undertaken to get more insight in the biological behavior of the pilocytic astrocytoma. Therefore MIB-1 Labeling indices were correlated with the behavior of the tumor as detected by follow-up CT- or MRI-scans of the brain. In this way we tried to answer the question wether MIB-1 expression reflects differences in biological behavior of pilocytic astrocytomas.

MATERIAL AND METHODS

Formalin fixed and paraffin embedded tumor material of 44 patients was used for this study. All histologic material was reviewed and classified according to the WHO-classification (revised version of 1993). From 6 patients two different tumor-samples were obtained from second operations because of residual or recurrent tumor. At the time of primary surgery the patients were all in the pediatric age group (0-16 years), except for 5 patients who were 18, 20, 20, 27 and 39 years of age, respectively. All patients were operated between 1975 and 1995 in the department of neurosurgery at the University Hospital Groningen, The Netherlands.

Among the 44 patients there were 28 cerebellar pilocytic astrocytomas, 1 pilocytic astrocytoma of the conus medullaris, 6 pilocytic astrocytomas of the optic nerve, 3 supratentorial hemispheric
pilocytic astrocytomas, 1 third ventricular pilocytic astrocytoma and 5 supratentorial low grade astrocytomas. All but two patients had postoperative CT- or MRI-scans. The interval between surgery and the last performed scan ranged from 7 months to more than 12 years with a mean follow-up time of 54 months. All scans were reviewed and judged on presence or absence of tumor and on size of present tumor. An increase in size on follow-up scans was interpreted as tumor progression, an unchanged size as stable residual tumor and a decrease or disappearance as regression of residual tumor. All patients were followed until the end of the study (May 1997) by interviewing the pediatrician or the family doctor. The results of these interviews are referred to as "clinical follow-up". The clinical follow-up of the two patients (nr.s 21 and 24) without postoperative neuro-imaging studies lasted more than 16 years in both cases. According to the postoperative notes of the surgeon they both had a cystic cerebellar tumor with macroscopic total resection at surgery.

In total 50 tumor samples were stained with the MIB-1 antibody. Routinely formalin fixed paraffine embedded tissue was used. Five micrometer sections were cut, floated in a water bath of 50 degrees Celsius and mounted on APES (Amino Propyl Ethoxy Silane) coated slides. They were placed on a slide warmer for 15 minutes at 60 degrees Celsius and dried overnight in an oven at 37 degrees Celsius. For antigen retrieval we used wet autoclaving. Wet autoclaving was performed as follows: After covering the slides with 300 microliter of 10mM citrate Ph=6,0, cover slips were placed to prevent drying-out of the tissue. They were placed in a metal box, able to contain 36 slides. This box was put in a preheated pressure cooker. After reaching a pressure of 115 Psi (+/-115 degrees Celsius) heating was continued for 10 minutes. After cooling off the metal box was removed from the cooker and held for 20 minutes at room temperature. This procedure was repeated twice. After antigen retrieval slides were incubated with 400* diluted primary MIB-1 antigen (Immunotech, France). Two step immunostaining was performed according to the manufacturer's procedure of the Biogenex kit, containing anti-mouse-biotin and alkaline phophatase conjugated streptavidin. BCIP-NBT (bromo-chloro-indolyl-phosphate - 4-nitro-blue-tetrazolium-chloride; Boehringer Mannheim) was used as substrate. A multi-tissue slide containing 24 mamma carcinoma samples was used as control.

Fifteen randomly chosen high power (x 400) microscopic fields were chosen from each tumor slice and the cells were counted. MIB-1 LI was calculated as the number of positive cells divided by the total number of cells.

RESULTS

Fifty tumor samples of 44 patients were studied; 39 patients had a pilocytic astrocytoma and 5 patients a low grade astrocytoma. MIB-1 LI's in the whole group ranged from 0% to 19% with a mean value of 4,2%. Table 1 lists the MIB-1 LI's for the different tumors and localizations.
TABLE 1. MIB-1 LI values for 50 samples of 39 pilocytic astrocytomas and 5 low grade astrocytomas.

<table>
<thead>
<tr>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>LI neg.</td>
<td>14</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
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<tr>
<td>LI 1-5%</td>
<td>6</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LI 5-10%</td>
<td>7</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>LI 10-20%</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>mean</td>
<td>4,4%</td>
<td>-</td>
<td>2%</td>
<td>5,3%</td>
<td>4%</td>
</tr>
</tbody>
</table>

Legend: piloc.astro.= pilocytic astrocytoma; LI= MIB-1 labeling index; neg.= negative, which means LI value smaller than 1%.

In 23 of the 44 patients post-operative CT- or MRI-scans showed residual tumor, which proved an incomplete resection. In order to relate the biological behavior of the tumor to the MIB-1 LI value, two subgroups of patients with post-operative residual tumor were determined. One group consisted of 12 patients with "benign" behavior, which means no progression of tumor or even regression during further neuro-radiological follow-up.

Ten of these patients had residual tumor after surgery without progression of this tumor during follow-up, whereas two patients showed regression of their residual tumor after radiation therapy (table 2). The mean MIB-1 LI was 3,3% in this group. The other group consisted of 11 patients with 12 tumor samples showing progression of their residual tumor during subsequent neuro-radiological follow-up. The mean MIB-1 LI in this group was 6,6%. These patients are listed in table 3. The remaining patients, who had complete tumor resection without recurrence during follow-up, are listed in table 4.

TABLE 2. Characteristics and MIB-1 LI of 12 patients with stable residual tumor or regression of residual tumor after radiation therapy (nr.’s 14 and 25).

<table>
<thead>
<tr>
<th>pat. nr.</th>
<th>age yrs.</th>
<th>s</th>
<th>tumor type</th>
<th>neurorad. f.u (months)</th>
<th>clinical. f.u (months)</th>
<th>RT</th>
<th>LI</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>M</td>
<td>c.p.a.</td>
<td>57</td>
<td>57</td>
<td>no</td>
<td>6%</td>
</tr>
<tr>
<td>10</td>
<td>11,7</td>
<td>M</td>
<td>c.p.a.</td>
<td>59</td>
<td>91</td>
<td>no</td>
<td>2%</td>
</tr>
<tr>
<td>13</td>
<td>14,5</td>
<td>F</td>
<td>c.p.a.</td>
<td>39</td>
<td>129</td>
<td>yes</td>
<td>neg. post-RT</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>M</td>
<td>c.p.a.</td>
<td>84</td>
<td>84</td>
<td>yes</td>
<td>10%</td>
</tr>
<tr>
<td>pat. nr.</td>
<td>age yrs.</td>
<td>s</td>
<td>tumor type</td>
<td>neurorad. f.u. (months)</td>
<td>clinical. f.u. (months)</td>
<td>RT</td>
<td>LI</td>
</tr>
<tr>
<td>----------</td>
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<td>------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>25</td>
<td>16,5</td>
<td>M</td>
<td>c.p.a.</td>
<td>27</td>
<td>106</td>
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<td>neg.</td>
</tr>
<tr>
<td>27</td>
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<td>F</td>
<td>c.p.a.</td>
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<td>12</td>
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</tr>
<tr>
<td>28</td>
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<td>c.p.a.</td>
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<td>19</td>
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</tr>
<tr>
<td>30</td>
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<td>o.n.p.a.</td>
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<td>133</td>
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</tr>
<tr>
<td>31</td>
<td>1,5</td>
<td>F</td>
<td>o.n.p.a.</td>
<td>81</td>
<td>81</td>
<td>no</td>
<td>8% chemo</td>
</tr>
<tr>
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<td>9,5</td>
<td>F</td>
<td>l.g.a.</td>
<td>60</td>
<td>105</td>
<td>yes</td>
<td>neg.</td>
</tr>
<tr>
<td>41</td>
<td>4,5</td>
<td>F</td>
<td>3d v. p.a.</td>
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<td>64</td>
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<td>5%</td>
</tr>
<tr>
<td>43</td>
<td>12,5</td>
<td>M</td>
<td>sp.p.a.</td>
<td>33</td>
<td>96</td>
<td>yes</td>
<td>neg.</td>
</tr>
</tbody>
</table>

Legend: pat.nr = patient number; age yrs. = age in years at time of operation; s = sex; time to progr. = time to detection of tumor progression on follow-up scans; neurorad. f.u. = duration of neuroradiological follow-up in months; clinical f.u. = duration of clinical follow-up in months; RT = radiation therapy; LI = MIB-1 labeling index; c. = cerebellar; p.a. = pilocytic astrocytoma; o.n. = optic nerve; l.g.a. = low grade astrocytoma; 3d v. = third ventricle; sp. = spinal; te. = temporal; par. = parietal; post-RT = post radiation therapy; chemo = chemotherapy; neg. = negative; metas = metastases in spinal canal and third ventricle; recurr = recurring tumor 6 years after “total” resection.

**TABLE 3.** Characteristics and MIB-1 LI of 12 tumor samples from 11 patients with progression of residual tumor, including one patient with recurrence (pat nr. 37).
### TABLE 4. Characteristics and Mib-1 LI of patients with total tumor resections without recurrences.

<table>
<thead>
<tr>
<th>pat nr</th>
<th>age</th>
<th>s</th>
<th>tumor type</th>
<th>neuro-rad f.u.</th>
<th>Clinical f.u.</th>
<th>RT</th>
<th>Mib-1 LI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8,5</td>
<td>M</td>
<td>c.p.a.</td>
<td>59</td>
<td>63</td>
<td>no</td>
<td>9%</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>M</td>
<td>c.p.a.</td>
<td>4</td>
<td>5</td>
<td>no</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>13,7</td>
<td></td>
<td>c.p.a.</td>
<td>30</td>
<td>82</td>
<td>no</td>
<td>3%</td>
</tr>
<tr>
<td>5</td>
<td>11,5</td>
<td>F</td>
<td>c.p.a.</td>
<td>33</td>
<td>60</td>
<td>no</td>
<td>1%</td>
</tr>
<tr>
<td>6</td>
<td>8,5</td>
<td>F</td>
<td>c.p.a.</td>
<td>62</td>
<td>98</td>
<td>no</td>
<td>4%</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>M</td>
<td>c.p.a.</td>
<td>15</td>
<td>117</td>
<td>no</td>
<td>neg.</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>F</td>
<td>c.p.a.</td>
<td>52</td>
<td>81</td>
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<td>9</td>
<td>5,5</td>
<td>M</td>
<td>c.p.a.</td>
<td>33</td>
<td>92</td>
<td>no</td>
<td>9%</td>
</tr>
<tr>
<td>11</td>
<td>8,5</td>
<td>F</td>
<td>c.p.a.</td>
<td>120</td>
<td>163</td>
<td>no</td>
<td>1,6%</td>
</tr>
<tr>
<td>12</td>
<td>3,5</td>
<td>F</td>
<td>c.p.a.</td>
<td>77</td>
<td>114</td>
<td>no</td>
<td>neg.</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>M</td>
<td>c.p.a.</td>
<td>15</td>
<td>104</td>
<td>no</td>
<td>8%</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>F</td>
<td>c.p.a.</td>
<td>126</td>
<td>130</td>
<td>no</td>
<td>neg.</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>M</td>
<td>c.p.a.</td>
<td>146</td>
<td>146</td>
<td>no</td>
<td>neg.</td>
</tr>
<tr>
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<td>14</td>
<td>M</td>
<td>c.p.a.</td>
<td>70</td>
<td>216</td>
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<td>neg.</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>F</td>
<td>c.p.a.</td>
<td>24</td>
<td>200</td>
<td>yes</td>
<td>10%</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td>M</td>
<td>c.p.a.</td>
<td>70</td>
<td>192</td>
<td>no</td>
<td>19%</td>
</tr>
<tr>
<td>23</td>
<td>17,5</td>
<td>M</td>
<td>c.p.a.</td>
<td>51</td>
<td>187</td>
<td>no</td>
<td>neg.</td>
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</tbody>
</table>
In the 39 patients with pilocytic astrocytomas, 43 tumor samples were investigated. Nineteen tumors were negative for MIB-1 staining and 24 were positive. In the MIB-1 negative group there were 6 patients with postoperative residual tumor, from which 1 showed progression during neuro-radiological follow-up. In the MIB-1 positive group there were 13 residual tumors from which 7 showed progression. There is a tendency of MIB-1 negative tumors showing fewer progressions compared to MIB-1 positive tumors (p=0.15, Fisher exact test).

Six patients had more than one surgical tumor resection and MIB-1 staining was performed on subsequent tumor specimens of the same patient (table 5). In 5 of these 6 patients the second sample had a lower MIB-1 LI than the first sample. The remaining patient had a malignant degeneration from low grade astrocytoma to high grade astrocytoma with a low MIB-1 LI in the first sample and a much higher MIB-1 LI in the second.

**TABLE 5.** Characteristics and MIB-1 LI’s of six patients with more than one tumor resection.

<table>
<thead>
<tr>
<th>pat nr</th>
<th>tumor type</th>
<th>RT</th>
<th>LI 1st</th>
<th>interval</th>
<th>RT</th>
<th>LI 2nd</th>
<th>Reason for second operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>c.p.a</td>
<td>no</td>
<td>9%</td>
<td>4</td>
<td>no</td>
<td>3%</td>
<td>residual tumor</td>
</tr>
<tr>
<td>10</td>
<td>c.p.a</td>
<td>no</td>
<td>7%</td>
<td>2</td>
<td>no</td>
<td>2%</td>
<td>tumor progression</td>
</tr>
<tr>
<td>13</td>
<td>c.p.a</td>
<td>no</td>
<td>3%</td>
<td>10</td>
<td>yes</td>
<td>neg.</td>
<td>tumor progression after RT</td>
</tr>
</tbody>
</table>

Legend: see legend Table 2.
<table>
<thead>
<tr>
<th>pat. nr.</th>
<th>tumor type</th>
<th>RT</th>
<th>LI 1st</th>
<th>interval</th>
<th>RT</th>
<th>LI 2nd</th>
<th>Reason for second operation:</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>c.p.a</td>
<td>no</td>
<td>18%</td>
<td>8</td>
<td>no</td>
<td>neg.</td>
<td>1st operation was recurrence from 10 yrs. before, 2nd operation was progr. of residual tumor, hereafter 11 yrs. free from tumor</td>
</tr>
<tr>
<td>36</td>
<td>l.g.a</td>
<td>no</td>
<td>3%</td>
<td>3</td>
<td>no</td>
<td>neg.</td>
<td>residual tumor</td>
</tr>
<tr>
<td>44</td>
<td>l.g.a</td>
<td>no</td>
<td>neg.</td>
<td>6</td>
<td>no</td>
<td>15%</td>
<td>progression to astro. gr III.</td>
</tr>
</tbody>
</table>

Legend: pat. nr.= patient number; RT= radiation therapy; interval=period between subsequent operations in months; LI=MIB-1 labeling index.; c.p.a. = cerebellar pilocytic astrocytoma; l.g.a.= low grade astrocytoma; neg.= negative, which means LI value smaller than 1%; progr.= progression.

**DISCUSSION**

The pilocytic astrocytoma is a peculiar neoplastic entity for it's capability of showing progression, stabilization and spontaneous regression. A possible explanation of this unpredictable behavior might be found in the cell kinetics of this tumor. An important previous study in this field was done by Ito et al (7). The percentage of cells in S-phase of the cell cycle, as determined by the bromodeoxyuridine labeling index (B UdR LI) in a group of 50 pilocytic astrocytomas was low, ranging from 0.22 to 4.3 %. This LI value did not correlate with the outcome of the patient. However, 4 tumors that recurred after partial resection had higher B UdR LI values than 32 tumors that did not recur after subtotal resection. Furthermore the B UdR LI tended to be lower in older patients, and these authors concluded that the tumor growth rate appeared to slow down with increasing age.

A disadvantage of the bromodeoxyuridine labeling technique is it's invasiveness regarding the obligatory preoperative intravenous infusion of B UdR into the patient. Among other methods to study cell kinetics the Ki-67 labeling technique has shown it's usefulness in quantifying the amount of proliferating cells (3,8,12). Onda et al have shown the usefulness of MIB-1 staining (12) and have compared the reliability of the MIB-1 proliferating cell index to the Ki-67 LI (11). The MIB-1 immunostaining was often superior to that of Ki-67 in individual tumors. Furthermore they showed a statistically significant linear correlation between the two indices in a group of 90 malignant gliomas. A significant relation between Ki-67 LI and grade of astrocytoma, and with length of survival, was given by Jaros et al (8) in their study of 43 astrocytomas of different grades. Patients with LI's higher than 5% had a reduced survival compared to those with LI's below 5%. Zuber et al (17) demonstrated a significant difference in Ki-67 LI values of the three astrocytoma types grade I, II and III. However, the Ki-67 LI alone did not significantly correlate with survival in this group of 51 glioma patients, but the 5 patients in this group that survived more than 40 weeks had low Ki-67 LI's (lower than 2.5%). Another recent study on astrocytomas grade II, III and IV demonstrated the Ki-67 LI, obtained by the use of the MIB-1 monoclonal antibody, to be a significant prognostic
factor (16). In the study presented here there is a trend towards fewer progressions from MIB-1 negative tumors compared to MIB-1 positive tumors. The MIB-1 LI values showed a wide range; supratentorial pilocytic astrocytomas tended to have lower values than cerebellar pilocytic astrocytomas. The optic nerve pilocytic astrocytomas had the highest values (table 1).

In previous reports the mean values of the Ki-67 LI ranged from 0,9% to 6% among 21 patients from different studies, as listed in table 6. MIB-1 labeling studies focusing on pilocytic astrocytomas have not been reported in the literature. Considering the more benign behavior of the pilocytic astrocytoma opposed to the grade II astrocytoma, one would expect a lower percentage of cells showing proliferative activity in this tumor. However, as can be seen from table 6, Ki-67 LI's from pilocytic astrocytomas are in the same range or higher than those of grade II astrocytomas. Ki-67 LI values given in this table, resulting from other studies, were never tested in relation to tumor proliferation. They were mostly related to histopathological grade, comparing the results for the different glioma grades. Only in the study of Zuber et al. all low grade astrocytoma patients were alive 3 to 42 months after treatment, the Ki-67 LI’s were low, ranging from 0,1 to 4,5% (17).

In pilocytic astrocytoma patients it is difficult to study the relation between proliferative potential and patient outcome as determined by survival time, since patient outcome after treatment is overall good or excellent with very long survival times. Therefore we distinguished two groups of patients with different biological behavior based upon neuroradiological follow-up. One group consisting of 11 patients with progression of tumor proven on follow-up CT- or MRI-scans, and the other consisting of 12 patients of whom the residual tumor after partial surgical resection did not show any tendency to grow or even showed regression on follow-up scans (tables 2 and 3). Duration of follow-up until detection of tumor progression among the 11 patients ranged from 2 to 74 months. In 9 patients progression appeared within one year after surgical treatment, in the other 2 patients after respectively 16 and 74 months. In the "quiescent" group follow-up time lasted from 12 to 133 months, with a mean of 56 months. MIB-1 LI values in the group with progression of tumor ranged from 0% to 18%, with a mean of 6,6%. In the group without progression these values ranged from 0% to 10%, with a mean of 3,3%. Group numbers are too small to give a significant relation between MIB-1 LI value and behavior of the tumor during neuroradiological follow-up. However, there is a tendency towards lower LI values in the group without tumor progression.

Comparing the results of MIB-1 labeling of two different tumor specimens in the re-operated patient shows in 5 of the 6 cases that the second sample, taken from residual tumor, has a lower MIB-1 LI than the first sample (table 5). Remarkably Ito et al (7) have found the same result in BUdR labeling of subsequent recurring tumor samples of the same patient. A speculative explanation might be the alteration of the micro-environment of the tumor after partial surgical resection. A decreased vascular feeding might be part of this alteration. The only case in which the second tumor sample had a higher MIB-1 LI value was where a malignant degeneration from astrocytoma grade 2 to grade 3 had appeared (patient number 44). This finding of decreased proliferative potential of a tumor remnant after partial surgical resection corresponds with the well known clinical observation that those tumor remnants may remain "quiescent" for many years.
TABLE 6. Mean Ki-67 LI values in previous studies of gliomas.

<table>
<thead>
<tr>
<th>Study:</th>
<th>ASTROCYTOMA</th>
<th></th>
<th></th>
<th>piloc.astro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grade 4</td>
<td>grade 3</td>
<td>grade 2</td>
<td>piloc.astro</td>
</tr>
<tr>
<td>Giangaspero 1987 (5)</td>
<td>10-40% (n=7)</td>
<td>1% (n=1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ostertag 1987(13)</td>
<td>8%</td>
<td>9,5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shibata 1988 (14)</td>
<td>9,8% (n=53)</td>
<td>4,3% (n=6)</td>
<td>1,8% (n=12)</td>
<td>0,9% (n=13)</td>
</tr>
<tr>
<td>Zuber 1988 (17)</td>
<td>11,1% (n=27)</td>
<td>3,5% (n=8)</td>
<td>1,0% (n=16)</td>
<td>4,5% (n=1)</td>
</tr>
<tr>
<td>Maier 1990 (10)</td>
<td>9,9% (n=18)</td>
<td>5,1% (n=4)</td>
<td>1,5% (n=6)</td>
<td>0,7% (n=1)</td>
</tr>
<tr>
<td>Hara 1990 (6)</td>
<td>5,2% (n=3)</td>
<td>3% (n=5)</td>
<td>0,5% (n=5)</td>
<td></td>
</tr>
<tr>
<td>Louis 1991 (9)</td>
<td>21% (n=7)</td>
<td>4,4% (n=8)</td>
<td>&lt;1% (n=4)</td>
<td>6% (n=1)</td>
</tr>
<tr>
<td>Tsanaclis 1991 (15)</td>
<td>10,1%</td>
<td>8%</td>
<td>&lt;1%</td>
<td>5,6% (n=1)</td>
</tr>
<tr>
<td>Jaros 1992 (8)</td>
<td>11,1% (n=20)</td>
<td>7,5% (n=13)</td>
<td>1,2% (n=7)</td>
<td>0,9% (n=3)</td>
</tr>
<tr>
<td>Coons 1993 (1)</td>
<td>7,2% (n=9)</td>
<td>0,8% (n=7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onda 1994 (11)</td>
<td>13,8% (n=17)</td>
<td>7,0% (n=1)</td>
<td>4,2% (n=7)</td>
<td>4,2% (n=43)</td>
</tr>
<tr>
<td>This study(MIB-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CONCLUSION

Pilocytic astrocytomas have low MIB-1 LI's compared to high grade astrocytomas, however these values are in the same range as or higher than those reported for the astrocytomas grade 2. Regarding the different outcome of patients treated for astrocytoma grade 2 opposed to pilocytic astrocytoma, the MIB-1 LI seems not to be an important predictive factor in patient outcome. However, according to previous reports, the MIB-1 LI does relate to patient outcome in high grade astrocytomas. It also reflects to a certain degree the biological behavior of the tumor within the group of pilocytic astrocytomas.

From all the patients with residual tumor after operation, those with tumor progression showed a tendency towards higher MIB-1 LI's than those with "quiescent" tumor during follow-up. Pilocytic astrocytomas that are negative for MIB-1 staining are very unlikely to show recurring tumor or progression of post-operative residual tumor.

MIB-1 LI's of residual tumor have lower values than those taken from the sample at first operation. Perhaps the proliferative potential of the tumor decreases after resection of a great part of the tumor and the vascular feeders.

MIB-1 staining might be an additional tool in deciding how long and how frequent a patient with residual pilocytic astrocytoma after operation needs to be followed and in the decision making about
possible further treatment. Series with larger numbers of patients and very long follow-up periods are needed to give more significant data about the clinical usefulness of the MIB-1 LI in pilocytic astrocytomas.

REFERENCES

CHAPTER 6

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

Dirven CMF, Hayes VM, Koudstaal J, Molenaar WM, Mooij JJA.
In progress for publication.
INTRODUCTION

The TP53 tumor suppressor gene is the most commonly mutated gene in human neoplasms (1,2). The normal function of the TP53 gene is to correct disturbances in cell replication. TP53 codes for the DNA-binding phosphonuclear protein, p53, involved in transcription, activation and regulation of the cell cycle (3,4). In case of DNA damage or disturbances in the replicative process p53 is involved in initiating DNA-repair mechanisms, blocking the cell cycle by inducing G1-arrest or triggering apoptosis (5). Furthermore, the gene plays an important role in the process of cellular differentiation (6). Disturbances in p53 function are strongly associated with carcinogenesis. The gene, located at band p13.1 of chromosome 17, consists of 11 exons of which exons 5 to 8 contain the evolutionary conserved region (7). Most TP53 mutation detection studies have therefore been restricted to this region. Among high grade astrocytomas p53 immunopositiveness and TP53 mutations are reported in a high frequency; in various studies ranging from 30%-90% (8-12). In adult low grade gliomas this frequency is 5%-60% (8,9,11-16). The involvement of the TP53 gene in malignant progressio of low grade astrocytomas to a higher grade is confirmed in several studies (15,17-20). These studies suggest that TP53 mutations are early events in astrocytoma formation. Despite the high incidence of p53 dysfunction, a relation between p53 status and survival of patients with high grade astrocytomas could not be established in most studies (16,19,21-24). However, in high grade astrocytomas of the brain stem TP53 mutation and p53 expression were associated with survival; patients with p53 immunopositive or mutated tumors having a worse prognosis (25). Two other studies among low grade astrocytomas in adults reported a better prognosis for p53 immunonegative tumors compared to immunopositive tumors (15,19). In low grade and pilocytic astrocytomas of childhood TP53 mutations seem to be a rare event, with only three mutations occurring in 107 (2.8%) previously investigated tumors (13,14,26-32). However, in 3 studies accumulation of p53 protein was detected in a high percentage of pilocytic astrocytomas (13,33,34). The purpose of this study is to investigate the role of the TP53 gene in pilocytic astrocytomas by looking at the following: one, the percentage of immunohistochemically positive cells; secondly, the mutation frequency of the TP53 gene; thirdly, to determine whether immunohistochemical detection of p53 is associated to TP53 mutation; and finally whether the determination of p53 status (staining or mutation rates) has a relation to tumor behavior. To achieve this we performed immunohistochemistry with a p53 monoclonal antibody on a series of 43 pilocytic astrocytomas and 5 low grade astrocytomas of childhood. For TP53 mutation analysis, we used a comprehensive denaturing gradient gel electrophoresis (DGGE) assay and sequence analysis of the entire coding region of TP53 in 12 of those tumors.
MATERIAL AND METHODS

Formalin fixed and paraffin embedded tumor material of 48 patients was used for this study. All histologic material was reviewed and classified according to the WHO-classification. At the time of primary surgery the patients were all in the pediatric age group (0-16 years), except for 5 patients who were 18, 20, 20, 27 and 39 years of age, respectively. All patients were operated between 1975 and 1995 in the department of neurosurgery at the University Hospital Groningen, the Netherlands.

Among the 48 patients there were 30 cerebellar pilocytic astrocytomas, 1 pilocytic astrocytoma of the conus medullaris, 6 pilocytic astrocytomas of the optic pathways, 3 supratentorial hemispheric pilocytic astrocytomas, 3 third ventricular pilocytic astrocytomas and 5 supratentorial low grade astrocytomas.

All but two patients had postoperative CT- or MRI-scans. The interval between surgery and the last performed scan ranged from 3 months to more than 10 years with a mean follow-up time of 50 months. All patients except one, were followed until the end of the study (February 1997) by screening the medical files and interviewing the pediatrician or the family doctor. The one patient (nr. 47) that was lost to follow-up lives abroad, the direct postoperative CT scan showed total resection of a third ventricular pilocytic astrocytoma. The clinical follow-up of the two patients without postoperative neuroimaging studies lasted more than 16 years in both cases. According to the postoperative notes of the surgeon they both had a cystic cerebellar tumor with gross total resection at surgery.

Immunohistochemical analysis.
In total 48 tumor samples were stained with the p53 monoclonal antibody. Routinely formalin fixed paraffin-embedded tissue was used. Five micrometer sections were cut, floated in a water bath and mounted on APES (Amino Propyl Ethoxyl Silane) coated slides. They were placed on a slide warmer for 15 minutes at 60 degrees Celsius and dried overnight in an oven at 37 degrees Celsius. Wet autoclaving was used for antigen retrieval and was performed as follows: After covering the slides with 300 microliter of a solution containing 0.15 M sodium chloride, 0.1 M malic acid and 0.2% SDS pH=6.0, cover slips were placed to prevent drying-out of the tissue. They were placed in a metal box, holding 36 slides. This box was placed in a preheated pressure cooker. After reaching a temperature of 115 degrees Celsius, heating was continued for 10 minutes. After cooling off the metal box was removed from the cooker and held for 20 minutes at room temperature. This procedure was repeated twice. After antigen retrieval, slides were incubated with 1:400 diluted primary p53 antibody (Clone BP53-12, Neomarkers, Freemont USA) for 45 minutes. Two step immunostaining was performed according to the manufacturer’s procedure of the Biogenex kit, containing anti-mouse-biotin and alkaline phosphatase conjugated streptavidin. BCIP-NBT (bromo-chloro-indolyl-phosphate -4-nitro-blue-tetrazolium-chloride; Boehringer Mannheim) was used as substrate. A multi-tissue slide containing 24 breast-carcinomas was used as control material, some strongly positive, some negative. Two observers (CMFD and JK) independently scored the number of immunopositive cells in 10 randomly chosen high power fields, where tumor tissue was present. Scoring was performed in a 4 grade scale: negative, slightly positive (less than
5% of cells), positive (5%-50% of cells), strongly positive (more than 50% of cells). When the results of the 2 observers were discordant, the tumor slides were re-evaluated until agreement was achieved. Tumors were regarded immunopositive when more than 5% of cells were stained by the antibody.

**Genetic analysis.**

DNA was extracted from 12 paraffin-embedded tumors: 10 pilocytic and 2 low grade astrocytomas using the method previously described by Wang et al (35). The entire coding region of *TP53* was amplified within 12 amplicons using nested PCR. Primer sets and PCR conditions were as will be described by Hayes et al, in submission (36). All amplicons had a GC-rich clamp attached to the one end of the sequence, thus creating a high stability domain appropriate for genetic analysis using denaturing gradient gel electrophoresis (DGGE). All amplicons were electrophoresed on a 9% polyacrylamide gel, containing 5% glycerol and a 35%-75% Urea-Formamide denaturing gradient, at 150V for 7.5 hours at 59 degrees Celsius (36). Gels were stained with ethidium bromide, homoduplex or heteroduplex aberrant DGGE bands were excised from the gel, incubated in distilled water overnight at 4 degrees Celsius and re-amplified. After purification of the PCR products, sequencing using single-stranded radioactive sequencing or single-stranded automated sequencing was performed using a non-GC-clamped primer. All mutations were confirmed either by sequencing from both ends or by restriction enzyme digestion.

**RESULTS**

**Clinical.**

All patients were categorized in 2 groups, one group of 26 patients who had total tumor resection after first operation, confirmed by postoperative MRI- or CT scanning and one group of 22 patients who had incomplete resections resulting in residual tumor on postoperative scans. One patient (nr. 37) had a recurrence of a low grade astrocytoma after initial total resection, this patient is included in the second group. This last group was followed by sequential follow-up scans and from these results tumor behavior was analyzed. Thirteen patients with residual tumor showed no progression of tumor on follow-up scans: 10 remained stable and 3 showed regression after radiation therapy. Duration of follow-up ranged from 12 to 133 months, with a mean of 59 months (Table 1). Eleven patients with residual tumor showed progression of the tumor on follow-up scans. The interval between operation and the first scan detecting tumor progression was less than 1 year in 9 patients. In 1 patient progression was detected on the first performed MRI scan during follow-up, which took place after 16 months and in 1 patient (nr.37) a low grade astrocytoma recurred 6 years after initial complete resection shown on direct postoperative MRI scan (table 2). Two patients with initial progressive residual tumor (nrs. 10 and 13) were operated a second time, and after this they again had residual tumor which remained stable. Those two patients are listed in table 1 as well as in table 2.
**TABLE 1.** Characteristics and p53 status of 13 patients with stable residual tumor or regression after radiation therapy (patientnrs. 14,25,39).

<table>
<thead>
<tr>
<th>nr</th>
<th>tumor</th>
<th>rad fu months</th>
<th>clin fu months</th>
<th>RT</th>
<th>p53-LI immunohistochemistry</th>
<th>TP53 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cpa</td>
<td>57</td>
<td>57</td>
<td>no</td>
<td>- (&lt;5%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>cpa</td>
<td>96</td>
<td>96</td>
<td>no</td>
<td>- (&lt;5%)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>cpa</td>
<td>39</td>
<td>129</td>
<td>yes</td>
<td>- (&lt;5%)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>cpa</td>
<td>84</td>
<td>84</td>
<td>yes</td>
<td>+ (5-50%)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>cpa</td>
<td>27</td>
<td>106</td>
<td>yes</td>
<td>- (0)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>cpa</td>
<td>12</td>
<td>12</td>
<td>no</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>28</td>
<td>cpa</td>
<td>19</td>
<td>19</td>
<td>no</td>
<td>+ (&gt;50%)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>onpa</td>
<td>133</td>
<td>133</td>
<td>yes</td>
<td>+ (5-50%)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>onpa</td>
<td>81</td>
<td>81</td>
<td>no</td>
<td>+ (&gt;50%)</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>lga</td>
<td>60</td>
<td>105</td>
<td>yes</td>
<td>- (0)</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>3vpa</td>
<td>64</td>
<td>64</td>
<td>yes</td>
<td>+ (&gt;50%)</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>sppa</td>
<td>33</td>
<td>96</td>
<td>yes</td>
<td>+ (5-50%)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>3vpa</td>
<td>20</td>
<td>32</td>
<td>no</td>
<td>+ (5-50%)</td>
<td>yes</td>
</tr>
</tbody>
</table>

*Legend tables 1,2 and 4:*  
*nr* = patient number; *tumor* = type and localization of tumor: *c* = cerebellar; *pa* = pilocytic astrocytoma; *on* = optic nerve; *sp* = spinal; *3v* = third ventricular; *lga* = low grade astrocytoma; *rad fu* = neuroradiological follow-up (in table 2: time to the first scan detecting progression of tumor); *clin fu* = clinical follow-up; *RT* = radiation therapy; *LI* = labeling index; *-* = negative; *+* = positive; *NED* = no evidence of disease.
TABLE 2. Characteristics and p53 status of 10 patients with progression of residual tumor, and 1 patient with a recurring low grade astrocytoma (patient nr 37).

<table>
<thead>
<tr>
<th>nr</th>
<th>tumor</th>
<th>rad fu months</th>
<th>clin fu months</th>
<th>RT</th>
<th>p53-LI immuno</th>
<th>TP53 mutation</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>cpa</td>
<td>12</td>
<td>49</td>
<td>no</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>10</td>
<td>cpa</td>
<td>2</td>
<td>96</td>
<td>no</td>
<td>- (0)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>cpa</td>
<td>10</td>
<td>129</td>
<td>yes</td>
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<td>23</td>
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<td>8</td>
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<td>no</td>
<td>- (&lt;5%)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>onpa</td>
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<td>22</td>
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<td>+ (&gt;50%)</td>
<td>no</td>
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<td>33</td>
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<td>- (&lt;5%)</td>
<td></td>
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<td>onpa</td>
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<td>- (&lt;5%)</td>
<td>no</td>
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<tr>
<td>37</td>
<td>lga</td>
<td>74</td>
<td>74</td>
<td>no</td>
<td>+ (5-50%)</td>
<td>no</td>
</tr>
<tr>
<td>40</td>
<td>lga</td>
<td>16</td>
<td>24</td>
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</tr>
<tr>
<td>44</td>
<td>lga</td>
<td>6</td>
<td>21</td>
<td>yes</td>
<td>+ (&gt;50%)</td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemical.
Among the 48 tissue samples 29 (60%) were positive for p53 immunostaining (more than 5% of positive staining cells) and 19 (40%) were negative (less than 5% of positive cells). From the 19 negative tumors 7 showed no staining at all and 12 were slightly positive with less than 5% of the cells being positive (table 3).

Among all 43 pilocytic astrocytomas 25 (58%) were p53-immunopositive and 18 (42%) immunonegative. Among the 5 low grade astrocytomas 1 showed no staining at all, and 4 were positive.

In the group of 13 stable or regressive residual tumors 5 were p53 immunonegative and 8 were positive. Among the 11 progressing residual tumors 6 were p53 immunonegative and 5 were positive. A relation between p53 immunostatus and tumor behavior could not be found.
TABLE 3. Results of p53 immunostaining for 48 samples of 43 pilocytic astrocytomas and 5 low grade astrocytomas.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>&lt;5%</td>
<td>5-50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Cerebellar pilocytic astrocytoma (n=30)</td>
<td>5</td>
<td>9</td>
<td>10</td>
<td>6</td>
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<tr>
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<td>-</td>
<td>3</td>
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<td>2</td>
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<td>-</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>spinal pilocytic astrocytoma (n=1)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>supratentorial low grade astrocytoma (n=5)</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>TOTALS:</td>
<td>7</td>
<td>12</td>
<td>19</td>
<td>10</td>
</tr>
</tbody>
</table>

*Genetical.*

Of the 10 pilocytic astrocytomas analyzed, 5 tumors showed 8 mutations and of the 2 analyzed low grade astrocytomas 1 showed a mutation (table 4).

The mutations at codons 123, 126, 320 and 379 are novel, which means that they have not been reported in any tumor or tissue previously. The intron 5 mutation, which is a 2 base pair deletion, is also novel. The mutations at codons 67, 132 and 218 have been reported previously, but, not in pilocytic astrocytomas. The codon 47 mutation is the only one that has been found in a pilocytic astrocytoma previously, but never in any other tumor.

In the pilocytic astrocytomas 3 mutations are probable causative to synthesis of a dysfunctional p53 protein, another 3 might be causative, whereas the remaining 2 are “silent” mutations, with no deleterious effect on protein synthesis (details will be reported separately). Mutation frequency from this series is 3 to possible 6 causative mutations in 10 pilocytic astrocytomas analyzed (30%-60%).
TABLE 4. Possible causative TP53 mutations and results of p53 immunohistochemistry in 10 pilocytic and 2 low grade astrocytomas.

<table>
<thead>
<tr>
<th>nr</th>
<th>tumor</th>
<th>resection</th>
<th>follow-up</th>
<th>p53-LI immuno</th>
<th>p53 mutation</th>
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</thead>
<tbody>
<tr>
<td>9</td>
<td>cpa</td>
<td>total</td>
<td>NED</td>
<td>- (&lt;5%)</td>
<td>exon 5 codon 126, exon 6 codon 218, exon 9 codon 320, exon 11 codon 379</td>
</tr>
<tr>
<td>4</td>
<td>cpa</td>
<td>residual</td>
<td>progression metastases</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>27</td>
<td>cpa</td>
<td>residual</td>
<td>stable</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>34</td>
<td>onpa</td>
<td>residual</td>
<td>progression</td>
<td>- (&lt;5%)</td>
<td>no</td>
</tr>
<tr>
<td>29</td>
<td>onpa</td>
<td>residual</td>
<td>progression</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>48</td>
<td>3vpa</td>
<td>residual</td>
<td>stable</td>
<td>+ (5-50%)</td>
<td>exon 4 codon 67</td>
</tr>
<tr>
<td>45</td>
<td>cpa</td>
<td>total</td>
<td>NED</td>
<td>+ (5-50%)</td>
<td>intron 5</td>
</tr>
<tr>
<td>46</td>
<td>cpa</td>
<td>total</td>
<td>NED</td>
<td>+ (5-50%)</td>
<td>exon 5 codon 132</td>
</tr>
<tr>
<td>26</td>
<td>cpa</td>
<td>total</td>
<td>NED</td>
<td>+ (&gt;50%)</td>
<td>no</td>
</tr>
<tr>
<td>37</td>
<td>lga</td>
<td>total</td>
<td>recurrence</td>
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<td>no</td>
</tr>
<tr>
<td>47</td>
<td>3vpa</td>
<td>total</td>
<td>lost to fu</td>
<td>+ (5-50%)</td>
<td>exon 4 codon 47</td>
</tr>
<tr>
<td>36</td>
<td>lga</td>
<td>total</td>
<td>NED</td>
<td>+ (5-50%)</td>
<td>exon 4 codon 123</td>
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</table>
DISCUSSION

Dysfunction of the TP53 gene is an early event in the formation of astrocytomas grade II-IV and TP53 mutations occur in both high grade and low grade astrocytomas (6). Furthermore, TP53 is involved in the progression of low grade astrocytomas to a higher grade (17,18). These observations suggest also a role for TP53 in pilocytic astrocytomas.

In previous reports however, a total of 107 pilocytic astrocytomas were molecular genetically screened for TP53 mutations and only 3 have been found (13,14,26-32). Most of these studies have only screened for mutations on part of the gene, mostly being the region of exons 5 to 8. One of the mutations was found on exon 7 at codon 248, but 2 others were located on exon 4 at codon 47 and on exon 9 at codon 324, respectively (13,14,27). Therefore, until now it is generally believed that the TP53 gene plays no role in pilocytic astrocytomas (37).

Previous immunohistochemical studies for p53 in pilocytic astrocytomas disclosed that 25% of tumors are positive. This information is derived from 8 previous studies in which a total of 77 pilocytic astrocytomas were analyzed, mostly using the monoclonal antibody pAb 1801 (11-13,31,33,34,38,39). We have found among the 43 studied pilocytic astrocytomas immunopositiveness, being defined as positive immunostaining in more than 5% of cells, in 25 tumors (58%). The antibody we have used in this study, BP53-12 was also used in one of the previous reports and compared to pAb 1801; they gave similar results and immunopositiveness was found in 70% of pilocytic astrocytomas (13). This antibody, as stated by the manufacturer, recognizes mutant as well as wildtype p53 protein.

The data from the literature indicate that there is a discrepancy between the incidence of TP53 mutations, being approximately 3%, and p53 immunopositiveness, being 25%. This can be explained by the fact that in most studies not the entire TP53 gene was screened, leaving the possibility for the presence of mutations on other parts of the gene. Furthermore, the observation of overexpression of the p53 protein, resulting in immunopositiveness, without the presence of a TP53 mutation is well known. Anoxia or DNA damage, for example, may induce up regulation of TP53, resulting in overexpression of wildtype p53 protein, being a physiological response to a disturbed cell replication process (5). This physiological response activates normal p53 function, which can be the repair of DNA by bringing the cell into G1-arrest or the induction of apoptosis. The very short half-life of wildtype p53 protein in normal cells prohibits detection by a monoclonal antibody. However, when the half-life of the protein is increased through binding to viral oncoproteins or to the product of the MDM-2 gene, the p53 accumulation will be detected by the antibody, and immunopositiveness will be the result (6). In this situation the p53 protein has probably lost its normal function. On the other hand mutations in the TP53 gene may occur without immunohistochemistry being positive. Namely, when a nonsense mutation in the gene has occurred the p53 protein may be absent or truncated, in both cases there will be no detection by a p53 antibody (6).

Another reason for the low incidence of TP53 mutations as determined by molecular biologic techniques such as single-strand conformation polymorphism analysis (SSCP) and sequencing may be that in heterogenous tumors, where less than 10% of cells harbor the mutation, due to sample errors, these techniques become unreliable (40). In a study on gliomas grade II-IV a good
correlation between p53 immunohistochemical and sequencing data was found when the percentage of abnormal cells within the tumor mass was greater than 5% (10). When the percentage fell below 5%, the correlation became unreliable and it was concluded that in those tumors the abnormal cell population can be better detected by immunohistochemical means. For reasons as stated above, the value of this conclusion should be doubted.

Mutation frequency in the literature is about 3% whereas in our material it is 30%-60%. This is possibly due to the fact that we have screened all exons of the gene, which was actually done in only 12 out of the 107 tumors studied by others (13,37). Most studies have focused on the well known “hot-spot” region of the gene, being exons 5-8. Of the 3 previously described mutations 2 have been found to occur outside this region. It appears that most of the TP53 gene mutations occurring in pilocytic astrocytomas are located outside this hot-spot region, since 5 of the 8 mutations we describe in this report are located on other exons.

The character of the mutations we have found is possibly causative for p53 protein dysfunction in 3 cases, and might be causative in another 3 cases. Two of the mutations are silent, having no effect on protein synthesis. The mutation found to occur at codon 47 (exon 4) has been previously reported to occur in a pilocytic astrocytoma and, as far as we know, never before in any other tumor type (13). One could therefore speculate that the codon 47 mutation may have resulted from an endogenous mutagen involved in the development of pilocytic astrocytomas. The numbers, however, are too low to come to any conclusion whether or not this is a hot-spot mutation for pilocytic astrocytomas. The mutation occurring at codon 132 (exon5) has been previously reported to occur within a glioma of the brain (7). The codon 218 mutation (exon 6) has previously been described in a melanoma of the skin, but this is the first report of this mutation occurring in pilocytic astrocytomas (7). It is uncertain if the novel intron 5 mutation will have an effect on the function of the p53 protein. Recently, intronic mutations of TP53 were also found in 6 pediatric malignancies: in 3 cases of acute lymphoblastic leukemia, 2 cases of rhabdomyosarcoma and in 1 brain tumor of unspecified origin (41). The mutation was also found in normal tissue of these patients, making it a germ line mutation. The brain tumor, one of the rhabdomyosarcomas and a normal lymph node of this patient all showed immunohistochemically elevated levels of p53 protein, whereas the remaining tissues were not tested. It was suggested that this mutation stabilizes wildtype p53 protein, resulting in its accumulation and predisposing to cancer.

Most of the studies that reported on the relation between p53 status and survival are done in high grade astrocytomas. In 2 of them immunopositiveness correlated with decreased survival (42,43). However, in 8 studies such a relation could not be found (11,16,19,21-24,38). In 3 studies of low grade gliomas p53 immunostatus had no relation to patient outcome (15,44,45). One study, comprising 24 grade II astrocytomas showed a trend towards better survival among p53 negative tumors compared to positive tumors, but this relation was not significant (19). Pollack et al reported that malignant childhood brainstem astrocytomas with TP53 mutations or p53 overexpression had a significantly shorter progression free survival (25). To our knowledge, there are no reports on the relation between p53 status and survival, prognosis, patient outcome or tumor behavior in pilocytic astrocytomas. Such studies are difficult to perform because patients with pilocytic astrocytomas have a very good prognosis and an excellent survival. Therefore, we related p53 status to tumor behavior by analyzing results of follow-up neuroimaging studies in patients with postoperative residual tumor.
Two groups, with different tumor behavior could be distinguished; one with progression of residual tumor and one with stable or regressing residual tumor. The results of p53 immunostaining did not differ among both groups. \(TP53\) mutations were found in 4 neuro-radiologically confirmed totally resected pilocytic tumors and in one stable residual tumor. They were not found in 3 pilocytic astrocytomas that showed progression of the residual tumor, neither in 1 low grade astrocytoma that recurred. Another stable residual pilocytic astrocytoma and a totally resected pilocytic astrocytoma lacked \(TP53\) mutations also. Although the numbers are small, these findings suggest that there is no relation between results of p53 immunolabeling or \(TP53\) mutation screening and behavior of residual tumor.

Four of the 5 tumors that showed \(TP53\) mutations were p53 immunopositive. The low grade astrocytoma with the \(TP53\) mutation was also p53 immunopositive. Only the pilocytic tumor with the 4 \(TP53\) mutations (nr. 9) was p53 immunonegative. Although the results of immunohistochemistry seem to be concordant to those of mutation analysis in the tumors that showed mutations, 4 other tumors that lacked a \(TP53\) mutation were also p53 immunopositive. These results confirm the notion that p53 immunopositiveness does not reflect the presence of a mutated \(TP53\) gene. The lack of a clear relation between results of immunohistochemistry and DGGE, makes p53 immunolabeling a useless technique for \(TP53\) mutation screening.

In conclusion, these results suggest that there is no clear relation between results of p53 immunohistochemistry and the results of screening for \(TP53\) mutations within the same tumors. Furthermore, the biological behavior of pilocytic astrocytomas is not related to p53 immunostatus and neither to the presence or absence of \(TP53\) mutations. Nevertheless, the high \(TP53\) mutation frequency in pilocytic astrocytomas as found in this study, suggests, in analogy to other astrocytomas, that \(TP53\) gene dysfunction plays an important role in the formation of pilocytic astrocytomas.
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CHAPTER 7

UP-REGULATION OF SPECIFIC \textit{NF1} GENE TRANSCRIPTS IN SPORADIC PILOCYTIC ASTROCYTOMAS

Platten M, Giordano MJ, Dirven CMF, Guttman DH, Louis DN
INTRODUCTION

Neurofibromatosis 1 (NF1) is a common, autosomal dominant disorder with an incidence of approximately 1 in 3000 individuals (1). The disease is characterized by neurofibromas, café-au-lait spots, hamartomas of the iris (Lisch nodules), axillary freckling, and distinct osseous lesions. Patients with NF1 are also predisposed to a host of tumors, including malignant peripheral nerve sheath tumors (neurofibrosarcomas), pheochromocytomas, rhabdomyosarcomas and gliomas. Of the gliomas in NF1 patients, the most characteristic and common is pilocytic astrocytoma of the optic nerve (optic nerve glioma).

The NF1 gene comprises 59 exons and spans more than 350 kb of genomic DNA on the long arm of chromosome 17 (17q11.2) (2-7). The approximately 13-kb NF1 mRNA transcript encodes neurofibromin, a protein with a central 400-amino-acid region that is homologous with human GTPase-activating proteins (GAPs). The GAP-related domain (GRD) of neurofibromin stimulates the intrinsic GTPase activity of p21-ras, implicating neurofibromin in the transduction of proliferating and differentiation signals (2-7). The NF1 gene is alternatively spliced, leading to multiple transcript isoforms, some of which are highly expressed in brain. For instance, the GRD region contains an alternatively spliced 63-nucleotide exon, designated 23a. NF1 transcripts that include exon 23a (type 2) and those that do not include exon 23a (type 1) are both expressed in normal brain (8-10). Another NF1 splice variant involves a 30-nucleotide exon between exon 9 and exon 10a, designated 9br (also termed 9a). Exon-9br-containing transcripts are specifically expressed in brain (11). Additional alternative splicing of the NF1 gene has been described, but these additional transcripts are not found in the central nervous system.

Loss of neurofibromin expression occurs in malignant peripheral nerve sheath tumors with NF1 gene inactivation (12) and is associated with elevated levels of p21-ras (13-15). Neurofibromin is also reduced or absent in other sporadic tumors, including NF1-related tumors such as pheochromocytoma and non-NF1-related tumors such as melanoma and neuroblastoma (16-18). Interestingly, exon-23a-containing isoforms may have reduced GAP activity, compared with those isoforms lacking this exon (19), and variable expression of these isoforms may correlate with malignant transformation and differentiation (8-10, 19-22).

Furthermore, transgenic mice heterozygous for NF1 gene inactivation have a higher incidence of malignant tumors than wild type mice (23). These observations all support the prediction that the NF1 gene functions as a tumor suppressor gene.

The association of optic nerve glioma with NF1 raises the hypothesis that the NF1 gene is involved in optic nerve glioma tumorigenesis. Optic nerve gliomas and histologically identical pilocytic astrocytomas of the cerebellum and third ventricular region occur in non-NF1 patients (24). These observations further raise the possibility that the NF1 gene is altered in sporadic (non-NF1-associated) optic nerve gliomas and other pilocytic astrocytomas. This hypothesis is supported by the finding of allelic loss of the NF1 region on chromosome 17q in NF1-associated and sporadic pilocytic astrocytomas (25). Direct analysis of the NF1 gene, however, has not been undertaken to confirm its role in pilocytic astrocytoma formation. To evaluate the role of the NF1 gene in these tumors, therefore, we examined quantitative and qualitative aspects of NF1 gene expression in a series of sporadic pilocytic astrocytomas.
MATERIALS AND METHODS

Fresh portions of 6 pilocytic astrocytomas and 3 glioblastoma multiforme (GBM) were obtained from the University of Groningen Hospital and the Massachusetts General Hospital. Of the 6 pilocytic astrocytomas, three were optic nerve gliomas (patient ages 5 months, 14 months and 4 years) and 3 were cerebellar pilocytic astrocytomas (patient ages 5, 11 and 13 years). None of the patients with pilocytic astrocytomas had a family history of NF1 or NF1-related skin lesions. Normal frozen postmortem brain specimens of the superior parietal lobe (5 samples) were obtained from the Brain Tissue Resource Center at McLean Hospital (Belmont, MA) and of the superior frontal lobe (2 samples) from MGH.

RNA POLYMERASE CHAIN REACTION (PCR) ANALYSIS.

RNA was extracted from histologically verified tumor or normal tissue using TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. NF1 cDNA was generated from 3 to 5 µg of RNA using 200 U of SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD), 0.1 µg of oligo-dT (Pharmacia, Piscataway, NJ), and 50 U of RNAase inhibitor (Perkin Elmer, Norwalk, CT) in a total volume of 50 µl containing 2.5 mmol/L of each dNTP, 0.1 mol/L dithithreitol, 5 µg bovine serum albumin, 250 mmol/L Tris-Hcl, pH 8.3, 375 mmol/L KCl, and 15 mmol/L MgCl at 37°C for 1 hour. For all reverse transcriptase reactions, a control was performed omitting the reverse transcriptase to exclude DNA contamination. NF1-GRD cDNA was amplified with primers 5'-GAATTCCCTCAGTCTGGAT3' and 5'-TGCTGCTAGCATCAAAGTTTGGCTTTCA3' for 30 cycles at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. NF1-GRD isoforms containing exon 23a (type 2) are 366 bp whereas those without exon 23a (type 1) are 303 bp in length. NF1-9br region cDNA was amplified for 30 cycles of 95°C for 45 seconds, 69°C for 3 minutes, and 72°C for 3 minutes using primers 5'-CTGAGCAATCTCTGGCATGTGACTGA3' (located in exon 10c) and 5'-TGGAAGTCTACGAAAAGCTCTTGCTGG3' (located in exon 7). The isoforms with and without exon 9br are 761-bp and 731-bp amplicons, respectively. As an internal control for the amount of RNA in each sample, cDNA from the cyclophilin gene was amplified using primers 5'-ATGGTCAACCCCACCGTGTT3' and 5'-CGTTGTAAGTCACCACCCT-3', which yield a 206-bp amplicon after 22 cycles of 95°C for 30 seconds, 55°C for 40 seconds, and 72°C for 30 seconds. Previous studies revealed linear template amplification under these circumstances for 25 cycles (26). PCR was performed in a programmable thermal cycler (MJ Research, Watertown, MA). The PCR mixes contained 0.05 mmol/L of each dNTP, 1 pmol of each primer, 20 mmol/L Tris, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl, and 1 U of Taq polymerase. The PCR products were radiolabeled with 1 µCi of [a-32P]dCTP, which was added before the DNA-denaturing step of the final 2 PCR cycles to provide linear incorporation of the radioisotope. The radiolabeled PCR products were electrophoresed on 6% polyacrylamide denaturing sequencing gels, visualized by autoradiography, and quantitated by densitometry using an LKB 2222-020 UltraScan XL laser densitometer (Pharmacia). Each experiment was repeated at least twice. NF1 gene expression level was defined as the ratio between the NF1-GRD and cyclophilin transcripts. In addition, as another control, NF1 gene expression was also evaluated by analyzing the ratio of the NF1-9br region transcript with cyclophilin.
**SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS.**

SSCP was performed on the 6 cases as detailed elsewhere (27). PCR of the NF1-GRD and NF1-9br region was performed as above, except for the addition of 1µCi of α-32PdCTP at the start of the reaction. The NF1-GRD product was digested with Hinf1 (New England BioLabs, Beverly, MA) to yield 238- (including exon 23a), 175-, and 128-bp fragments to improve sensitivity of the SSCP. Similarly, the NF1-9br region SSCP products were digested with Hinf1 and PstI and yielded 238-, 211-, 175- (including 9br), and 137-bp fragments. The digested products were run on 6% and 8% nondenaturing polyacrylamide gels containing 10% glycerol at 4 and 6 W overnight. The gels were dried and visualized autoradiographically.

**IMMUNOHISTOCHEMISTRY.**

Immunohistochemistry was performed on 5 of the 6 specimens, as detailed elsewhere (28), using an antibody (B3A.1) directed against the carboxyl-terminal region of neurofibromin. Twelve-micron-thick frozen sections were placed on SuperFrost-Plus slides (Fisher Scientific, Pittsburgh, PA) and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.2, for 10 minutes. After incubation with 10% goat serum in 0.1 mol/L PBS containing 0.02% Triton X-100 for 60 minutes, the sections were incubated overnight at 4°C in a 1:500 dilution of rabbit polyclonal B3A.1. The slides were then washed in PBS and incubated in a 1:200 dilution of anti-rabbit biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 60 minutes. After PBS washes, the reaction was visualized using the Vectastain Elite kit (Vector) and diaminobenzidine. The sections were then dehydrated and mounted in 50% DPX:xylene without counter staining. Positive controls include immunohistochemistry for glial fibrillary acidic protein (Sigma Chemical Co, St. Louis, MO), and negative controls involved omission of the primary antibody.

**RESULTS**

Of the 7 specimens of normal brain, 5 showed predominant expression of the type 1 GRD transcript and 2 (both from the parietal lobe) had type 2 isofrom predominance. On the other hand, all 6 pilocytic astrocytomas showed marked type 2 predominance (table 1). Densitometric analysis on the pilocytic tumors revealed two- to fivefold excess of type 2 transcripts whereas the 3 GBMs showed four- to fivefold excess of type 2 transcripts (table 1). Six of the 7 normal brain specimens showed the 9br-containing transcript in addition to the 9br-negative transcript, whereas only 1 pilocytic astrocytoma and 1 GBM expressed the 9br-containing transcript (table 1). All controls with omission of reverse transcriptase were negative.

The mean ratio of NF1-GRD to cyclophilin transcripts was 0.72 for the normal brain samples (range, 0.26 to 1.17). All tumor specimens had increased NF1 gene expression; relative expression levels ranged from 1.4 to 4.2 times that of the normal brain mean ratio (table 1). In general, NF1
gene expression was somewhat higher in the pilocytic astrocytomas than in the GBMs and higher in the cerebellar pilocytic astrocytomas than in the optic nerve tumors (table 1). The mean ratio of NF1-9br region to cyclophilin transcripts paralleled the NF1-GRD/cyclophilin ratios. No SSCP migration shifts were noted in any of the fragments from tumors or from normal cDNA controls. The controls with no DNA, done to exclude PCR contamination, were negative. Immunohistochemistry revealed strong cytoplasmic neurofibromin positiveness in all 5 tumors studied, implying the presence of full-length neurofibromin. Control reactions for glial fibrillary acidic protein showed a similar strong cytoplasmic reaction, and negative controls showed no staining.

**TABLE 1.** Clinical and NF1 gene expression data.

<table>
<thead>
<tr>
<th>Case</th>
<th>age</th>
<th>site</th>
<th>relative NF1 expression</th>
<th>type 1/2 ratio</th>
<th>9br expression</th>
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<td>1.9</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>5 months</td>
<td>optic nerve</td>
<td>1.9</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>14 months</td>
<td>optic nerve</td>
<td>1.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>13 years</td>
<td>cerebellum</td>
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<td>0.3</td>
<td>+</td>
</tr>
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<td>4.2</td>
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</tr>
<tr>
<td>29</td>
<td>4 years</td>
<td>optic nerve</td>
<td>1.4</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
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<td>0.3</td>
<td>-</td>
</tr>
<tr>
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<td>temporal lobe</td>
<td>1.5</td>
<td>0.2</td>
<td>+</td>
</tr>
<tr>
<td>GBM 1520</td>
<td>59 years</td>
<td>temporal lobe</td>
<td>1.4</td>
<td>0.2</td>
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</table>

**DISCUSSION**

The NF1 gene functions as a tumor suppressor gene in a variety of tumors in both NF1 and non-NF1 patients (12-14,16,17,26). Because of the close association between pilocytic astrocytomas and NF1 (1) and because allelic losses at the NF1 region occur in these tumors (25), the NF1 gene was also hypothesized to act as a tumor suppressor in pilocytic astrocytomas. We therefore predicted that NF1 gene and neurofibromin expression would be reduced or absent in pilocytic astrocytomas. Surprisingly, however, NF1 gene transcripts were elevated up to fourfold in the tumors when compared with NF1 transcript levels in normal brain. Increased expression or accumulation of other tumor suppressors, such as p53 (29), occurs in human tumors. Such accumulation, however, may have different causes and sequelae, depending on whether the protein is mutant or wildtype. For example, most tumors that accumulate p53 protein have missense mutations that inactivate the DNA-binding ability of the molecule (30,31). To evaluate whether the NF1 gene was mutant in our cases, we screened for approximately 1 kb of
coding sequence, including the critical GRD region. Although we did not detect sequence alterations, our negative screening of less than 8% of the coding sequence does not exclude mutations in other regions of the gene. We therefore evaluated the same tumors immunohistochemically using an antibody directed against the carboxy terminus of the protein, as most NF1 gene mutations lead to a truncated protein (2) that would not be detected by a carboxyl-terminal antibody. Consequently, the presence of full-length neurofibromin on immunohistochemical analysis in all 5 of the studied pilocytic tumors argues that the accumulating protein is most likely wildtype.

These observations raise the possibility that wildtype neurofibromin is being overexpressed as part of a physiological response. Such a situation may be similar to the accumulation of wildtype p53 in malignant astrocytomas and other tumors, which presumably reflects a physiological response by p53 to genomic damage or deregulated proliferation (32,33).

In the case of the NF1 gene, overexpression in tumors may represent an intact mechanism to reduce p21-ras-mediated proliferative signals. Recent data on the NF1 gene in malignant diffuse, fibrillary astrocytomas support this assumption (34). In a study of 21 malignant astrocytomas, all showed high levels of NF1 gene expression. Moreover, increased neurofibromin correlated with elevated levels of activated p21-ras, and reduction of activated p21-ras in vitro yielded a reduction in the levels of neurofibromin. In addition, neurofibromin expression may also be increased in activated non-neoplastic astrocytes, which may indicate an epigenetic mechanism of NF1 gene regulation (35). Combined, the findings imply that non-neoplastic, benign neoplastic, and malignant neoplastic astrocytes may share a common pathway in which the NF1 gene is up-regulated, although the precise significance of this up-regulation remains unclear.

Because NF1 isoforms may have different tumor suppressor capabilities, we examined pilocytic astrocytomas for those alternative transcripts that are normally expressed in the central nervous system. Early studies suggested that type 1 isoforms (without the exon 23a insert) are the predominant variant in fetal brain and neuroectodermal tumors, whereas the type 2 isoform was more common in differentiated cells (8). Other studies, however, showed type 1 predominance in normal brain and type 2 predominance in brain tumors (9). Our examination of 7 normal brain specimens showed type 1 isoforms as the more common transcript in 5 cases and type 2 in 2 cases. Although NF1 expression may be elevated in reactive astrocytes (35) and astrocytes predominantly express type 2 isoforms (36), the 2 brains in our study with type 2 predominance did not have evidence of reactive astrocystosis. All of the pilocytic astrocytomas, however, had conspicuous type 2 transcript predominance, as had been noted in malignant astrocytomas as well (this study and ref. 9). Type 2 predominance has also been demonstrated in a series of 12 pilocytic astrocytomas studied qualitatively at the GRD region (D.H. Gutmann and R.L. Heidemann, unpublished results).

Although it is tempting to postulate that the type 2 isoforms have reduced tumor suppressor activity, it is equally possible that the type 2 predominance merely reflects a solely astrocytic phenotype rather than a neuronal or mixed neuronal/astrocytic phenotype as would be seen in normal brain. In fact, the type 1 isoform is predominantly expressed in neurons in rat adult brain tissue whereas type 2 transcripts are expressed in rat glial cells (36). Thus, type 2 predominance may also reflect a physiological response that is intact in both normal and neoplastic astrocytes.

Expression of the NF1 transcript containing the 9br exon was detected in 6 of 7 normal brain tissues but was reduced or absent in the pilocytic tumors. Malignant astrocytomas also do not
express the 9br-containing transcripts (this study and ref. 11). Again, however, it is unclear whether this represents oncogenic expression or a less active molecule or physiological expression of an astrocytic isoform. Recent studies of 9br isoforms have shown that 9br-containing transcripts are expressed only by neurons (D.H. Gutmann, unpublished results), suggesting that the lack of expression of 9br-containing isoforms in astrocytomas may also simply reflect an astrocytic phenotype.

NF1 is phenotypically highly variable, even within individual families. Interestingly, monozygous twins display much closer phenotypes than distant affected relatives, which has suggested that the NF1 phenotype is modified by other genes (37). Such modification may be mediated through potential transcriptional regulatory elements in the promotor region of the NF1 gene (38). Alternatively, phenotypic variation may be mediated by mRNA editing, a phenomenon that has recently been suggested for the NF1 gene (3). Finally, genes embedded within the NF1 gene (e.g., EV12A, EV12B, and OMGP) may play a role in NF1 gene expression or in modulating the NF1 phenotype (2). The complexities of NF1 gene expression in normal and tumor cells may imply that neurofibromin has multiple functions that modify cell differentiation and proliferation. In this regard, better understanding of NF1 gene regulation will no doubt contribute to explaining the up-regulation of specific NF1 gene transcripts in pilocytic astrocytomas.

REFERENCES


23. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumor predisposition in


CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS
COMMENTS ON THE DETERMINATION OF PROLIFERATIVE POTENTIAL.

It appears that none of the two proliferation markers tested in this study, MIB-1 labeling and AgNOR staining, by itself have absolute predictive value for the behavior of the residual tumor after incomplete resection of a pilocytic astrocytoma. Tumors that are negative for MIB-1 staining, are very unlikely to show progression of the residual tumor. Only 1 out of 6 residual tumors that were negative for MIB-1 staining showed progression (pat.nr. 17). Tumors with positive MIB-1 LI can either progress, remain stable or even regress. The AgNOR study shows the same result: low-scoring residual tumors remain stable or regress, whereas high scoring tumors can either progress or remain stable. Since AgNOR surface area measurements have not been performed previously in normal astrocytes, nor in pilocytic astrocytomas, the definition of “low” and “high” AgNOR scores can not be made on a more objective basis than the one that is used in this study, where the median score of the whole group was used as a threshold between low and high scores. For each cell three optional pathways exist: The cell may proliferate continuously, may stay alive without further division or it may die by apoptosis. The growing of a tumor is the result of the fact that the pool of proliferating cells exceeds the number of cells that die by apoptosis. The proliferative potential of a tumor is determined by the number of dividing cells in that tumor (the growth fraction) and the speed at which these dividing cells run through the cell cycle on one hand and the extent of cell-death by apoptosis on the other.

As stated previously in this thesis (chapter 3) Ki-67 or MIB-1 labeling is a reliable method to determine the growth fraction in a cell population, which is the amount of cells that are in an active phase of the cell cycle. AgNOR expression reflects the rapidity of the cell cycle, an increased AgNOR number correlates to fast dividing cells (chapter 3). As stated in the paper of Hostadter et al, proliferative activity (PA) can be defined as growth fraction (GF) divided by generation time (GT) (1). The generation time is inversely related to speed of the cell cycle. This leads to the following equation: PA=GF/GT. Or in terms of proliferation markers: proliferative activity=Ki-67 LI x AgNOR. From this equation it becomes clear that assessment of the proliferative potential cannot be performed by testing just one of the two mentioned proliferation markers. For example, a high AgNOR score, reflecting that cells which are dividing do this in a fast way, in the presence of a very low or almost negative Ki-67 LI, which means that only very few cells are dividing, results in a very low proliferative potential. It must be reminded that AgNOR staining is positive in all cells, also in resting cells and that increased AgNOR activity only reflects increased protein synthesis, not necessarily used for cell proliferation. Consequently, a high growth fraction as determined by a high Ki-67 LI combined with a very slow cell cycle also results in low proliferative potential of the tumor. However, further study is needed to define more precisely the boundaries of the terms “low” and “high”.

Therefore, it is worthwhile to test the combined results of MIB-1 labeling and AgNOR staining, in relation to tumor behavior. In table 1 the results of the different studies for residual tumors are summarized, including proliferative potential as a function of MIB-1 LI and AgNOR. The first 13 patients listed have stable or regressive residual tumor, the remaining patients show progression of residual tumor.
### TABLE 1. Results of MIB-1, AgNOR, p53-immunohistochemistry, TP53 screening for mutations, flowcytometry and NF1 studies in stable or regressive (pat. nrs. 14,25,39) and progressive tumors.

<table>
<thead>
<tr>
<th>nr</th>
<th>age</th>
<th>tumor</th>
<th>MIB-1</th>
<th>AgNOR</th>
<th>prolifer</th>
<th>p53 imm.</th>
<th>TP53 mut.</th>
<th>flow</th>
<th>NF1 expr</th>
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<td>19</td>
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<td>dipl</td>
</tr>
<tr>
<td>13</td>
<td>14.5</td>
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<td>cpa</td>
<td>39</td>
<td>neg</td>
<td>0</td>
<td></td>
<td>neg</td>
<td></td>
</tr>
<tr>
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<td>cpa</td>
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<td>10%</td>
<td>2.8</td>
<td>28</td>
<td>pos</td>
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</tr>
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<td>cpa</td>
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<td>neg</td>
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<td>81.2</td>
<td>0</td>
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</tr>
<tr>
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<td>19</td>
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<td>175</td>
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</tr>
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<td>10</td>
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<td>onpa</td>
<td>133</td>
<td>neg</td>
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<td>0</td>
<td>pos</td>
<td>dipl</td>
</tr>
<tr>
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<td>81</td>
<td>8%</td>
<td>15.9</td>
<td>127</td>
<td>pos</td>
<td>dipl</td>
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<tr>
<td>39</td>
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<td>lga</td>
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### PROGRESSIVE RESIDUAL TUMORS

<table>
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<th>tumor</th>
<th>MIB-1</th>
<th>AgNOR</th>
<th>prolifer</th>
<th>p53 imm.</th>
<th>TP53 mut.</th>
<th>flow</th>
<th>NF1 expr</th>
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<td>5</td>
<td>M</td>
<td>cpa</td>
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<td>18.2</td>
<td>36</td>
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<td>11.5</td>
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<td>cpa</td>
<td>2</td>
<td>7%</td>
<td>48</td>
<td>336</td>
<td>neg</td>
<td>1.13</td>
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<td>cpa</td>
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<td>3%</td>
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</tr>
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<td>cpa</td>
<td>7</td>
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<td>0</td>
<td></td>
<td>neg</td>
<td></td>
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<tr>
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<td>17</td>
<td>M</td>
<td>cpa</td>
<td>6</td>
<td>18%</td>
<td></td>
<td></td>
<td>neg</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>4</td>
<td>F</td>
<td>onpa</td>
<td>3</td>
<td>15%</td>
<td>39.1</td>
<td>586</td>
<td>pos</td>
<td>no 1.4</td>
</tr>
<tr>
<td>33</td>
<td>0.5</td>
<td>M</td>
<td>onpa</td>
<td>2</td>
<td>4%</td>
<td>57.8</td>
<td>231</td>
<td>neg</td>
<td>1.9</td>
</tr>
<tr>
<td>34</td>
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<td>onpa</td>
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<td>5%</td>
<td>64</td>
<td>320</td>
<td>neg</td>
<td>no dipl</td>
</tr>
<tr>
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<td>12.1</td>
<td>121</td>
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<td>no dipl</td>
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<td>M</td>
<td>lga</td>
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<td>44.1</td>
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<tr>
<td>44</td>
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<td>F</td>
<td>lga</td>
<td>6</td>
<td>15%</td>
<td>38.1</td>
<td>572</td>
<td>pos</td>
<td>1.75</td>
</tr>
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</table>
Legend table 1.
1 = patient number; 2 = age at operation; 3 = sex; 4 = type and location of tumor: cpa = cerebellar pilocytic astrocytoma, onpa = optic nerve tract pilocytic astrocytoma, 3vpa = third ventricle pilocytic astrocytoma, sppa = spinal medullary pilocytic astrocytoma, lga = low grade astrocytoma. 5 = duration of neuro-radiological follow-up in months, in group of progressive tumors: time to detection of progression on CT- or MRI-scan. 6 = Mean MIB-1 LI. 7 = mean AgNOR in square micrometer per cell. 8 = proliferative potential as determined by MIB-1 LI x AgNOR. 9 = p53 immunostaining with monoclonal BP53-12 antibody, neg = negative (number of cells showing staining is less than 5%), pos = positive (more than 5% of cells show immunostaining). 10 = p53 mutation present? 11 = results of flowcytometry, dipl= diploid, if not diploid the S-phase fraction is given. 12 = results of neurofibromatosis 1 gene screening, relative amount of overexpression of neurofibromin compared to normal brain.

When the above mentioned equation of proliferative activity, being a function of the combined MIB-1 LI and AgNOR surface area measurement, is applied to the two groups of tumors of our interest, being the postoperative residual tumors that either regress and remain stable, or show progression, a much more convincing difference in proliferative activity between the two groups is found (table 2). In this way the proliferative activity (PA) could be assessed in 3 regressive, in 9 stable and in 9 progressive tumors. In case of a negative MIB-1 labeling result, the value for MIB-1 LI is set at 0, which is a mathematical simplification. PA values among these 21 tumors ranged from 0 to 586, with a mean of 131, all values are in mean square micrometer per cell. The 3 regressive tumors had MIB-1 LI values of 0, 0 and 28 respectively, with a mean of 9. In the stable group 4 tumors had a value of 0, the highest value is 175 and the mean is 52. In the progressive group only 2 tumors have a value of 0, most other values are very high and the mean is 245.

**TABLE 2.** Proliferative potential, as determined by the multiplication of results of AgNOR staining and MIB-1 labeling, of residual tumors related to behavior of tumor during follow-up.

<table>
<thead>
<tr>
<th></th>
<th>prol. pot. : individual values</th>
<th>mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole group (n=21)</td>
<td>0-586</td>
<td>131</td>
</tr>
<tr>
<td>regressive (n=3)</td>
<td>0,0,28</td>
<td>9</td>
</tr>
<tr>
<td>stable (n=9)</td>
<td>0,0,0,0,19,28,127,165,175</td>
<td>52</td>
</tr>
<tr>
<td>progressive (n=9)</td>
<td>0,0,36,121,231,320,336,572,586</td>
<td>245</td>
</tr>
</tbody>
</table>
Of the several functions of the TP53 and NF1 genes, some are shared in common. Both gene products have an effect on cell cycle regulatory processes (fig. 1). Neurofibromin keeps p21ras, the gene product of the ras proto-oncogene, in its inactive state. In the signal transduction pathway, in which several growth factors (such as PDGF, TGF and EGF) exert their growth stimulating effect on the cell cycle, the activated p21ras plays a key role in transducing these growth stimuli from the cell membrane to the nucleus (2). Activated p21ras deregulates the cell cycle, possibly by overexpression of proteins called cyclins, which, together with cyclin-dependent kinases (CDK), control passage through the various stages of the cell cycle and the induction of S-phase entry. CDK promote the phosphorylation of the product of the retinoblastoma tumor suppressor gene (Rb gene), which is the final step to bring the cell from the G1- into S-phase. Neurofibromin, due to its GAP-like function, is able to inactivate p21ras, which leads to inhibition of proliferative signals. The p53 protein stimulates the p21waf1 gene product. Being a strong CDK inhibitor this gene product is also a critical regulator of the cell cycle, resulting in G1-arrest. The p21waf1 protein is developmentally expressed in a variety of cells where it is involved in cell differentiation (3). Also neurofibromin plays a critical role in differentiation processes during embryogenesis (4). Both p53 protein and neurofibromin are elevated in astrocytes after ischemia, probably these gene products not only have a function in protecting against carcinogenic events, but also in recovery after brain ischemia (2). These functions of both the TP53 and NF1 genes support the view that pilocytic astrocytoma formation is not primarily the result of malfunctioning of such tumor suppressor genes but that the abnormal proliferative capacity of tumor cells induces activation of these genes. Because of their strong influence on cell cycle regulation, it is possible that these genes become activated as a result of a physiological response, when disturbances in the cell cycle occur, such as abnormal replication, for example because of DNA-damage. It is plausible that many carcinogenic events during the life of a cell are corrected by the “recruitment” of such genes. The possibilities for p53 to correct such events are twofold: either to block the cell cycle and bring the cell to a G1-arrest, subsequently followed by inducing DNA-repair mechanisms (stimulating the GADD genes), or, if this is not sufficient, by promoting cell death via apoptosis (by stimulating the Bax-gene and down regulating the Bcl-2 gene), in this way prohibiting the further clonal expansion of such a deregulated cell (see figure 1 in chapter 3). The possibilities for the NF1 gene to correct carcinogenic events are less well understood, but the GAP-like function of neurofibromin, causing inactivation of p21ras and blocking of entry into the S-phase of the cell cycle, is probably one of those functions (5). However, when the tumor suppressor gene itself is damaged by a mutation, or the functional gene product becomes ineffective for other reasons, these protective actions of the tumor suppressor genes will be lacking and the deregulated cell can outgrow and multiply unhindered. In this view dysfunction of the TP53 and NF1 genes can not be regarded as early steps in the formation of pilocytic astrocytomas. More likely these genes are up-regulated as a result of abnormal cell proliferation when tumor formation already has occurred. Since the presence of a TP53 mutation in these tumors does not seem to influence tumor behavior, it is unlikely that a TP53 gene mutation adds to the carcinogenic events that occur in pilocytic astrocytomas.
In line with this view the results of our p53 study can be interpreted as follows: It is expected that all proliferating tumor cells show up-regulation of TP53. This may result in overexpression of wildtype p53 (in case of a normal functioning gene), in the production of mutant p53 (in case of a missense TP53 mutation) and in absence of the protein or the production of a truncated protein (in case of a nonsense mutation). The antibody we have used in our study recognizes mutant as well as wildtype p53, therefore, the first two possibilities will lead to immunopositiveness and the latter to immunonegativity.

In non-TP53 mutated tumors a positive reaction due to an overexpression of wildtype p53 protein is expected. A positive immunoreaction was indeed found in all 6 tumors that lacked a TP53 mutation; in 4 tumors the immunoreaction was very intense (>50% of cells), in 1 tumor intermediate, and in 1 tumor very low (<5% of cells).

Of the 6 tumors that showed a TP53 mutation, 5 had intermediate positive (5%-50% of cells) and 1 very low positive (<5% of cells) immunoexpression. These tumors probably have a missense mutation resulting in the production of a protein with an extended half-life. The one case with very low immunoexpression contains 4 different TP53 mutations, possibly in this tumor hardly any p53 protein is produced, or the protein produced is altered in such a manner that it has become undetectable for the antibody used. Of the 5 mutant tumors with intermediate, probably mutant, p53 expression, 4 were totally resected, therefore, no conclusions about tumor behavior can be drawn. One tumor was incompletely resected and the residual tumor remained stable during follow-up. The 6 non-mutant tumors with presumed wildtype p53 overexpression, showed a more aggressive behavior than the mutant tumors; 3 showed progression of residual tumor, 1 recurred and 1 was totally resected. Since it is expected that the TP53 mutant tumors behave more “malignant” than the non-mutant tumors, because they lack the “protective” function of p53, which is clearly not the case in our material, apparently, TP53 status has no influence on tumor behavior. This could implicate that the mechanisms which cause the tumor cells to proliferate in pilocytic astrocytomas can not be “corrected” by p53.

Figure 1. Regulatory influence of the gene products of TP53 and NF1 on the cell cycle.
Since Ki-67 protein is only expressed in cells in the G1, S, G2, and M phases of the cell cycle, and not in Go, a negative MIB-1 LI, as seen in 6 stable residual tumors, possibly indicates that very few or no cells are proliferating. This is in concordance with the observation that these tumors do not progress.

The positive MIB-1 LI in stable residual tumors, theoretically implies that these tumors contain proliferating cells, or, assuming that cells in the G1-phase of the cell cycle express Ki-67 antigenic activity, the tumor cells are in G1-arrest. In case these tumors do contain proliferating cells there is probably an increased cell death via apoptosis, keeping the total number of cells in the tumor in balance. Four of the 6 stable residual tumors with positive MIB-1 LI had high and 2 had low p53 overexpression. Assuming that the p53 is wildtype, this overexpression might reflect an adequate physiological response, indicating an activation of p53. Possibly, in these stable tumors the p53 response either forces the cells into G1-arrest or, the abnormal cell replication is compensated by an increased apoptotic activity, both resulting in stable residual tumor with positive MIB-1 labeling.

The behavior of the 3 regressive tumors can be explained in the same line: Two of those had a negative MIB-1 LI as well as a negative p53 immunorespnes, reflecting absence of cell proliferation and therefore, no need for p53 activation (nrs. 25 and 39). The remaining regressive tumor (nr.14) had a positive MIB-1 LI and a positive p53 immunorespne, this might reflect a successful p53 mediated “G1-arresting” or apoptotic response that “corrects” the abnormal cell proliferation.

NF1 gene screening in this study has only been performed in 6 tumors out of the whole group, 4 of them had residual tumor of which one remained stable and 3 showed progression. All 6 tumors showed overexpression of the gene product neurofibromin, ranging from 1.4 to 4.2 times the value of neurofibromin expression in normal brain. Mutations in the GAP-related domain of the NF1 gene were not found. In line with the view as stated above this overexpression may represent a physiological response to abnormal cell proliferation, which is confirmed by the fact that the antibody reactions, specific to full length neurofibromin, were all positive. The neurofibromin overexpression can be regarded as an attempt of the cells to interfere with the p21ras mediated signal transduction pathway in order to block the abnormal proliferative signals.

The pilocytic astrocytoma with stable residual tumor (pat.nr.27) has the highest level of neurofibromin overexpression. This case is of interest because p53 is overexpressed in the absence of a TP53 mutation, suggesting the p53 to be wildtype. Furthermore, the negative MIB-1 LI indicates the absence of proliferating cells. In this tumor p53 and neurofibromin possibly are both activated. The lower levels of overexpression in the 3 tumors with progressive residual tumor (pat. nrs. 29,33,34) might reflect an insufficient physiological response resulting in tumor progression. It seems that the NF1 gene, like TP53, is not capable of correcting all tumorigenic events that have occurred in pilocytic astrocytomomas.
CLINICAL IMPLICATIONS.

Based on the results of studies in this thesis, the algorithm for follow-up of patients surgically treated for a pilocytic astrocytoma, as stated in chapter 2, can be more specified (figure 2).

In this algorithm the principal question is whether patients have postoperative residual tumor. From the group studied in chapter 6, 24 patients with pilocytic astrocytomas had total resections and after mean follow-up of 50 months, no recurrence occurred. In the retrospective study of chapter 2 among 73 patients, 1 recurrence occurred. Therefore, when there is no residual tumor on postoperative MRI-scan and neither on a control MRI-scan after 1 year, the patient needs no further neuroradiological surveillance.

Since the TP53 and NF1 genes have no relation to tumor behavior, results of immunohistochemical and genetic analyses of these genes can not be used for deciding how to treat and follow a patient with postoperative residual pilocytic astrocytoma. However, a negative result of MIB-1 labeling, and a low AgNOR staining value are informative regarding behavior of residual tumor. In the algorithm it is suggested to use both parameters for determining the frequency of follow-up MRI-scans. Residual tumors with negative MIB-1 LI in combination with low AgNOR need a MRI follow-up after 1 year and 4 years and when remaining stable can be discharged from further controls. The duration of 4 years is chosen because in the retrospective study most reoperations appeared to take place within an interval of 0.5-4 years after the first operation. More than 50% of the residual tumors that show a positive MIB-1 labeling progress during follow-up. Therefore, patients with such tumors should be followed by yearly MRI scans. How long this follow-up needs to be continued remains unsure. In our retrospective study 7 patients were re-operated for progressing residual tumor after a time interval of 6-17 years, in the literature “recurrences” after even a longer period are reported (6). Therefore, it is suggested to maintain neuroradiological surveillance in patients with MIB-1 positive residual tumor life-long.

Since radiation therapy has never been proven to be beneficial in the treatment of cerebellar pilocytic astrocytomas, the therapy for progressing residual tumor needs to be surgical. However, some reports claim a response to chemotherapy and stereotactic radiation therapy of pilocytic astrocytomas, mostly located at other places in the brain (see chapter 1). Therefore, these additional treatment modalities can considered to be used for residual tumor after re-operation at surgically inaccessible sites.
FIGURE 2. Algorithm for the follow-up of patients with a surgically treated pilocytic astrocytoma.
CONCLUSIONS

1. The incidence of residual tumor in patients surgically treated for cerebellar pilocytic astrocytomas is 31%. Progression of residual tumor and/or recurrence during follow-up occurs in 21% of cases treated for a cerebellar pilocytic astrocytoma.

2. Postoperative residual pilocytic astrocytoma may show progression, remain stable or even regress. Tumor-, treatment- or patient-related factors that influence the behavior of the residual tumor could not be found.

3. When pilocytic astrocytomas are negative for MIB-1 staining, it is very unlikely that the residual tumor will show progression. MIB-1 positive residual tumors can either progress or remain stable.

4. Pilocytic astrocytomas with a low AgNOR score did not show progression of residual tumor. High scoring tumors can either remain stable or progress. Further study is needed to define the threshold between low and high scores.

5. The combined results of MIB1 labeling and AgNOR staining, multiplied to assess proliferative potential, may have a predictive value for tumor behavior.

6. In contrast to results of previous reports, TP53 mutations do occur in pilocytic astrocytomas in a high frequency. It is unlikely that these mutations are involved in the formation of pilocytic astrocytomas.

7. Results of p53 immunohistochemistry do not relate to p53 function and neither to tumor behavior.

8. The presence of TP53 mutations does not indicate that residual tumors are likely to show progression.

9. The NF1 gene is unlikely to be mutated in pilocytic astrocytomas. A tumor suppressor function of the NF1 gene could not be established in pilocytic astrocytomas.

10. The NF1 gene product, neurofibromin, is up-regulated in the studied pilocytic astrocytomas, probably as the result of a physiological response.

11. A patient, surgically treated for a pilocytic astrocytoma, showing no residual tumor on two MRI-scans, one directly postoperative and one after 1 year, needs no further control.
REFERENCES

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
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 p21ras 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 p21ras mediated proliferative signals. This is supported by the finding of elevated neurofibromin levels together with elevated levels of activated p21ras in high grade astrocytic tumors.
These data illustrate that pilocytic astrocytomas overexpress specific $NF1$ gene transcripts, perhaps as a regulatory response to growth stimuli. The role of the $NF1$ 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 $NF1$ gene suggests that this gene is not involved in pilocytic astrocytoma formation. The fact that tumors with $TP53$ mutations do not behave more malignant than those lacking mutations, makes it unlikely that $TP53$ gene mutations are an early event in tumorigenesis of pilocytic astrocytomas. Furthermore, it can be questioned whether the $TP53$ gene is involved in the formation of pilocytic astrocytomas. It is more likely that in pilocytic astrocytomas the $TP53$ and $NF1$ 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.
INLEIDING

Dit proefschrift handelt over een zeldzaam, goedaardig, vooral bij kinderen voorkomend hersengezwel, genaamd het "pilocytaire astrocytoom".
Ieder gezwel, ofwel tumor, is een opeenhoping van niet-normale lichaams cellen die een verhoogde groeineiging vertonen. Deze groeineiging uit zich in het zich versneld delen en vermenigvuldigen van de cellen. Het groegedrag van tumoren kan uiteen lopen van zeer aggressief tot zeer mild. Aggressieve, kwaadaardige of "maligne" tumoren, vertonen ingroei in het omliggende normale lichaamswervsel en kunnen op deze wijze bloedvaten infiltreren. De tumorcellen worden in de bloedbaan meegevoerd naar andere plaatsen in het lichaam, en kunnen zodoende uitzetting veroorzaken. De uitzettingsen kunnen elders in het lichaam nieuwe tumoren vormen; de zgn. "metastasen". Wanneer men in het algemeen over "kanker" spreekt, worden dergelijke, zich uitzettingende tumoren bedoeld, die dan ondermeer uit kunnen gaan van de long, de borst, de darm, de prostaat, of de baarmoederhals. De term "hersenkanker" wordt nooit gebezigd, de reden hiervan is waarschijnlijk gelegen in het feit dat tumoren uitgaande van de hersenen zelden uitzetting vertonen.

Goedaardig of "benigne" tumoren vertonen geringere neiging tot ingroei in het omgevende weefsel, waardoor zelden uitzetting ontstaat. Een dergelijke tumor kan vergeleken worden met een zich traag vergrotende, bolvormige celmassa die de omgevende weefsels wegduikt. Wanneer een kwaadaardige tumor door middel van een operatie behandeld wordt, zal behalve de tumor zelf, ook een deel van het omliggende normale lichaamswervsel, waarin zich de voor het blote oog onzichtbare "in-gegroeide" tumorcellen bevinden, weggenomen dienen te worden. Daarentegen kan een goedaardige tumor door de chirurg vaak in zijn geheel verwijderd worden. In het eerste geval is het vaak onzeker of na de operatie daadwerkelijk alle kwaadaardige cellen weggenomen zijn, hetgeen de reden vormt voor een "nabehandeling" in de zin van bestraling of chemotherapie. Indien de kwaadaardige cellen hiervoor ongevoelig zijn kan de tumor terug keren. Echter wanneer een goedaardige tumor volledig verwijderd is, kan de patient meestal genezen worden verklaard. Goedaardige tumoren, gelegen in de hersenen, kunnen een ernstige bedreiging voor de patient vormen. Enerzijds, omdat de hersenen zich in de harde schedel bevinden waardoor er geen "uitwijk-mogelijkheid" bestaat voor het weggedrukte hersewervsel, met als gevolg een snel toenemende druk op het omringende hersenweefsel bij tumorgroei. Anderzijds, omdat het gezwel zich diep in de hersenen kan bevinden, waardoor het voor de neurochirurg onmogelijk is deze totaal te verwijderen. "Astrocyten" zijn cellen die zeer talrijk voorkomen in het hersenweefsel. Zij hebben een stervormig uiterlijk en vormen de bedding voor de eigenlijke hersencellen, die neuronen genoemd worden. De astrocyten vormen het steunweefsel voor de hersenen en zij voorzien de neuronen van "voeding". Normale astrocyten vertonen geringe groei, echter wanneer een dergelijke cel "ontregeld" is, kan snelle deling ontstaan. Uit deze cel onstaat dan een zich vermenigvuldigende groep cellen die een tumor vormen, genaamd "astrocytoom".
Van de vele soorten hersentumoren die voorkomen vormt de groep van de astrocytomen de grootste. Bepaalde typen astrocytomen zijn uitgesproken kwaadaardig en andere zeer goedaardig. De astrocytomen worden dan ook ingedeeld in 4 klassen of “graden”, die van graad I tot graad IV toenemen in mate van aggressiviteit. De pilocytaire astrocytomen behoren tot graad I. Zij groeien traag, vertonen nauwelijks neiging tot ingroei in het omliggende hersenweefsel, en zelden uitzetting. Indien een pilocytaire astrocytoom in zijn geheel chirurgisch verwijderd kan worden is de patient “genezen”. Vaak echter kunnen deze tumoren niet geheel en soms geheel niet verwijderd worden; bijvoorbeeld omdat een deel van de tumor zeer diep in de hersenen gelegen is en chirurgische verwijdering ernstige schade aan de omliggende hersenenstructuren zou veroorzaken. Voor patienten met dergelijke goedaardige, maar door de ligging zeer bedreigende, tumoren is een goede behandeling niet voor handen. Bestraling en chemotherapie geven mogelijk remming van de tumorgroei, maar hebben ernstige bijwerkingen, met name bij kinderen. Het is bekend dat sommige resttumoren na operatie jarenlang stabiel aanwezig kunnen blijven zonder groei te vertonen, en soms zelfs spontaan kunnen verdwijnen. Daar tevoren niet bekend is hoe de resttumor zich zal gedragen, ziet de chirurg zich vaak voor een dilemma gesteld bij het bepalen van de uitgebreidheid van de operatie. Die kan enerzijds bestaan uit een risicovolle operatieve ingreep die gericht is op totale verwijdering van de tumor. Anderzijds kan de ingreep meer "behoudend" zijn, bestaande uit veilige gedeeltelijke tumorverwijdering, gevolgd door een afwachtend beleid, in de hoop dat de tumorrest niet verder zal uitgroeien of zal verdwijnen. Het in dit proefschrift beschreven onderzoek omvat de volgende doelstellingen:
- Het vaststellen van de frequentie van voorkomen van resttumor na een operatieve behandeling wegens een pilocytaire astrocytoom, alsmede van het groeigedrag van de resttumor.
- Trachten te voorspellen welke tumoren verdere uitgroei zullen vertonen en welke stabiel zullen blijven of verdwijnen. Hiertoe werd gezocht naar eigenschappen van het tumorweefsel die de verschillen in groeigedrag kunnen verklaren. Groeiende cellen bevatten bepaalde eiwitten die niet-groeien de cellen missen. Ook kunnen bepaalde verstoringen of veranderingen (mutaties) in het genetische materiaal (de genen) van de tumorcel er toe leiden dat die cel versnelde groei vertoont. Van een aantal patienten die langdurig volledig bekend zijn en waarvan het groeigedrag van de tumor inmiddles bekend is, werd het tumor weefsel onderzocht op aanwezigheid van twee soorten eiwit en op veranderingen in twee onderdelen van het genetische materiaal (genen). Gekeken werd of er een relatie bestond tussen het groei-gedrag van de tumor enerzijds en het gehalte van de twee soorten eiwit, alsmede de veranderingen in de twee genen, anderzijds.
- Het opstellen van een behandelschema waarin vastgelegd is hoe vaak patienten met resttumor door middel van controle hersenfoto’s vervolgdi, en hoe de groeiende resttumor behandeld dient te worden en wanneer gebruik gemaakt dient te worden van chemotherapie of bestraling.

Onderstaand volgt een korte samenvatting van de verschillende hoofdstukken.

Hoofdstuk 1

Een historisch overzicht wordt gegeven vanaf de ontdekking in 1932 door Harvey Cushing, tot aan de plaatsing van pilocytaire astrocytomen binnen de huidige hersentumor classificatie zoals

Het stellen van de diagnose "pilocytair astrocytoom" gebeurt door de (neuro-)patholoog, die hiertoe het verwijderde tumorweefsel, na uitvoeren van verschillende kleurtechnieken, onder een microscoop bekijkt. Op grond van het uiterlijk van de tumorcellen, zoals de afmeting, de vorm en de rangschikking ten opzichte van elkaar alsmede op grond van andere kenmerken van het weefsel kan de tumor herkend worden als zijnde een pilocytair astrocytoom. Omdat niet alle pilocytaire astrocytomen hetzelfde groeigedrag vertonen is in het verleden vaak geprobeerd om het groeigedrag in verband te brengen, of te koppelen aan bepaalde weefselkenmerken. Deze pogingen zijn echter altijd onsuccesvol gebleven.

Uit de vele patientenseries die in het verleden in de medische literatuur beschreven zijn komt naar voren dat indien de tumor volledig verwijderd is de patient vrijwel altijd genezen verklaard kan worden. Bij niet volledige verwijdering is het verdere beloop echter onvoorspelbaar: De tumor kan opnieuw uitgroeien, kan levenslang stabiel aanwezig blijven zonder enige hinder voor de patient te veroorzaken en soms kan de tumor spontaan verdwijnen. Sommige studies tonen een gunstig effect aan van bestraling op de tumorrest, terwijl andere dit ontkennen. Recentelijk zijn enige gunstige effecten van chemotherapie gerapporteerd. Echter beide aanvullende behandelingen kennen ernstige bijwerkingen, met name bij jonge kinderen. Vooralsnog geldt de chirurgische verwijdering van de tumor als behandeling van eerste keus.

Hoofdstuk 2

Onderzocht werd hoe patienten na een operatie functioneren, hoeveel patienten er zijn die nog resttumor hebben, en hoe de resttumor zich in de opvolgende jaren gedraagt. De gegevens van 73 patienten, geopereerd wegens een pilocytair astrocytoom in de hersenen, en vervolgd met een gemiddelde duur van 8,2 jaar, werden geanalyseerd. Bij 80% van de patienten bestond na de operatie een geheel of vrijwel geheel normaal functioneren, 14% was ernstig neurologisch beschadigd en 6% was overleden als gevolg van de tumor of de operatie. Op grond van verrichtte hersenfoto's (CT- en MRI scans) na de operatie, bleek bij 31% van de geopereerde patienten nog resttumor aanwezig te zijn. Verder vervolg van deze groep met controle scans, vertoonde bij 60% opnieuw uitgroei van de resttumoren, bij 32% een groei stilstand en bij 8% alsnog spontane verdwijning. Ter bepaling van het verdere behandelbeleid is vaststelling van de aanwezigheid van eventuele resttumor noodzakelijk. Hiervoor kan niet vertrouwd worden op het oordeel van de chirurg, daar dit in meer dan 30% van de gevallen niet overeenstemde met het resultaat van de na de operatie
verrichtte hersen-scan. Daarom wordt geadviseerd direct na de operatie altijd een scan te verrichten. Indien hierop resttumor zichtbaar is zal de patient regelmatig met controle scans vervolgd dienen te worden om te bezien hoe deze tumor zich gedraagt. Indien er geen resttumor aanwezig is wordt geadviseerd nog eenmaal, na 4 jaar, een controle scan te verrichten. Indien de resttumor groeit dient opnieuw geopereerd te worden. Wanneer dit niet mogelijk is kan overwogen worden als aanvullende behandeling gebruik te maken van chemotherapie of bestraling.

Hoofdstuk 3

In dit hoofdstuk wordt een inleiding gegeven op vier verschillende methoden van tumorweefsel onderzoek, waarvan bij eerdere toepassing op andere tumoren, gebleken is dat deze een mogelijks voorspellende waarde hebben ten aanzien van het groeigedrag van die tumor. De technische aspecten van deze onderzoek methoden worden uiteen gezet. De resultaten van eerdere onderzoeken, gepubliceerd in de medische literatuur, zowel uitgevoerd op pilocytaire astrocytomen als op andere (hersen) tumoren, worden geanalyseerd. In de volgende hoofdstukken worden de resultaten van toepassing van deze vier technieken op een groep pilocytaire astrocytomen beschreven.

Hoofdstuk 4

Tumoren van 26 geopereerde kinderen werden onderzocht op de mate van aanwezigheid van een bepaald eiwitcomplex, genaamd “NOR” (Nucleolar Organizer Regions). Deze eiwitcomplexen zijn aanwezig in iedere cel, maar in snel delende cellen verhoogd aanwezig. Door het tumorweefsel te impregneren met een zilververbinding kunnen de NORs microscopisch zichtbaar worden gemaakt. Zij worden dan AgNORs genoemd en kunnen in aantal of oppervlakte eenheid gemeten worden. Tumoren met een lage score vertoonden groei-stilstand en soms spontane verdwijning van de resttumor. Die met een hoge score vertoonden zowel uitgroei als groeistilstand. Vanwege het lage aantal tumoren dat onderzocht kon worden ontbrak de mogelijkheid om statistisch onderbouwde conclusies te leveren. Echter, er is een duidelijke tendens die aangeeft dat het zeer onwaarschijnlijk is dat laag scorende resttumoren verdere uitgroei vertonen.

Hoofdstuk 5

De mate van aanwezigheid van een bepaald eiwit (genaamd Ki-67 ofwel MIB-1) werd onderzocht in het tumorweefsel van 44 geopereerde patienten. Dit eiwit is slechts aanwezig in cellen die groei ofwel deling vertonen, en niet in rustende cellen. Door middel van een zogenaamde “immuunkleuring” kan dit eiwit microscopisch in de individuele tumorcellen aangekleurd en zichtbaar gemaakt worden. Theoretisch bevinden cellen die het eiwit bevatten zich in een groei- of delingsfase. Naarmate een tumor meer delende cellen bevat zal deze snellere groei vertonen. Echter bij goedaardige hersentumoren is zelden en bij pilocytaire astrocytomen nooit tevoren onderzocht in hoeverre de aanwezigheid van dit eiwit een relatie vertoont met het groeigedrag van de tumor. De 12 patienten met een tumorrest die bij vervolgonderzoek geen uitgroei vertoonde hadden gemiddeld een geringer aantal eiwit bevattende cellen in de tumor dan de resttumoren die wel uitgroeiden. In 6 resttumoren bleken in het geheel geen eiwit bevattende cellen aanwezig te zijn; 5
van deze tumoren vertoonden geen en 1 vertoonde wel uitgroei. Deze resultaten suggereren voor tumoren met een laag Ki-67-eiwit gehalte een geringe kans op uitgroei. Echter, tevens blijkt dat deze eiwit bepaling voor de individuele geopereerde patient met een resttumor geen absoluut voorspellende waarde heeft ten aanzien van het toekomstige groei-gedrag van die tumor.

**Hoofdstuk 6**

In dit onderzoek, op het weefsel van 48 geopereerde patienten, werden de functie en de structuur van een deel van het genetische materiaal van de tumorcellen geanalyseerd. Het genetische onderzochte materiaal betrof het “p53-gen”, waarvan bekend is dat het in normale lichaamszellen een beschermende functie heeft tegen abnormale celgroei. Dit gen treedt in werking wanneer het, voor iedere cel voorgeprogrammeerde, delingsproces verstoord is. Het produceert dan een eiwit dat verdere celdeling blokkeert, opgetreden fouten in het delingsprogramma kan herstellen, of wanneer dit allemaal niet voldoet, de cel te gronde richt. Hiermee wordt voorkomen dat een enkele “ontregelde” cel uit kan groeien tot een tumor. Uit andere onderzoeken is gebleken dat dit gen bij vrijwel alle kwaadaardige tumoren in het menselijke lichaam niet goed functioneert; het gen is dan gemuteerd en het geproduceerde eiwit functioneert niet goed. Tevens is gebleken dat tumoren met het gemuteerde p53-gen zich agressiever gedragen dan die met een intact gen. In de huidige theorieën over het ontstaan van kanker wordt aan dit gen dan ook een belangrijke oorzakelijke rol toegeschreven.

Ook bij hooggradige, kwaadaardige astrocytomen blijkt dit gen vaak gemuteerd, echter bij de pilocytaire astrocytomen zijn in het verleden zelden mutaties aangetoond. Er wordt dan ook verondersteld dat het p53-gen geen rol van betekenis speelt bij het ontstaan van pilocytaire astrocytomen.

Dit onderzoek toont aan dat het gen wel degelijk in een hoog aantal onderzochte tumoren gemuteerd is en blijkbaar een rol speelt bij het ontstaan van pilocytaire astrocytomen. Echter er bleek geen duidelijke relatie aanwezig te zijn tussen het al dan niet aanwezig zijn van een mutatie en het gedrag van de resttumor na een operatie.

**Hoofdstuk 7**

In dit onderzoek werd een ander deel van het genetische materiaal van de tumorcellen onderzocht. Nu werd het zogenaamde "NF-1 gen" geanalyseerd. Mutatie van dit gen veroorzaakt een van de meest frequent voorkomende aangeboren ziekten: Neurofibromatosis 1, ofwel de ziekte van "von Recklinghausen”. Patienten met deze ziekte blijken vaak pilocytaire astrocytomen te ontwikkelen. Dit gegeven heeft geleid tot de vraag of het NF-1 gen betrokken is bij het ontstaan van pilocytaire astrocytomen.

Van 6 patienten werd het verwijderde tumorweefsel onderzocht op mutaties in het NF-1 gen, deze bleken niet aanwezig te zijn. Voorts bleek dat het genproduct, het eiwit "neurofibromine", waarvan bekend is dat het in normale cellen een "groeiernemmende" werking heeft, in verhoogde mate
aanwezig was. Uit deze bevindingen werd geconcludeerd dat het NF-1 gen geen rol speelt bij het ontstaan van pilocyttaire astrocytomen. Het lijkt waarschijnlijk dat het gen geactiveerd is in reactie op de tumorvorming, hetgeen mogelijk gezien kan worden als een poging van de cel om de abnormaal hoge groeisnelheid te remmen.

Hoofdstuk 8

Uit eerder onderzoek is gebleken dat iedere tumor bestaat uit 3 soorten cellen met een verschillende levensloop: Cellen die zich oneindig lang vermenigvuldigen, cellen die zich in een rustfase bevinden en nooit tot deling komen, en cellen die afsterven. Indien er overwegend cellen voorkomen die zich delen zal de tumor groeien. Indien er overwegend cellen zijn die zich in de rustfase bevinden, en er evenveel cellen zijn die delen, als die sterven, zal de tumor stabiel in grootte blijven. In het geval er meer cellen sterven dan delen, zal de tumor spontaan verdwijnen. Het groeigedrag van een tumor is dus afhankelijk van de balans tussen het aantal delende cellen en het aantal stervende cellen in die tumor. Voorts is voor de mate van groei van belang de snelheid waarmee de cellen zich delen. Het vaststellen van het aantal stervende cellen in een tumor is momenteel nog niet goed mogelijk. De gebruikte eiwitbepalingen, zoals toegepast in de onderzoeken van hoofdstuk 4 en 5, vormen echter een goede maat voor het vaststellen van de snelheid van celdeling en, respectievelijk, voor het aantal delende cellen in een tumor. Het combineren van de waarden van deze beide eiwitbepalingen voor iedere tumor bleek een meer betrouwbare indicatie voor het groeigedrag van de tumor te bieden dan iedere waarde afzonderlijk. Met andere woorden: tumoren waarin beide eiwitten laag aanwezig of afwezig waren, vertoonden nooit uitgroei van de resttumor. Van de twee bestudeerde genen kon geen invloed op het groeigedrag van de tumor vastgesteld worden.

Het protocol voor behandeling en vervolg van patienten met een resttumor kan met behulp van de resultaten van de twee eerder genoemde eiwit-bepalingen bijgesteld worden: Indien de tumor van de patient waarbij een rest is achtergebleven de beide eiwitten niet of in een lage hoeveelheid bevat, behoeft levenslange controle door middel van hersenscans niet meer plaats te vinden. Voorts kan de chirurg op grond van deze bepalingen beslissen om de operatieve ingreep te beperken tot een gedeeltelijke, veilige tumor verwijdering, ofwel om meer risico’s te nemen en te trachten de tumor geheel te verwijderen.
Nawoord

Curriculum Vitae

1972-78: Secondary school, Prof. ter Veen Lyceum, Emmeloord, Gymnasium-B diploma.
1978-87: College and medical school at the "Katholieke Universiteit" in Nijmegen, the Netherlands.
1982-84: Student-research project at the dept. of neurophysiology, Academic Hospital in Nijmegen.
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1988: Internship at the dept. of neurosurgery, University Hospital, Vrije Universiteit, Amsterdam (Prof. dr. H.A.M. van Alphen).
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