The pilocytic astrocytoma
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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>sex</th>
<th>tumor</th>
<th>MIB-1</th>
<th>AgNOR</th>
<th>prol</th>
<th>p53 imm.</th>
<th>TP53 mut.</th>
<th>flow</th>
<th>NF1 expr</th>
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**STABLE AND REGRESSIVE RESIDUAL TUMORS**

**PROGRESSIVE RESIDUAL TUMORS**
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>Group</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>
GENETIC ASPECTS.

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 immunonegativeness.

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) immunoeexpression. 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 immunoeexpression 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 immunoreponse, 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 immunoresponse, 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 p21_{ras} 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 astrocytomas.
**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
