Usefulness of markers of cell proliferation in the management of pituitary adenomas

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ABSTRACT

1. Pituitary adenomas are benign and slow-growing tumours whose clinical manifestations depend mainly on the secretory activity of the adenomatous cells. Except for prolactinomas, surgical removal of the tumour is the therapy of choice.

2. Despite extensive research on their clinical and pathophysiological aspects, few studies have explored the oncological characteristics of these rare lesions. Among these, the growth fraction of pituitary adenomas has been determined by different methods, of which the most useful are those performed in archival material.

3. The results reported in the literature show that adrenocorticotropic hormone-secreting tumours seem to be characterized by a higher proliferation index than the other types of pituitary adenomas, despite their usually small tumour size.

4. In small series of patients radiotherapy and medical treatment with dopaminergic drugs and octreotide were associated with a lower proliferation index than untreated tumours. Tumour size was not correlated with the growth fraction of the pituitary tumours, whereas invasiveness was correlated in most studies.

5. From a clinical point of view, however, the more promising utilization of the proliferation index seems to be in predicting the potential of recurrence of the tumour, thus allowing a more rational approach to follow-up and further treatment of patients with pituitary adenomas.

INTRODUCTION

Pituitary adenomas are benign neoplasms that account for about 10–15% of all intracranial tumours. The simplest classification of these tumours takes into consideration the clinical presentation of the patient. Thus, pituitary adenomas are usually subdivided into those secreting growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH) and thyrotrophic hormone (TSH), and non-functioning tumours. The latter is a rather broad category comprising gonadotropin-secreting, null cell, silent corticotropinoma, silent somatotropinoma and oncocyctic adenomas, where clinical presentation is due exclusively to compression effects on neighbouring structures or destruction of the normal pituitary gland. Surgical removal of the tumour is the therapy of choice in all types of pituitary adenomas, with the exception of PRL-secreting tumours which are usually responsive to medical treatment with dopaminergic drugs. As recurrence of the pituitary lesion after incomplete removal of the tumour is common, adjuvant radiotherapy is often advised in such patients.

Key words: cell proliferation, growth fraction, pituitary neoplasm, pituitary surgery.
Abbreviations: AgNOR, argyrophilic nucleolar organizer regions; ACTH, adrenocorticotropic hormone; BUdR, bromodeoxyuridine; GH, growth hormone; PCNA, proliferating cell nuclear antigen; PRL, prolactin; TSH, thyrotrophic hormone.
Correspondence: Dr M. Losa.
Pituitary adenomas are histologically benign, but some tumours may show clinical aggressiveness that is reflected by relentless progression despite multiple surgical, medical, and radiotherapeutic interventions. Curiously, histological analysis of pituitary adenomas does not always distinguish between aggressive and non-aggressive lesions [1]. Tumour invasion into surrounding structures, dura, bone or normal pituitary gland is frequently detected, especially in large adenomas [1], but by itself this cannot be considered, as it often is in other types of neoplasm, typical of malignant transformation. Indeed, the diagnosis of pituitary carcinomas is very uncommon [2] and based more on clinical than pathological data, in particular the presence of metastases. Nevertheless, histological analysis of pituitary adenomas may identify particular subgroups of patients at risk of tumour regrowth. For example, aggressive behaviour of some types of pituitary tumours – sparsely granulated GH-secreting adenoma, sparsely granulated corticotrophic adenoma, mixed GH and PRL adenoma, and acidophil stem cell adenoma – has been already recognized. In summary, routine histological analysis of pituitary adenomas has, with little exception, not proven to be capable of predicting exactly their aggressiveness. Thus, alternative and prognostically informative tools to assess the clinical behaviour of pituitary tumours and their potential to regrow have long been sought.

Different expression or mutation of oncogenes and tumour suppressor genes in neoplastic tissue is predictive of tumour relapse and survival in several malignancies. However, convincing evidence of the usefulness of these markers in the clinical management of patients' adenoma is still lacking. Thus, accumulation of p53 protein in pituitary adenomas, although uncommon, correctly identified pituitary carcinomas but did not reliably distinguish invasive from non-invasive tumours [3,4]. Similar results have been reported also for mutations of the ras oncogene [4,5], the retinoblastoma suppressor gene [6], and the protein kinase-C gene [7], whereas loss of heterozygosity at the multiple endocrine neoplasia-1 tumour suppressor gene locus has been found in a few cases of sporadic pituitary adenoma [8].

Based on the theoretical assumption that aggressive tumours should be characterized by faster growth, methods to assess the proliferation rate of pituitary adenomas could provide valuable information about their long-term outcome. In the present paper we review the published data about the measurement of the growth fraction in pituitary adenomas.

**CELL CYCLE AND GROWTH FRACTION**

Pituitary adenomas, like all other tissues, consist of two cell populations: one of resting cells and the other of cells that undergo active replication through the mitotic cycle. The rate of growth of pituitary adenomas depends theoretically on the doubling time of the adenomatous cells. The normal cell cycle takes about 2 days, but in almost all types of cancer is prolonged [10]. Cell doubling time is slowed by many factors, among which the most important are the death of some tumour cells by apoptosis (programmed cell death), ischaemic or haemorrhagic events, and the presence of a pool of quiescent cells that do not enter the mitotic cycle.

Assessment of tumour growth and/or recurrence by MRI or CT scans is of paramount importance in clinical decision making, especially for patients with non-functioning tumours. However, since most pituitary adenomas should be treated quickly after diagnosis and their growth is unpredictable, it is not advisable to infer the growth fraction of pituitary adenomas by comparing intervalled neuroradiological studies. For this reason, histological techniques capable of determining the growth fraction of neoplastic cells have been considered a potentially useful tool in understanding the growth characteristics of pituitary tumours.

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**Figure 1 Schematic representation of the cell cycle**

Resting cells (G0) can enter the replication phase under proper stimulation. The mitotic cycle comprises four different stages: G1, S, G2, and M. The G1 phase is of highly variable duration and is followed by the S phase, during which DNA synthesis and replication occur. After completion of the S phase cells go through the G2 phase preceding cell division which occurs in the M phase. At the end of mitosis cells can re-enter another mitotic cycle or join the other resting cells in G0 phase. Ki-67 antigen is present at a constant level during the entire mitotic cycle; PCNA is present from the G1 through to the G2 phase, but is maximally expressed during the S phase, whereas the BUDR method can recognize replicating cells only when they are in the S phase.
METHODS TO MEASURE THE GROWTH FRACTION

Direct counting of cell mitoses in slowly growing neoplasms, such as pituitary adenomas, is unreliable because mitoses occupy only about one-fifth of the entire cell cycle and are thus correspondingly rare in biopsy specimens [11]. DNA synthesis takes place only in proliferating cells. Accordingly, several methods have been applied to histological preparations to identify the cell kinetics of excised tumours (Table 1). Autoradiography of tumours exposed to tritiated thymidine in vivo [12] or in vitro [13] is time-consuming and requires the use of relatively high amounts of radioactivity. A second method employs monoclonal antibodies against bromodeoxyuridine (B UdR), a thymidine analogue incorporated during DNA synthesis, which can be used to determine the growth fraction of the excised tumour. With this method, patients receive a slow intravenous infusion of B UdR shortly before surgery and the percentage of cells that have incorporated B UdR is detected by immunohistochemistry on formalin-fixed specimens [14,15]. However, this method is able to pick up cells only during the S phase of the mitotic cycle (Figure 1).

Antigens expressed only during the mitotic cycle can be detected by immunohistochemistry (Table 1). The Ki-67 monoclonal antibody was developed during studies attempting to produce an antibody specific for the nuclei of Reed–Sternberg cells. It recognizes a nuclear protein found in normal proliferating cells, whose function is at present still unknown. Analysis of its reactivity in replicating cells during the various phases of the cell cycle has demonstrated that the Ki-67 antigen is expressed in replicating cells during the G¿, S, G¿ and M phases but not in resting cells (Figure 1). Calculation of the Ki-67 antigen-labelling index, obtained by counting the number of Ki-67-labelled nuclei in a representative sampling of the tumour and expressing it as a percentage of the total number of cells counted, permits direct measurement of the growth fraction in fresh frozen specimens of tumours [16,17]. A novel monoclonal Ki-67 antibody (MIB-1) can be used for formalin-fixed, paraffin-embedded tissue sections. Proliferating cell nuclear antigen (PCNA), an acidic nuclear protein that is involved in DNA replication by acting as a cofactor of DNA polymerase δ, is synthesized at the highest concentration during the late G¿ and the entire S phases of the cell cycle, whereas declining concentrations are detected in the G¿ phase (Figure 1). The PCNA method can be used for deparaffinized and rehydrated formalin-fixed tissue sections [18]. A further proliferation-associated nuclear antigen (p105) can also be detected in formalin-fixed tissue sections [19].

A fourth method involves the measurement of argyrophilic nucleolar organizer regions (AgNOR), representing loops of ribosomal DNA transcribed by RNA polymerase I [20]. Silver staining techniques are necessary to demonstrate AgNOR in replicating cells.

Flow cytometry allows the rapid determination of the ploidy level of tumour cells, from which the different phases of the cell cycle can be calculated [11]. However, chromosomal abnormalities can be detected in pituitary tumours without other characteristics of increased proliferation [19].

GROWTH FRACTION AND ADENOMA CHARACTERISTICS

Before reviewing the published data on the determination of the growth fraction in pituitary adenomas, it must be stressed that variation of the measurement method, sampling material and technical details may account for some of the differences noted among the results of the various studies.

Regardless of the method used, no relationship has emerged linking age, sex or duration of symptoms to the growth fraction of pituitary adenomas. Analysis of the secretory activity of the tumour shows a higher Ki-67 labelling index in patients with ACTH-secreting adenomas (Cushing’s disease) in most but not all studies (Table

<table>
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<th>Method</th>
<th>Type of tissue</th>
<th>Reproducibility</th>
<th>Routine applicability</th>
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<tr>
<td>Mitotic count</td>
<td>Paraffin embedded</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Fresh frozen or paraffin embedded</td>
<td>Good</td>
<td>Good</td>
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<tr>
<td>Thymidine labelling index</td>
<td>Fresh tissue (vital)</td>
<td>Good</td>
<td>Poor</td>
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<td>BUdR</td>
<td>Fresh tissue (vital)</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>AgNOR</td>
<td>Paraffin embedded</td>
<td>Dubious</td>
<td>Good</td>
</tr>
<tr>
<td>PCNA</td>
<td>Paraffin embedded or fresh frozen</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Fresh frozen</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>MIB-1</td>
<td>Paraffin embedded</td>
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In the study by Shibuya et al. [15] the same result was obtained with the DNA polymerase method, whereas the BUdR and AgNOR values for ACTH-secreting tumours were not different from other types of adenoma. These findings have been interpreted as indicating a high proliferative potential of ACTH-secreting adenomas, a hypothesis also supported by the known rate of relapse of the disease after apparently complete removal of the tumour. On the other hand, almost 90% of ACTH-secreting tumours are microadenomas and the duration of symptoms before diagnosis averages more than 4 years [21], observations not easy to reconcile with a high proliferative potential of ACTH-secreting adenomas. ACTH-secreting adenomas may possess a higher intrinsic growth fraction than other adenomas, but this characteristic may be counterbalanced by factors such as high cortisol levels that increase fractional cell loss and thus retard the progression of tumour growth to the macroadenoma stage.

The relationship between the growth fraction and prior medical or radiation therapy has been investigated in detail in some studies. Dopaminergic drugs are the treatment of choice in patients with PRL-secreting adenomas, not only because they lower PRL levels but also because they cause substantial tumour shrinkage in the majority of patients [22]. The Ki-67 labelling index was significantly lower in 18 patients with PRL-secreting adenomas who had been treated with bromocriptine until surgery (0.6%) than in 7 untreated patients (1.06%) or in 16 patients who discontinued the drug at least 2 months before surgery (1.49%) [23], suggesting that dopaminergic drugs have a direct antiproliferative effect that vanishes soon after their withdrawal. In another study, however, Shibuya et al. [15] did not detect any difference between treated and untreated patients with PRL-secreting tumour, although it was not stated whether patients continued treatment until the day of surgery or discontinued it days or weeks before. Details on changes of tumour size during therapy with bromocriptine were not reported in both studies as well as the reason (intolerance to dopaminergic drugs, lack of efficacy, or personal choice of the patient) for performing surgery. Therefore, without this information, caution should be exercised in interpreting the effect of dopaminergic therapy on the labelling index of PRL-secreting adenomas. A similar study was later performed in acromegalic patients pretreated with octreotide for 1 to 2 months before surgery: the 16 patients who received the drug had a lower Ki-67 value (0.50%) than the 36 previously untreated patients (1.0%) [24]. Again, clinical details before and during octreotide therapy were not provided and it is thus impossible to determine whether the lower value of the labelling index in octreotide-treated adenomas correlated with inhibition of GH secretion or decrease in tumour size. Conventional radiotherapy did not modify the Ki-67 value of six recurrent adenomas (0.68%) compared with five patients who also had recurrence of the pituitary tumour but who did not undergo post-operative radiation (0.70%) [24]. However, since the mean interval to recurrence was significantly longer in irradiated patients (13.8 years) than in those who had not undergone radiotherapy (4.0 years), it was suggested that radiotherapy may slow tumour regrowth by increasing the cell loss fraction rather than by diminishing the cell growth fraction.

Tumour size has not been found to correlate with the markers of tumour growth. Ki-67 expression was not different when tumours were classified into microadenoma (n = 74) or macroadenoma (n = 115) [25]. No correlation between tumour diameter and Ki-67 value was also found by Knosp et al. [26] in 62 patients, by Shibuya et al. [15] in 64 patients, and in our preliminary study in 55 patients [27]. It is thus likely that other mechanisms regulate the growth of pituitary adenomas.

Invasiveness of pituitary adenoma has been defined according to intraoperative observation by the surgeon (as usually reflecting gross invasion of the tumour into the surrounding dura or bone), neuroradiological studies (erosion of bone on CT scan, growth into the cavernous sinus or the sphenoid sinus on MRI), or histological examination of a specimen of the surrounding dura (allowing assessment of the presence of microscopic

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<tr>
<td></td>
<td>Ki-67 (n = 61)</td>
<td>Ki-67 (n = 64)</td>
<td>Ki-67 (n = 191)</td>
<td>Ki-67 (n = 22)</td>
<td>Ki-67 (n = 68)</td>
</tr>
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<td>1.11% (n = 12)</td>
<td>0.69% (n = 10)</td>
<td>0.81% (n = 50)</td>
<td>0.25% (n = 4)</td>
<td>2.15% (n = 30)</td>
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<tr>
<td>PRL-secreting</td>
<td>1.20% (n = 17)</td>
<td>0.91% (n = 20)</td>
<td>0.94% (n = 80)</td>
<td>1.10% (n = 5)</td>
<td>6.61% (n = 8)</td>
</tr>
<tr>
<td>Cushing’s disease</td>
<td>0.65% (n = 2)</td>
<td>2.49% (n = 7)</td>
<td>2.98% (n = 10)</td>
<td>0.90% (n = 1)</td>
<td>3.84% (n = 7)</td>
</tr>
<tr>
<td>Non-functioning</td>
<td>0.98% (n = 20)</td>
<td>0.82% (n = 24)</td>
<td>0.62% (n = 50)</td>
<td>0.29% (n = 21)</td>
<td>2.09% (n = 20)</td>
</tr>
<tr>
<td>TSH-secreting</td>
<td>–</td>
<td>0.87% (n = 3)</td>
<td>0.80% (n = 1)</td>
<td>–</td>
<td>3.78% (n = 3)</td>
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<tr>
<td>Nelson’s syndrome</td>
<td>2.10% (n = 2)</td>
<td>–</td>
<td>1.00% (n = 1)</td>
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GROWTH FRACTION AND THE RISK OF RECURRENTITY OF PITUITARY ADENOMAS

Proliferation index in pituitary adenomas

Figure 2 Comparison of the proliferation index between invasive and non-invasive pituitary adenomas

The numbers on the abscissa indicate the number of patients in each category. The studies listed correspond to [28], [26], [15] and [29] respectively. The method used to classify the invasiveness of the tumour is shown in parentheses. Statistical significance: *P < 0.05 and **P < 0.01.

Figure 3 Comparison of the proliferation index between recurrent and non-recurrent pituitary adenomas

The numbers on the abscissa indicate the number of patients in each category. The studies listed correspond to [15], [18] and [27] respectively. The method used to determine the proliferation index in each study is shown in parentheses. Statistical significance: *P < 0.01. Hsu et al. [18] reported the proliferation index at both primary and second operation of recurrent tumours; the value reported here refers to the second operation for comparison with the other reports.

infiltration not visible by other methods). As shown in Figure 2, the Ki-67 value was higher in patients with invasive tumours in 3 of the 4 studies that reported such analyses [26,28,29]. However, in our preliminary study on 55 patients with non-functioning adenomas the MIB-1 value did not differ between invasive and non-invasive tumours, as assessed by MRI signs of invasiveness [27]. No difference in the expression of Ki-67 and PCNA was found in 10 pituitary adenomas invading the sphenoid sinus, compared with 10 non-invasive adenomas [30].

Oncoprotein expression by pituitary adenomas was not correlated to measures of the cell growth fraction. In the study performed by Ikeda and Yoshimoto [31] the percentage of cells positive for the c-myc gene, considered fundamental to cell proliferation, was a good indicator of the biological activity of the pituitary tumours, in contrast to the results obtained in the same adenomas with BUdR. Raghavan et al. [32] assessed the presence of the cellular oncogenes c-fos, c-jun, and c-myc in 33 pituitary adenomas. Again the presence of one or all of the oncoproteins was not correlated to the Ki-67 labelling index. On the other hand, p53 protein, the product of a tumour suppressor gene mutation which confers a capacity for abnormal cell growth, was expressed preferentially in the tumours with the highest Ki-67 values [3].

Clinically important recurrence of the tumour. Identification of a more specific prognostic factor would greatly assist the management of such patients. In several studies determination of the growth fraction has revealed a significantly higher value of the growth fraction in recurrent compared with non-recurrent tumours (Figure 3). Shibuya et al. [15] found that the growth fraction in 7 recurrent pituitary adenomas was significantly higher than in 50 primary tumours, independent of the method used to determine the growth fraction (Ki-67 expression, BUdR, AgNOR and DNA polymerase). We also found [27] that the MIB-1 value of 8 recurrent non-functioning pituitary adenomas was significantly higher than the value in 47 non-recurrent tumours. However, these studies are not prospective, making it difficult to assess the real prognostic value of the growth fraction index in predicting tumour recurrence. Only Hsu et al. [18] reported the PCNA index in 30 pituitary adenomas of various types that later recurred and compared it with the value determined in 32 tumours that did not relapse during a mean follow-up of 6.6 years. The tumours that later recurred had a significantly higher PCNA index than the non-recurrent tumours. Moreover, stepwise multivariate regression analysis identified a high PCNA index as a greater contributor to the risk of late relapse than the other factors (tumour size, extrasellar extension, incomplete surgical removal and cystic lesions) found to be predictive by univariate analysis. From a theoretical point of view, tumours with a high growth fraction should be more likely to recur rapidly after incomplete removal. Indeed, Hsu et al. [18] found an inverse correlation between the PCNA index and the interval to
relapse in their group of 30 patients with recurrent pituitary adenomas.

**CONCLUSION**

Determination of the growth fraction of pituitary adenomas has been accomplished in different ways. The availability of simple methods that can be used even in archival material has allowed the study of the proliferation index in selected series of patients with pituitary adenoma, thus providing additional information about the pathophysiology and the growth characteristics of the tumour. From a clinical point of view, the more promising utilization of the proliferation index seems to be in the prediction of the potential of recurrence of the tumour, thus allowing a more rational approach to follow-up and further treatment of patients with these rare tumours.

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