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PCNA, Ki-67 and hTERT in Residual Benign Meningiomas

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Abstract. *Background: Relapse in individual patients after incomplete/residual removal of meningiomas cannot be predicted by histology alone as re-growth occurs even in histologically benign meningiomas. Materials and Methods: Proliferating cell nuclear antigen (PCNA), Ki-67 and human telomerase reverse transcriptase (hTERT) labelling indices were measured in histological sections derived from residual meningiomas in 37 patients to assess their relationship to relapse. The labelling index (LI) expressed the percentage of tumour cell nuclei immunoreactive for PCNA, Ki-67 or hTERT in 1,000 tumour cells counted per section. The histological specimens comprised the following 2 groups: (i) stable for at least 10 years after initial partial resection of residual meningiomas: 20 specimens; (ii) relapsing between 11 and 145 months after initial resection of residual meningiomas: 17 specimens. Results: The proliferative activity and hTERT expression do not directly correlate with every relapse. The PCNA LI significantly differed in the relapsing group (10.8%) compared to the stable group (5.5%) ($p=0.08$). The Ki-67 LI also was higher in the relapsing group (2.5%) than in the stable group (2.0%), but not statistically significantly ($p=0.9$). hTERT LI was significantly higher in the relapsing group (27.8%) than in the stable group (7.2%) ($p<0.01$). Conclusion: The mean PCNA, Ki-67 and hTERT LI were higher in the relapsing group of residual meningiomas than in the stable group, although no statistical difference was found for PCNA and Ki-67. On the other hand, a statistical difference between the two groups of meningiomas was found for hTERT; however, it is no absolute predictor for relapse at the individual patient level.*

Meningiomas are classified as benign, atypical and malignant by the World Health Organization according to their histopathological features (1). For the group of residual meningiomas after surgery, this grading system fails to give information on the putative relapse and biological behaviour of benign tumours. Techniques based on the detection of antigens specifically expressed by proliferating cells have been developed. Two of these techniques are proliferating cell nuclear antigen (PCNA) and Ki-67 immunolabelling. PCNA functions as a co-factor for DNA polymerase delta in the S-phase of the cell cycle and is also involved during DNA synthesis associated with DNA damage repair mechanisms (2). Ki-67 is expressed during the late G1-, S-, G2- and M-phases of the cell cycle, although its function is unknown (3). On the other hand, telomerase activity has been linked to an indefinite lifespan of tumour cells by elongation of shortened telomeric DNA during cell division. The enzyme complex is composed of different proteins including two major subunits, namely, an integral RNA subunit and a reverse transcriptase (hTERT) (4, 5). The present study was undertaken to gain more insight into the biological behaviour of residual meningiomas. The presence of PCNA, Ki-67 and hTERT at the first intervention were compared with the potential relapse of the meningioma after partial resection. In this way, we tried to answer the question of whether PCNA, Ki-67 or hTERT expressions reflect differences in biological behaviour in relation to the relapse of residual meningiomas.

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Materials and Methods

Clinical data and histopathological classification. Archival material of 37 patients, with intracranial meningiomas, who had undergone surgery at the University Hospital, Ghent, Belgium between 1984 and 1995, with a follow-up of at least 10 years, was examined. The median patient age was 54.1 (range 21-77 years) and there were 18 women and 19 men. All selected patients had undergone incomplete tumour resection according to Simpson's grade III and IV classification (6) based on the surgeon's assessment at the time of surgery and on post-operative scanning. Relevant clinical data were obtained from the patients' medical records. The classification and grading of the tumours was based on WHO criteria (1). Selected cases were stained immunocytochemically for vimentin (an intermediate filament), glial fibrillary acidic protein (GFAP) for the cytoplasmic filament of astrocytomas, S-100 protein for glial and neural tumours and for neuron-specific enolase (NSE), in order to confirm the diagnosis. The benign meningiomas included 20 of

meningothelial type, 2 fibroblastic, 7 transitional, 7 psammomatous and 1 angiomatous (Tables I and II). The presence of relapse of meningiomas was based on the surgeons' clinical and technical records and confirmed by histopathological data.

Immunohistochemistry. Sections (4 μm) of paraffin-embedded tissue were labelled with mouse mononclonal antibodies against PCNA (1:50 dilution, Novocastra, Newcastle, UK), Ki-67 (1:50 dilution, Novocastra) and hTERT (1:50 dilution, Novocastra), diluted 1:50 in BSA/PBS. An antigen retrieval method was applied for antigen Ki-67 and hTERT. The slides were heated in sodium citrate buffer (10 mM, pH 6.0) for 30 min in a microwave oven at the highest power (700 W, Amana, Newton, IA, USA). Immunostaining was performed with the standard streptavidin-biotin-peroxidase method and diaminobenzidine (DAB). Samples were incubated with biotinylated rabbit anti-mouse, 1:200 for 30 min, followed by the third incubation with the streptavidin-biotin-peroxidase complex, 1:200 for 30 min. Visualization of the complex was realized with DAB and the sections were counterstained with haematoxylin. A glioblastoma multiforme, positive for hTERT, was used as a positive control, whereas normal brain tissue and antigen-free buffer of the positive control were used as negative controls. Labelling indices (LI) were calculated as mean percentage values by counting the nuclei of tumour cells in 3 positive areas of the histological sections for 1,000 cells.

Statistical analysis. All statistical analyses was carried out using SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL, USA). A Mann-Whitney test was used to analyse the associations for the different variables between the 2 groups of meningiomas and a Spearman analysis for the correlation. The categorized variables entered into the analysis were re-growth, PCNA LI, Ki-67 LI and hTERT LI. The values were expressed as mean±SD and as percentages when appropriate. The level of significance was set at a p-value of less than 0.05. All reported p-values are two-tailed.

Results

Seventeen patients with intracranial residual tumour classified as a meningioma showed re-growth, whereas the other group of 20 patients with residual tumour remained stable for at least 10 years. This total group of 37 cases was analysed for the expressions of PCNA, Ki-67 and hTERT. The results are summarized in Tables I and II.

Analysis of proliferation markers PCNA and Ki-67 (Figure 1). PCNA immunoreactivity was localized in the nucleus of tumour cells. The PCNA LI in the whole group ranged from 1.0% to 45.0%, with a mean value of 7.9% (SD=10.3). Table I and Table II list the PCNA LI for patients of the 2 different groups with stable and re-growing meningiomas after initial partial surgery. The mean PCNA LI was 5.5% (SD=8.4) for the group with stable residual meningioma, while it was 10.9% (SD=11.8) for re-growing residual meningiomas.

Immunohistochemical analysis of tumour cell proliferation was accomplished with the Ki-67 monoclonal antibody, which recognizes a nuclear antigen expressed during the late G1-, S-, G2- and M-phases of the cell cycle (3). The percentages of proliferating cells in the 37 meningiomas, with a mean of 2.2% (SD=2.5) and ranging from 0.1% to 14.0%, are given in Tables I and II. In stable residual meningiomas, the mean Ki-67 LI was 2.0% (SD=1.6) and in relapsing residual meningiomas 2.5% (SD=3.3).

Analysis of hTERT: the human telomerase reverse transcriptase subunit (Figure 1). hTERT forms the reverse transcriptase subunit of the telomerase enzyme complex, which compensates for the loss of telomere DNA in dividing cells, and is thought to be expressed during the S- and early G2-phases of mitosis (7). hTERT immunoreactivity limited to tumour nucleoli was strong and distinct in the nucleoli, with a distribution ranging from a total absence of positive nuclei in 15 of the 20 stable residual meningiomas (75%) to numerous labelled tumour nuclei, either uniformly distributed or appearing in clusters in 12 out of 17 re-growing residual tumours (71%). The mean hTERT LI was 7.2% (SD=15.0) for the stable residual meningiomas and 27.8% (SD=32.6) for the re-growing residual meningiomas.

Relationship between the different variables (Figure 2). Although the labelling indices for both Ki-67 and PCNA were higher in the relapsing residual meningiomas than in the stable group, no significant correlation could be found between PCNA and Ki-67 ($p=0.14$). In contrast, a significant correlation was seen between PCNA and hTERT ($p<0.01$), but no significant correlation was found between Ki-67 and hTERT ($p=0.64$).

A Mann-Whitney test was used to analyse the difference between the group of stable and relapsing meningiomas for the variables. No significant difference was found for the Ki-67 proliferation marker ($p=0.9$) or for PCNA ($p=0.08$). On the other hand, a difference was found for hTERT ($p=0.008$). However, at the individual tumour level, none of the 3 tested markers had an absolute value.

Discussion

Telomerase expression was demonstrated to vary significantly in stable and relapsing meningiomas. Although the Ki-67 proliferation marker and PCNA were higher in the relapsing group of residual meningiomas in comparison to the stable ones, the difference was not significant. In general, the factors most predictive for relapse (6, 8) are the extent of resection and the histological type. The Simpson grade at initial surgery reportedly influences recurrence or relapse (6). Histological parameters have been described as predictive for recurrence of meningiomas (9). Hypercellularity, loss of architecture, nuclear pleomorphism, an increased mitotic index, tumour necrosis and brain invasion have been considered to be indicative of malignancy or progressive behaviour (10, 11). Few studies have been specifically designed to compare the

Table I. *Characteristics, PCNA, Ki-67 and hTERT of patients with no re-growth of a residual tumour.*

No.	Id	Gender	Age	Histopathology	Resection	PCNA LI	Ki-67 LI	hTERT LI
1	9	M	62	Psammomatous meningioma	Simpson III	5	3	19.9
2	36	M	66	Psammomatous meningioma	Simpson III	5	4.5	0
3	55	M	61	Psammomatous meningioma	Simpson III	40	1	0
4	66	F	59	Meningothelial meningioma	Simpson III	8.5	5	0
5	91	M	54	Meningothelial meningioma	Simpson III	3	3	0
6	112	F	61	Transitional meningioma	Simpson III	2	1	0
7	145	F	56	Transitional meningioma	Simpson III	8.5	4	28.3
8	168	F	56	Psammomatous meningioma	Simpson III	2	2	0
9	170	M	57	Fibromatous meningioma	Simpson IV	1	0.5	0
10	172	M	44	Transitional meningioma	Simpson III	6	5	57.5
11	202	F	52	Transitional meningioma	Simpson IV	1	0.1	26.1
12	208	F	61	Transitional meningioma	Simpson III	4.5	3	0
13	348	F	66	Meningothelial meningioma	Simpson IV	4.8	0.7	0
14	393	M	36	Meningothelial meningioma	Simpson IV	1	0.3	13.1
15	409	F	44	Psammomatous meningioma	Simpson III	1	2	0
16	507	M	57	Psammomatous meningioma	Simpson III	2	1	0
17	575	M	54	Meningothelial meningioma	Simpson IV	4	2	0
18	741	F	47	Meningothelial meningioma	Simpson IV	3	0.1	0
19	743	M	77	Meningothelial meningioma	Simpson IV	3	0.5	0
20	765	F	62	Fibromatous meningioma	Simpson III	4	2	0

Table II. *Characteristics, PCNA, Ki-67 and hTERT of patients with re-growth of a residual tumour.*

No.	Id	Gender	Age	Histopathology	Resection	PCNA LI	Ki-67 LI	hTERT LI
1	6	M	62	Transitional meningioma	Simpson III	6.6	0.5	18.3
2	10	M	25	Angiomatous meningioma	Simpson III	45	2.8	18.2
3	92	F	56	Meningothelial meningioma	Simpson III	3	14	0
4	100	F	46	Meningothelial meningioma	Simpson III	3.5	3	0
5	165	F	67	Meningothelial meningioma	Simpson III	11	4.6	42
6	187	F	65	Meningothelial meningioma	Simpson III	7	3.1	99
7	248	M	36	Meningothelial meningioma	Simpson III	25	0.8	22.3
8	255	M	64	Psammomatous meningioma	Simpson III	24.7	1.5	27.7
9	279	M	59	Transitional meningioma	Simpson III	4	0.6	96
10	301	M	55	Meningothelial meningioma	Simpson III	12	2.8	34.5
11	401	F	62	Meningothelial meningioma	Simpson III	2	0.6	0
12	465	M	32	Meningothelial meningioma	Simpson III	20	0.1	13.1
13	485	M	37	Meningothelial meningioma	Simpson III	1	3	0
14	588	F	62	Meningothelial meningioma	Simpson III	3	1.3	74.6
15	670	F	39	Meningothelial meningioma	Simpson IV	1	0.5	0
16	302	F	43	Meningothelial meningioma	Simpson III	12	0.1	6.4
17	1103	M	61	Meningothelial meningioma	Simpson III	4	2.5	20.4

histopathological features between relapsing residual meningiomas and stable meningiomas after initial partial surgery (12, 13). Meningioma, as a primary tumour of the coverings of the central nervous system, is known to be slow growing. Our purpose was to identify molecular markers to predict possible re-growth in residual meningiomas. Tumour cell proliferation and telomerase activity were thought to be important parameters and were assessed by immunolabelling of PCNA, Ki-67 and hTERT.

The proliferative activity of meningiomas was considered to be predictive for recurrence (10, 13). The cell can regulate ordered activation of protein complexes, triggering initiation and advancement through checkpoints. The assessment of cell proliferation in meningiomas is a very important tool in diagnosis and especially prognosis. It can be performed by calculating the LI of Ki-67 and PCNA. However, the usefulness of different LIs in establishing prognosis in individual cases is affected by

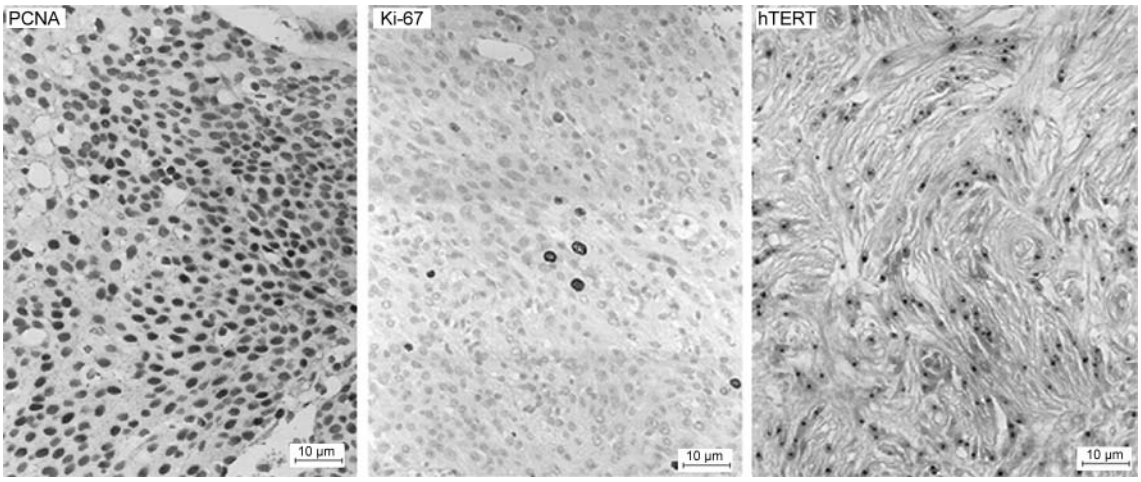

Figure 1. *Immunohistochemical staining showing diffuse and intense nuclear staining for PCNA, intense staining of some cell nuclei for Ki-67 and strong nucleolar staining for hTERT.*

sampling error, heterogeneity of the proliferation potential and the wide overlap of LI ranges between benign and malignant variants (10, 14).

The Ki-67 antibody recognizes a nuclear antigen that is present in the nuclei of proliferating cells (3, 15). In our study, the Ki-67 LI increased in the group of relapsing meningiomas, but had no prognostic value for some patients. These data indicate that proliferative activities or aggressiveness do not always develop with every relapse in residual meningiomas. There was no statistically significant difference between the Ki-67 LIs in relapsing and stable residual meningiomas, although there was a higher mean Ki-67 LI in the group of relapsing meningiomas (2.5%) compared with those that did not relapse (2.0%). Extensive overlap in the Ki-67 LI ranges occurred between these 2 groups.

In our results, PCNA was immunostained in a larger fraction of cells than Ki-67. PCNA has a longer half-life than Ki-67 and is needed for DNA repair in addition to DNA replication processes, in contrast to Ki-67, the expression of which is restricted to cells engaged in the proliferation cycle (3, 16). PCNA is distributed throughout the cell cycle with the highest concentration found in the late G1- and entire S-phases, and the lowest concentrations detected during the G2-phase. Levels of PCNA are very low in the M-phase, and undetectable in the G0-phase (2, 15). PCNA may also be expressed in damaged cells in relation to the amount of DNA alterations within the cancer cells. Tumours with a high PCNA index but low Ki-67 could be slowly growing, but rich in genetic aberrations and prone to developing aggressive clones. Although no significant difference for PCNA immunolabelling was seen, PCNA still has value as a proliferation marker in indicating a more malignant behaviour for meningiomas with a higher risk of relapsing or recurring.

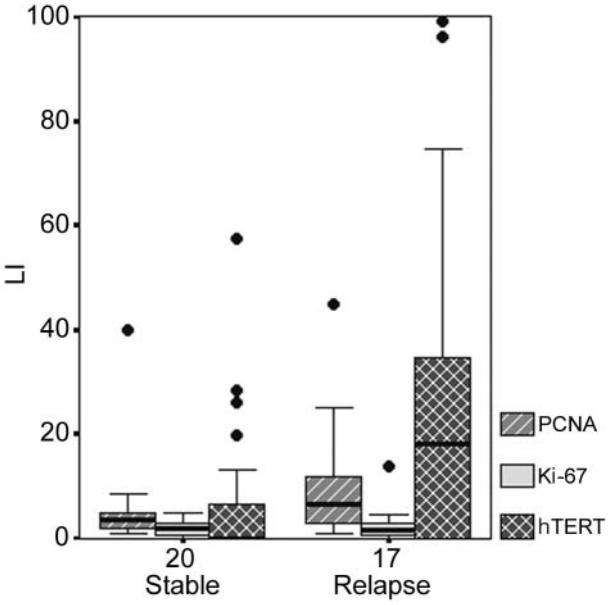

Figure 2. *PCNA, Ki-67 and hTERT labelling indices (LI) between the group of 20 stable and 17 relapsing residual benign meningiomas.*

It seems that telomerase is active on chromosomes during the S- and early G2-phases of mitosis, the time at which human telomeres are thought to be replicated and that the active telomerase complex is formed within the nucleolus, where it is sequestered, protected and prevents chromosomes from nonsense transcriptions throughout the rest of the cell cycle (7). Telomerase activity has been linked with an indefinite lifespan, with continuous proliferative capacity leading to a clinically malignant behaviour for meningiomas

(7, 14, 17-20). Our results support this more malignant behaviour because of the significantly higher presence of the hTERT protein within the group of relapsing residual meningiomas.

The significant correlation between PCNA and hTERT was of interest. Both are active in the S-phase of the cell cycle (2, 7), the time at which the DNA is duplicated. PCNA exists in 2 forms in the cell. One bound to the sites of DNA replication and another soluble in the nucleoplasm. PCNA has a dual function in that it is involved in DNA replication, as well as in DNA repair. Its role in DNA replication is as an activating factor for the enzyme polymerase δ, which is responsible for the replication of chromosomal DNA. PCNA seems to be involved in DNA excision and mismatch processes (21). The fact that PCNA and hTERT are active on DNA could be a reason for this significant correlation and a possible interaction with each other's function. Whether these 2 proteins influence each other should be further researched.

In summary, determining which meningiomas will relapse and which will not remains a difficult task. In reality, combinations of clinical factors along with the biological potential of the tumour play influential roles in the development of meningiomas. Our study indicated the potential role for proliferation markers, such as Ki-67 and especially PCNA. Further, the telomerase protein, hTERT, seems to be of value to indicate which residual meningiomas are aggressive and show a higher tendency to relapse.

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References

1 Kleihues P and Cavenee WK: WHO Classification of Tumors. Pathology and Genetics. Tumours of the Nervous System. Lyon: IARC Press, 2000.
2 Maga G and Hübscher U: Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci *116*: 3051-3060, 2003.
3 Scholzen T and Gerdes J: The Ki-67 protein: from the known and the unknown. J Cell Physiol *182*: 311-322, 2000.
4 Masutomi K, Kaneko S, Hayashi N, Yamashita T, Shirota Y, Kobayashi K and Murakami S: Telomerase activity reconstituted *in vitro* with purified human reverse transcriptase and human telomerase RNA component. J Biol Chem *275*: 22568-22573, 2000.
5 Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner, Kim NW, Trager JB, Taylor RD, Carlos R, Andrews WH, Wright WE, Shay JW, Harley CB and Morin GB: Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat Genet *17*: 498-502, 1997.
6 Simpson D: The recurrence of intracranial meningiomas after surgical treatment. J Neurochem *20*: 22-39, 1957.
7 Wong JM, Kusdra L and Collins K: Subnuclear shuttling of human telomerase induced by transformation and DNA damage. Nat Cell Biol *4*: 731-736, 2002.
8 Philippon J and Cornu P: The recurrence of meningiomas. *In*: Meningiomas (Al-Mefty O, ed.). Raven Press New York, pp. 87-105, 1991.
9 de la Monte SM, Flickinger J and Linggood RM: Histopathologic features predicting recurrence of meningiomas following subtotal resection. Am J Surg Pathol *10*: 836-843, 1986.
10 Abramovich CM and Prayson RA: Histopathologic features and MIB-1 labeling indices in recurrent and nonrecurrent meningiomas. Arch Pathol Lab Med *123*: 793-800, 1999.
11 Kim CH, Cheong JH, Bak KH, Kim JM and Oh SJ: Expression of the proliferating cell nuclear antigen and clinicopathological features in intracranial meningiomas. J Clin Neurosci *8*: 44-48, 2001.
12 Ho DM, Hsu CY, Ting LT and Chiang H: Histopathology and MIB-1 labeling index predicted recurrence of meningiomas: a proposal, of diagnostic criteria for patients with atypical meningioma. Cancer *94*: 1538-1547, 2002.
13 Takeuchi H, Kubota T, Kabuto M, Kitai R, Nozaki J and Ymashita J: Prediction of recurrence in histologically benign meningiomas: proliferating cell nuclear antigen and Ki-67 immunohistochemical study. Surg Neurol *48*: 501-506, 1997.
14 Cabuy E and de Ridder L: Telomerase activity and expression of telomerase reverse transcriptase correlated with cell proliferation in meningiomas and malignant brain tumors *in vivo*. Virchows Arch *439*: 176-184, 2001.
15 Cavalla P and Schiffer D: Cell cycle and proliferation markers in neuroepithelial tumors. Anticancer Res *17*: 4135-4143, 1997.
16 Mathews MB, Bernstein RM, Franza BR and Garrels JI: Identification of the proliferating cell nuclear antigen and cyclin. Nature *309*: 374-376, 1984.
17 Boldrini L, Pistolesi S, Gisfredi S, Ursino S, Lupi G, Caniglia M, Pingitore R, Basolo F, Parenti G and Fontanini G: Telomerase in intracranial meningiomas. Int J Mol Med *12*: 943-947, 2003.
18 Kalala JPO, Maes L, Vandenbroecke C and de Ridder L: The hTERT protein as a marker for malignancy in meningiomas. Oncol Rep *13*: 273-277, 2005.
19 Maes L, Lippens E, Kalala JPO and de Ridder L: The hTERT-protein and Ki-67 labelling index in recurrent and non-recurrent meningiomas. Cell Prolif *38*: 3-12, 2005.
20 Simon M, Park TW, Leuenroth S, Hans VH, Loning T and Schramm J: Telomerase activity and expression of the telomerase catalytic subunit, hTERT, in meningioma progression. J Neurosurg *92*: 832-840, 2000.
21 Kelman Z: PCNA: structure, functions and interactions. Oncogene *14*: 629-640, 1997.

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