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Immunohistochemical study of cell cycle regulatory proteins in intraductal breast carcinomas—a preliminary study

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Abstract

The aim of this study was to assess the levels of cell cycle regulatory proteins $p21^{waf1}$ (p21), p53, Cyclin A, Cyclin D1 and Ki-67 to see whether they correlated with recurrence-free survival (RFS). From 1982 to 1996, 50 patients aged less than 51 years underwent lumpectomy followed by radiotherapy for a pure ductal carcinoma *in situ* (DCIS). For each case, the following immunohistochemical stains were carried out: Ki-67, Cyclin A, Cyclin D1, p53 and $p21^{waf1}$ (p21). The percentage of positive nuclei was assessed. Multiple combinations of these factors were performed; in particular, we called the sum of Ki-67 and Cyclin A a global proliferation factor (GPF). Correlations with classical clinicopathological data were assessed. After a multivariate analysis, only GPF, Van Nuys Prognostic Index (VNPI) grade and mitotic index were independent predictive factors of recurrence in the whole population. In the population with close surgical margins, when the GPF level was less than the 25th percentile or more than the 75th percentile recurrence was low. In this preliminary study, GPF seems to be of interest to help in the decision process in the post-surgical management of the patient.

Keywords: Breast; Ductal carcinoma *in situ*; Cyclin A; Cyclin D1; Ki-67; p53; $p21^{waf1}$; VNPI grade

1. Introduction

Intraductal breast carcinomas carry a high risk of local recurrence. In half of the cases, this relapse occurs as an infiltrating ductal carcinoma. This underscores the importance of the first treatment decision that should be based on the potential risk of recurrence.

Mastectomy, which is a radical treatment, allows for a cure rate of approximately 98% [1]. Despite the fact that intraductal breast carcinomas are galactophoric diseases, the lesion is often localised and can be detected on mammograms by the microcalcifications. This is why a conservative surgical (lumpectomy) approach is often performed, with or without radiotherapy [1–4]. Numerous studies have aimed to identify predictive factors of relapse, especially histological parameters described according to several classifications [5–7]. Among them size, surgical margin status, architecture, nuclear grade and necrosis are the most often quoted [3,7,8]. These factors were studied by Silverstein and colleagues who defined a histological classification [7], and then the Van Nuys Prognostic Index (VNPI).

In this study, we tried to complete this clinicopathological approach with a semiquantitative analysis of some cell cycle-associated proteins, for which antibodies, which can be applied on paraffin sections, were available. These included:

- Ki-67, one of the most studied today, a protein present from the G1 to S-phase,
- Cylins A and D1, regulatory subunits for the cyclin-dependent kinases (cdks) and key cell cycle regulators. The binding of several inhibitors, such as $p21^{waf1}$, also modulates their activity.

Cyclin Dl exhibits a short half-life and serves as an activating partner for cdk4 and cdk2 in the mid-G phase. Its expression is induced by oestrogen and growth factors, and it acts as a cellular sensor for their

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presence [9]. Cyclin A appears during the late G1-phase and is often classified as a G2 cyclin [9].

The *TP53* gene is a tumour suppressor gene located on the short arm of chromosome number 17, band 13. It codes for a nuclear phosphoprotein that plays an important role in the regulation of the cell proliferation through the control of the progression from the G to S phase [10]. When numerous mutations are present in a cell, p53 stops the cell cycle at the so-called restriction point preventing cells from crossing into S phase. This allows the cell to repair DNA lesions through the transcription of the gene coding the p21 protein [11].

There are many studies in the literature examining correlations between the different prognostic clinicopathological and biological parameters. But, to the best of our knowledge, none have studied the impact of the factors involved in the cell cycle on the relapse-free survival (RFS). Thus, the aim of our study was to try to specify the relationship between the expression of several cell-cycle associated proteins and RFS.

2. Patients and methods

Our population consisted of 50 patients who had undergone the same conservative treatment (lumpectomy followed by radiotherapy: 50 Gy with a 10 Gy boost) for a pure ductal carcinoma *in situ* (DCIS) without any infiltrative focus. They were treated between 1982 and 1996 at the Centre François Baclesse (Caen, France). We chose to focus our study on patients younger than 51 years to obtain a homogeneous population with regard to hormonal status and treatment, the 51 year old threshold was chosen because the menopausal status was not always specified for all of the cases.

The clinicopathogical data were reviewed in a previous study described in Ref. [12]. Principal characteristics of our population are presented in Table 1.

For each case, a representative slide was selected, on which immunohistochemical reactions for the following were carried out: Ki-67, Cyclin A, Cyclin D1, p53 and $p21^{waf1}$ (p21).

Results of the stains were expressed quantitatively after two independent readings. The definitive value retained was observed by a third review of the discordant cases. Assessment of the percentage of marked nuclei was carried out on 300–400 nuclei in the most positive foci for every antibody used.

A statistical analysis was performed for the results for each antibody in all of the cases.

The percentage of positive nuclei for Ki-67 (MIB-1), Cyclin A, Cyclin D1, p53, p21 were assessed. Multiple combinations of these factors were performed. We retained those that we felt had a biological or prognostic interest. We calculated a global proliferation factor (GPF) that was the sum of the Ki-67 and Cyclin A results.

Table 1
Clinicopathological data

	Global population	Non-optimal surgical margins
Age (years)		
minimum	27	27
maximum	50	50
mean	43	43
Sample size (mm)		
minimum	20	20
maximum	180	100
mean	55	55
Tumour size[a]		
<15 mm	26	8
$\geqslant 15$ and $\leqslant 40$ mm	19	13
>40 mm	5	4
No. of blocks (nb)		
minimum	2	2
maximum	15	15
mean	7	8
Main architecture[a]		
comedo	23	9
non-comedo	27	16
VNPI grading[a]		
3–4	5	1
5–7	12	7
8–9	33	17
Mitotic index (number/hpf)		
minimum	0	0
maximum	7	6
mean	2.65	2
Surgical margins[a]		
$\geqslant 10$ mm	6	0
>1 and <10 mm	19	0
$\leqslant 1$ mm	25	25

No., number; VNPI, Van Nuys Prognostic Index; hpf, high power field.

[a] Number of patients are given in columns.

For the different antibodies, correlations between positivity and the following data were assessed: patient's age, tumour size, margin widths, architectural type, nuclear grade, maximal mitotic activity, VNPI grade.

We also studied the frequency of local recurrence according to the immunohistochemical results in the total population and also in the population with surgical margins equal to or less than 1 mm ($n=25$). All of these patients underwent a postoperative radiotherapy. The follow-up of these patients ranged from 50 to 140 months with a mean of 82 months.

2.1. Immunohistochemical techniques

Reactions were performed on paraffin-embedded 4 μm thick sections, that were dried all night at 56 °C. The slides were then deparaffinised and rehydrated. Heat

enhanced antigen retrieval was then performed in 10 mM pH 6 citrate buffer for 30 min in a microwave oven for Ki-67, Cyclin A and p53, and in 10 mM p H8 ethylene diamine tetra acetic acid (EDTA) buffer for 3 min in the pressure cooker for Cyclin D1.

The following antibodies were used: Ki-67 Immunotech, MIB1 clone diluted 1/5, Novocastra Cyclin A clone 6EE diluted 1/50, p53 DAKO clone DO7 diluted 1/25, Cyclin D1 Novocastra clone P2D11F11 diluted 1/800.

For Ki-67, cyclin A and p53, we used the biotin–streptavidin peroxidase revealing system. The primary antibody incubation time was 60 min, 20 min for the secondary biotinylated antibody, 20 min for the biotin–streptavidin peroxidase complex and 10 min for the diaminobenzidine (DAB) chromogen. This was performed at room temperature in an immunohistochemistry automate Optimax plus Biogenex®.

For Cyclin D1, we used the catalysed signal amplification Dako system Ref K1500. Endogenous biotin activity was blocked with 3% (vol/vol) hydrogen peroxide in methanol for 5 min, then blocking of proteins was followed by the primary and secondary antibodies and the biotin–streptavidin complex. Amplification was performed using biotinylated thyramide and hydrogen peroxide. The final steps were streptavidin peroxidase and then the DAB chromogen. Every step was for 15 min, except the last chromogen step that took 10 min. This technique was performed manually.

2.2. Statistical analysis

Statistical analysis was performed using the Medlog® and Stata® softwares. Differences between the distribution of the variables among the patient groups were tested using the Fisher's Exact test, the analysis of variance, or correlation coefficients, as appropriate. Survival curves were estimated with the Kaplan–Meier technique: the endpoint was the date of relapse or last point of follow up. Comparisons between survival curves were performed using the log rank test. Multivariate analysis was performed using the Cox model. For every marker and association of markers, correlations were studied according to quartiles.

3. Results

3.1. Global population

3.1.1. Frequency of positivity for each antibody.

We observed totally negative results for p21 and p53 in only 24 and 12% of the cases, respectively. In the other cases, we observed a great variation in positivity between the different markers, with rather similar values for Ki-67, Cyclin A and p21 and 2- to 3-fold higher values for Cyclin D1 and p53. Values ranged from 0 to 50% (mean of 12%, median of 5.5%) for p21 to 0–90% (mean of 37% and median of 30%) for Cyclin D1.

Because of an increased sensitivity due to the different staining method used, Cyclin D1 had a low threshold of positivity in comparison with the other markers. So, we used percentiles to compare cases with high versus low expression of the different markers.

3.1.2. Quantitive study of the reproducibility

In order to test the reproducibility of the labelling, we tested the correlation coefficients between the first and the definitive interpretations. This test defined the variability as 2% for p21 and p53, 3% for Cyclin D1 and Ki-67 and 5% for Cyclin A.

3.1.3. Study of the correlations between the markers

A positive correlation between Cyclin A and Ki-67 ($P=0.02$), between p21 and Cyclin D1 ($P=0.002$) and between p21 and Cyclin A ($P=0.004$) was observed. No other correlations between the markers were found.

3.1.4. Correlations between the markers and clinicopathological data

The correlations between the markers and clinicopathological data were studied using the 75th percentile of every parameter.

There was no relationship between the different levels of expression and age, with the exception of a relationship between women less or equal than 40 years and a high GPF value (Ki-67 + cyclin A) ($P=0.05$).

Tumours smaller than 1.5 cm showed a significantly higher p53 value, but this parameter did not correlate with any other marker.

Cyclin A and p21 were significantly higher in the group of comedo DCIS versus the non-comedo group. Such a relationship did not exist for the other markers.

There was a trend, that did not reach statistical significance, for lower levels of Cyclin A, Ki-67 and p21 in tumours with a lower nuclear grade. High maximal mitotic activity was related significantly with a high Cyclin A level, but not with the other markers.

All patients had a lumpectomy followed by radiotherapy. Results of the univariate analysis showed that the VNPI grade ($P=0.04$), surgical margin status ($P=0.02$), GPF using two thresholds (25th and 75th percentiles and singling out the group with mean values, $P=0.008$) were correlated with recurrences. Tumour size was linked to the risk, but without reaching statistical significance ($P=0.06$).

This relationship between GPF and recurrences was rather paradoxical: the population with intermediate values experienced significantly more recurrences (11/27) than the rest of the population 0/12 in the highest quartile and 1/11 in the lowest quartile, respectively ($P=0.03$) (see Fig. 1).

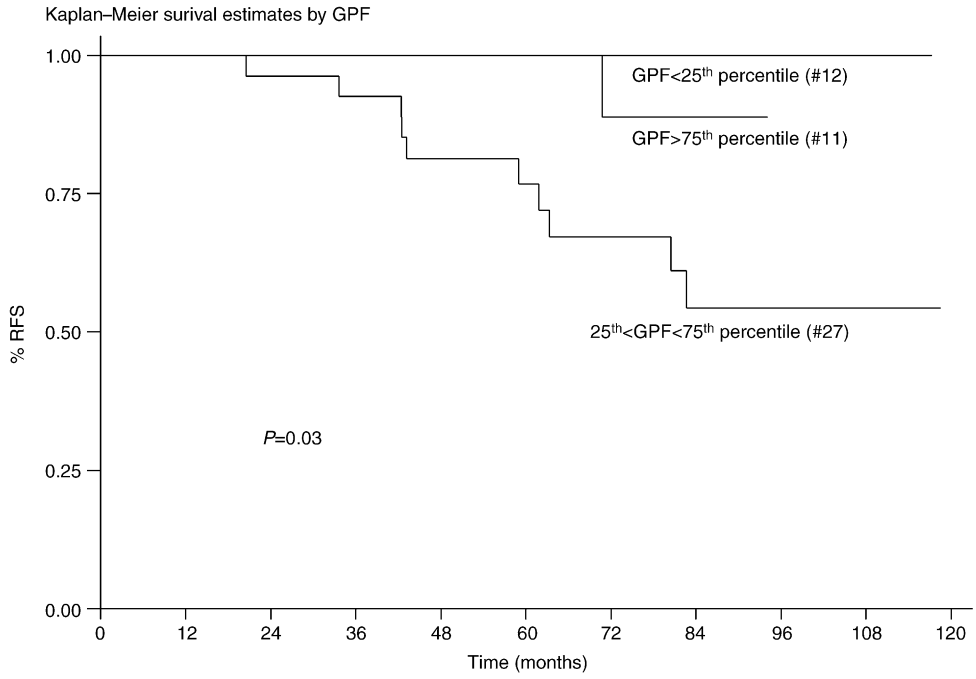

Fig. 1. Global population (50 cases)—recurrence-free survival according to global prolieration factor (GPF) (Cyclin A + Ki-67).

Concerning the incidence of recurrences in relation to the surgical margins, the RFS curve showed a significant difference between patients with margins greater than 1 mm (recurrences in 2/25 cases) and with margins less than or equal to 1 mm (10 recurrences/25 cases, $P = 0.02$).

After multivariate analysis according to the Cox model, including VNPI grade, tumour size, mitotic index, surgical margins and GPF, only GPF, VNPI grade and mitotic index were independent predictive factors of recurrence. Because the VNPI grade includes the parameter of surgical margins we decided to focus our study on the 25 cases with surgical margins less than or equal to 1 mm.

3.2. Population limited to patients with non-optimal surgical excision ($\leqslant 1$ mm)

This population included 25 patients. The frequency of positivity for every antibody, the study of correlations between the different markers and between these markers and the clinicopathological data did not show any real differences with results in the global population.

A multivariate analysis including age, DCIS architecture, nuclear grade, VNPI grade, tumour size, mitotic index, and the five biological markers showed that only GPF correlated with the number of recurrences ($P = 0.009$) in this group.

For this selected group, the intermediate category (when divided according to GPF) presented the highest risk of recurrences ($P = 0.02$), (Fig. 2).

A compound variable 'p21-cyD1' regrouping Cyclin D1 and p21 was defined as follows: concordant (high/high or low/low values) versus discrepant (high/low or low/high values), with the threshold at the median value. This variable shows a trend, that did not reach statistical significance because of the low number of patients, one recurrence in 9 patients with concordant values versus nine in 16 patients with discrepant values ($P = 0.07$) (Fig. 3).

4. Discussion

Numerous clinicopathological studies have been dedicated to the assessment of the risk of recurrences after lumpectomy for DCIS. These studies evidenced the predictive value of the following factors concerning the risk of recurrences: surgical margins [2,4,8,13], age [8,13], architectural type [8,13,14], nuclear grade [13,15], postlumpectomy radiotherapy [1,2,13], tumour size [12,16] and VNPI grade [7,12]. However, there remains some debate about the optimal treatment after lumpectomy for an infraclinic lesion detected on mammography. Should the patient be submitted to a second surgery (lumpectomy or even mastectomy?) and in the case of conservative surgery, is radiotherapy necessary?

Two approaches seem possible today to try to answer these questions: firstly, standardisation and precise quantification through imaging analysis of pathological parameters [17] and second, detection and quantification of biological parameters.

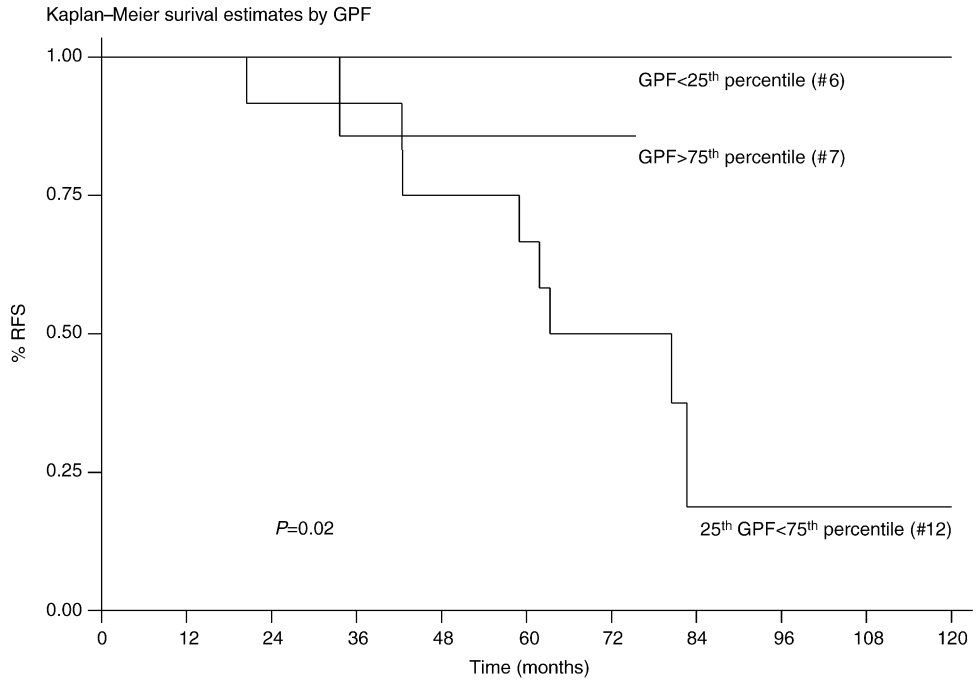

Fig. 2. Selected population (25 cases) — recurrence-free survival curve according to GPF.

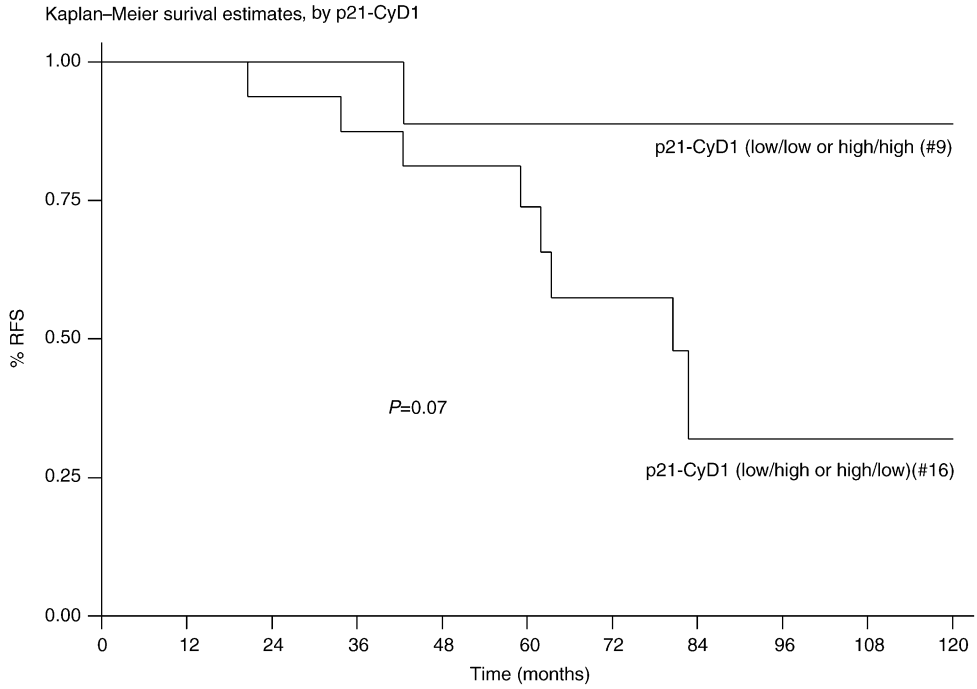

Fig. 3. Selected population (25 cases)—recurrence-free survival curve according to p21-CyD1 status.

Thus, we tried to estimate the prognostic value of the expression of the biological factors acting at different phases of the cell cycle.

The abnormally high expression observed for some of these factors is the result of either an increase in cellular activity, of an increased half-life of the protein or accumulation of these regulators because of functional or structural anomalies of the proteins. Immunohistochemistry does not allow us to differentiate between these different phenomena.

The results of the whole population including 50 patients allowed us to assess the relationship between the different factors studied.

The results of the 25 cases with unsatisfactory surgical margins permitted us to assess the recurrence risk according to the factors studied as all of the patients

had had the same treatment, namely lumpectomy followed by radiotherapy.

For Ki-67, despite differences in the interpretation of the results reported in the literature, the frequency and intensity of the staining seems to be linked to grade and architectural type of the tumour. Positivity ranges from 10 to 28% in low grade lesions and from 18 to 100% in high grade lesions [18–20]. In our study, the median value of marked nuclei was 13% (with a maximum value of 70%), no significant correlation was noted with the clinicopathological parameters studied.

To the best of our knowledge the literature concerning Cyclin A is scarce, with only one publication [21]. The previous study found no significant differences between low grade DCIS and normal breast tissue, but a significantly higher value in high grade DCIS versus normal tissue. In our study, Cyclin A was correlated only with Ki-67 and p21.

Studies concerning Cyclin D1 and DCIS have involved more than 500 patients [21–23]. The results of these studies, despite a significant increase in expression in DCIS versus normal tissue or simple hyperplasia, showed no such differences between low and high grade DCIS. Although our staining technique for Cyclin D1 was more sensitive than the method for the other markers, we could not find any correlations with clinicopathological or the other biological factors.

The role of p21 has only rarely been addressed in the literature [24,25]. In a study by Oh and colleagues [24], positive p21 was correlated with a low grade DCIS, in another study [25], like in ours, p21 was more highly expressed in high grade DCIS, but there was no relationship with clinicopathological or other biological factors.

A spate of publications exists concerning p53 and DCIS [26]. Most of the studies, used the DO7 clone as an antibody. Two of these studies [27,28] did not observe (as in our study) a correlation between high p53 expression and high grade DCIS, but the others showed this correlation.

Among the results presented, some are of prognostic interest:

(i) GPF: the sum of the percentage of positive nuclei for Cyclin A and Ki-67, is correlated with age, significantly so for patients less or equal than 40 years, this can be explained by the relationship between young age and comedo-type DCIS as evidenced by Goldstein and colleagues [29], and by the relationship between high grade DCIS and Cyclin A as shown in our results.

Recurrences were more frequent when the GPF was between the 25th and 75th percentiles of the population, for all 50 cases and also for the selected 25 cases with surgical margins less than or equal to 1 mm. In this selected subgroup, there were nine recurrences in 12 patients if the GPF was between the 25th and 75th percentiles and one recurrence in the other 13 patients. Despite the low number of patients, this difference was significant ($P = 0.009$) comparing these two groups. When we compare this population in three groups as in Fig. 2, $P = 0.02$. This homogeneous population included women aged between 27 and 50 years who underwent a lumpectomy with surgical margins less than or equal to 1 mm and secondary radiotherapy with doses of 50 Gy with a boost of 10 Gy centered on the tumour bed.

The multivariate study showed that GPF was the sole remaining significant prognostic factor in the subgroup with close margins, and remained significant, along with VNPI and mitotic index, in the whole population. The reason why, in the subgroup analysis, the mitotic count was not statistically significant is not straightforward. It could either be a statistical bias, because of the small number of patients included, thus only the strongest prognostic factors can be evidenced in such an analysis. Another possible explanation is that the mitotic count is a different method to assess proliferative activity other than, for example, flow cytometry or biological markers such as Cyclin A and Ki-67. The mitotic count is also dependent on technical factors [30] and on the duration of mitosis.

(ii) Cyclin D1 and p21: RFS curves showed a trend between concordant and discordant values of these two factors ($P = 0.07$).

These results need to be confirmed, but are nevertheless promising. Their practical interest will be validated only after standardisation of the techniques, allowing reproducible results between different investigators.

This study enabled us to define three different populations according to their GPF status. We propose the following hypotheses, which will need to be confirmed by other studies. For patients with a low GPF (Cyclin A + Ki-67) level, radiotherapy is probably of limited value, because of the low proliferative activity. For patients with intermediate values, with a high recurrence risk, radiotherapy seems to provide little benefit (10 recurrences in 12 patients despite radiotherapy). Finally, in those cases with high GPF values, radiotherapy seems promising (with a low recurrence risk despite involved surgical margins).

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