

## Immunohistochemical detection of cell proliferation in gastric carcinomas with the monoclonal antibody Ki-67. A study of 24 cases.

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**Summary.** The proliferative activity in 24 gastric carcinomas was determined by an immunohistochemical method using monoclonal antibody Ki-67 (ABC method). Immunostained nuclei were counted by two observers through a Nchet NS 1000 image numeriser. Three grades were defined according to stained nuclei percentage (proliferation index  $P_i$  = percentage of cells engaged in cellular cycle outside  $G_0$ ): grade 1 ( $P_i < 20\%$ ); grade 2 ( $20\% < P_i < 40\%$ ); grade 3 ( $P_i > 40\%$ ). About 60% of tumours were in grade 1 and 10% in grade 3.

No correlations were observed between  $P_i$  and the following parameters: histological differentiation; parietal extension; presence or absence of metastasis.

These results may be compared to the two other available studies of Ki-67 antibody in gastric cancers. Our study also showed a heterogeneous distribution of immunostained nuclei, within each single tumour and from one tumour to another, which has been noted in one previous study and in a similar one we made on colorectal carcinoma. This heterogeneity is the consequence of the variability of carcinomatous cell proliferative activity; an important biological factor in the evaluation of tumoral process.

The proliferative activity in gastric carcinomas provides an estimation of tumour dynamics that might be a prospective criterium for tumoral process evaluation.

**Key words:** Gastric carcinomas, Cell proliferation, Growth fraction, Monoclonal antibody Ki-67

### Introduction

Recently, several nuclear proteins have been described in proliferating cells. Ki-67 is a monoclonal antibody which recognizes a nuclear antigen expressed

in all phases of the cell cycle except  $G_0$ . Recent studies using Ki-67 in colorectal adenocarcinoma and adenoma have shown that indices of cellular proliferation have no prognostic significance but allow a precise evaluation of cellular growth fractions (Hoang et al., 1989).

The purpose of the present study was to analyse gastric carcinomas for the presence and distribution of Ki-67. Results of staining for Ki-67 were correlated with histological features of the neoplasms, depth of invasion of the cancer, vascular spread and presence of metastases.

Our present paper aims to determine some controversial previous results (Yonemura et al., 1990b; Porschen et al., 1991); unlike other published studies, we used a semi-automated image analyser processor, allowing a precise count of Ki-67 nuclear labelling, with preserved architecture.

### Materials and methods

Twenty-four cases of gastric carcinomas were obtained from routine surgical procedures. For light microscopy, three micron sections from paraffin-embedded specimens were stained with haematoxylin-eosin-saffran and periodic acid Schiff. For immunohistochemistry fresh tumour samples were snap-frozen at  $-170^\circ\text{C}$  in isopentane with liquid nitrogen. Six micron cryostat sections were examined with the monoclonal antibody Ki-67 (Dakopatts, Glostrup, Denmark) diluted at 1:25. The avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector, Burlingame, USA) (Hsu et al., 1981) was used with 3,3'-diaminobenzidine tetrahydrochloride, and appropriate negative controls were performed.

After counterstaining with eosin and strong haematoxylin, each slide was evaluated for Ki-67 positivity in areas of neoplasms. Cells were considered as positive for Ki-67 only when definite brown staining of the nucleus was identified. Staining of stromal cells was easy to distinguish from nuclear staining of tumour cells, as the former showed cytoplasmic staining.

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A quantitative method for recording Ki-67 positivity was used by two observers, ignorant of pathological findings. Sections were examined under a x 40 objective, with a semi-automated image analysis processor (Nachet NS 1000, Nachet-Vision, Evry, France). Fields were randomly selected through each section. A mean of 9 fields and 600 cells were counted in all the tumours. The mean percentage of stained nuclei in the different fields of a tumour provided a Ki-67 index or proliferation index (Pi). The mean Pi was compared in the different groups of neoplasms with a «t»-test of variance analysis. A three stage grading (grade 1: Pi <20%, grade 2: Pi from 20 to 40%, grade 3: Pi > 40%) was determined and compared with the other variables by a Chi 2 method.

The tumours were examined for morphological features according to the WHO classification (Watanabe et al., 1990). Three groups of tumours were individualized: carcinomas with liver, diaphragm, peritoneum, coeliac and infra pyloric lymph node metastases (group A); carcinomas with paragastric lymph node extension (group B); and carcinomas localized to the gastric wall (group C).

Without taking into consideration the group, tumours were separated according to the presence or absence of serosa extension (respectively S+ or S-) and to the histological type and degree of differentiation. Carcinomas of groups A and B, except one case, had vessel invasion (lymphatic or blood vessels within the tumour or the distant gastric wall). This invasion was absent in group C. Three tumours were located to the cardia.

## Results

In all tumours nuclear staining was present, Pi varied from 0.5% to 94.11% (mean 23.33%, standard deviation 23.54%; median 15.4%) (Fig. 1). Almost 60% of the tumours fell into grade 1 (Fig. 2), the remaining cases (Fig. 3) being equally distributed in grades 2 (Fig. 4) and 3 (Fig. 5).

In each case, the labelling index apparently varied at random from field to field. For example, four cases showed a great erratic pattern (Fig. 6), by opposition to

three other tumours (one from each grade) with a relatively even repartition of labelling (Fig. 7).

Pi seemed to increase regularly from group A to C, and the repartition between grades looked different in group C compared to A and B (a majority of grade 3 in

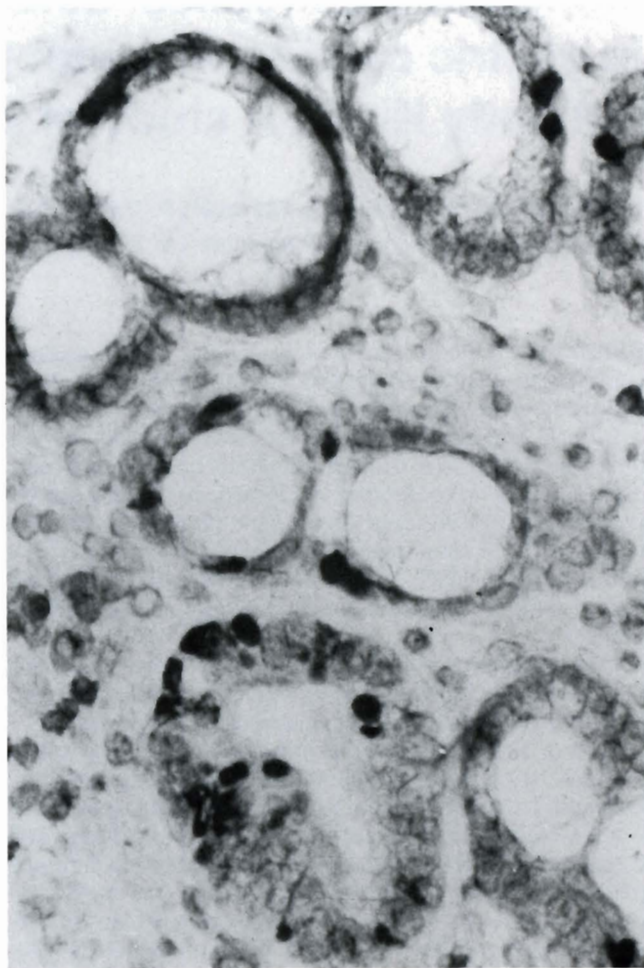


Fig. 2. Immunoperoxidase staining with Ki-67 antibody. Adenocarcinoma showing grade 1 proliferative index (positive nuclei < 20%), x 234

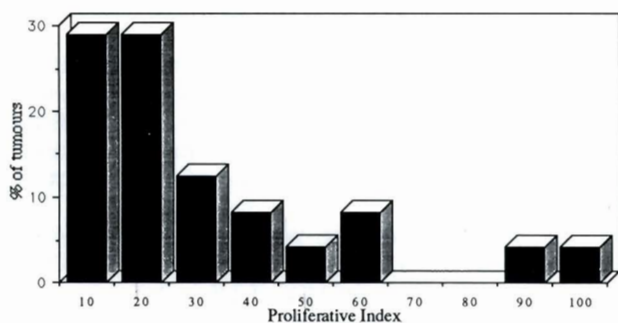


Fig. 1. Repartition of proliferative index (Pi) in 24 tumours. Pi varied from 0,5% to 94.11%. Near to 60% of tumours showed a lower index (<20%).

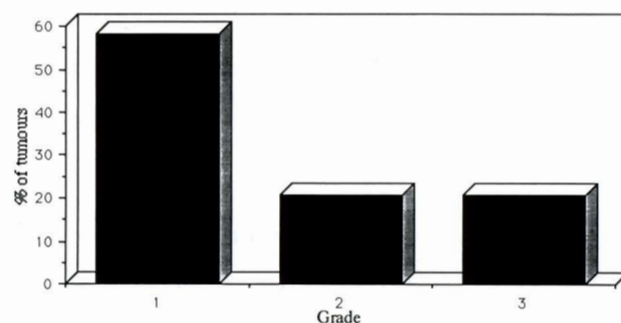


Fig. 3. Repartition of grades in 24 tumours. Near to 60% of tumours were in grade 1, the other cases equally divided in grade 2 and 3.

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group C, of grade 1 in A and B) (Table 1) but the differences failed to reach statistical significance.

No difference could be observed between S+ and S- tumours; their Pi was very near to the mean value, as was their grading (Table 2). Moderate and well-

differentiated carcinomas were more often of high grade than poorly differentiated ones, though without significance, and had a higher Pi ( $31.6 \pm 17.12\%$  versus  $21.41 \pm 11.64\%$ ), but test showed no significance ( $P > 0.3$ ) (Table 3). One adenosquamous carcinoma,



Fig. 4. Immunoperoxidase staining with Ki-67 antibody. Adenocarcinoma showing grade 2 proliferative index (positive nuclei = 20 - 40%) x 234

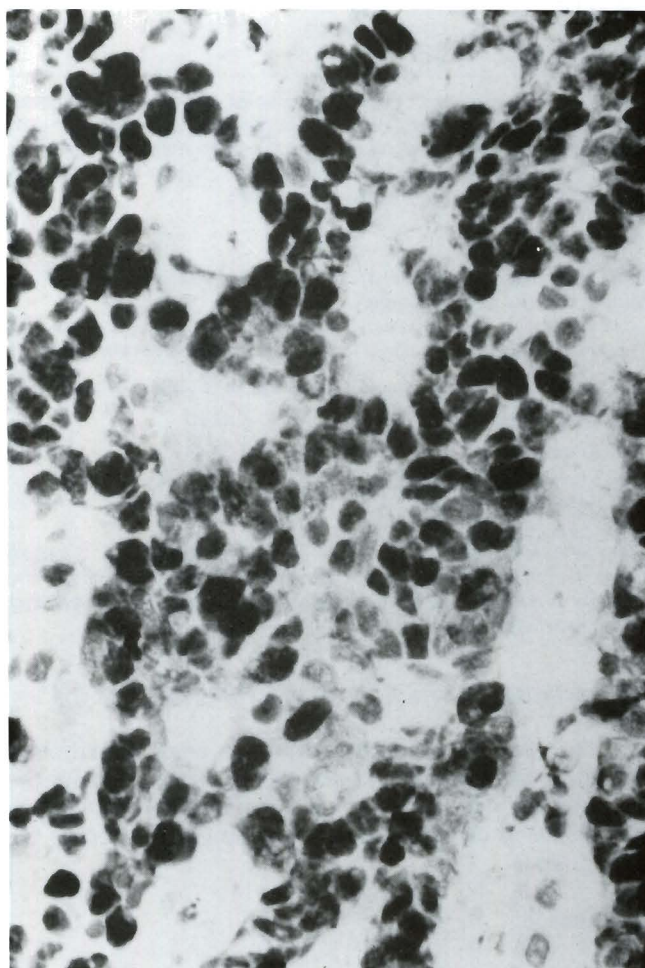


Fig. 5. Immunoperoxidase staining with Ki-67 antibody. Adenocarcinomas showing grade 3 proliferative index (positive nuclei > 40%) x 234

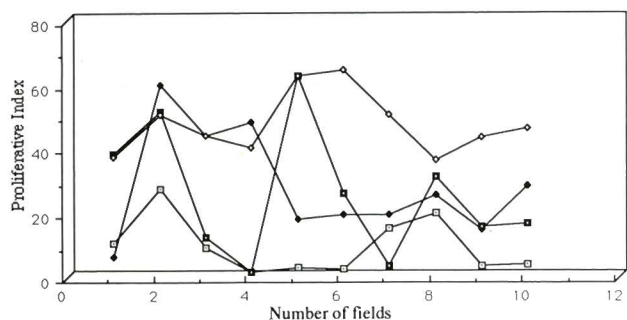


Fig. 6. Proliferative index (Pi) in four cases. Each line represents one case. In these four cases, distribution of Pi was erratic.

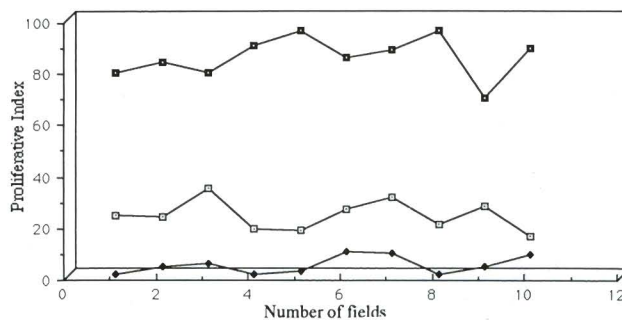


Fig. 7. Proliferative index (Pi) in three cases. Each line represents one case. In these three cases, distribution of Pi was relatively even.

### Antibody Ki-67 in gastric carcinomas

**Table 1.** Comparison of Ki-67 immunoreactivity in groups A, B and C; correlation with Ki-67 derived grade : number of cases in each category.

CARCINOMAS	Proliferation index		Grade 1	Grade 2	Grade 3
	m	sd			
GROUP A	18.482	15.823	8	2	2
GROUP B	25.842	25.076	5	2	1
GROUP C	43.249	38.423	1	1	2
ALL TUMOURS	23.33	23.54	14	5	5

m, mean value measured in the different fields; sd, standard deviation; group A, carcinomas with metastases; group B, carcinomas with paragastric lymph node extension; group C, carcinomas localized to the gastric wall; grade 1, proliferation index <20%; grade 2, proliferation index 20-40%; grade 3, proliferation index >40%.

**Table 2.** Comparison of Ki-67 immunoreactivity in carcinomas respectively with and without serosal extension; correlation with Ki-67 derived grade : number of cases in each category.

SEROSA	Proliferation index		Grade 1	Grade 2	Grade 3
	m	sd			
S+	25.44	26.206	12	3	4
S-	23.428	16.237	2	2	1
ALL TUMOURS	23.33	23.54	14	5	5

m, mean value measured in the different fields; sd, standard deviation; S+, carcinomas infiltrating the serosa; S- carcinomas without serosa extension; grade 1, proliferation index <20%; grade 2, proliferation index 20-40%; grade 3, proliferation index >40%.

**Table 3.** Comparison of Ki-67 immunoreactivity according to the histological type and degree of differentiation. Correlation with Ki-67 derived grade : number of cases in each category.

CARCINOMAS	Proliferation index		Grade 1	Grade 2	Grade 3
	m	sd			
ADENOSQUAMOUS	43.67		0	0	1
MUCINOUS	36.84	33.46	1	2	1
UNDIFFERENTIATION AND POOR DIFFERENTIATION	13.018	9.277	8	2	0
MODERATE AND GOOD DIFFERENTIATION	31.157	26.196	5	1	3

m, mean value measured in the different fields; sd, standard deviation; grade 1, proliferation index <20%; grade 2, proliferation index 20-40%; grade 3, proliferation index >40%.

with metastases and serosa invasion was of grade 3 (Pi = 44%).

The four mucoid type tumours were distributed within the three grades; one of them showed the highest Pi of all the cases with a value of 94.11%. The three carcinomas of cardia region (2 in group A, 1 in group C) were grade 1.

#### Discussion

Various recent methods have been used to determine a more precise prognosis in gastric carcinomas, with the aid of parameters differing from classical histological criteria. Sometimes two or more parameters have been investigated: DNA ploidy (determined by flow cytometry) and Bromodeoxyuridine labelling index (Brd-U Li) (Yonemura et al., 1988, 1990a; Ohya et al., 1990); DNA ploidy, Brd-U Li and Ki-67 reactivity (Yonemura et al., 1990b); epidermal growth factor receptor (EGF-R) status and Brd-U Li (Yonemura et al., 1989). One parameter has also been studied: DNA ploidy pattern (Aretxabala et al., 1988); Brd-U Li in early gastric cancers (Kamata et al., 1989); and proliferating cell nuclear antigen (PCNA) also known as cyclin (Robbins et al., 1987). Search for Brd-U Li, Ki-67 reactivity, EGF-R status and PCNA were performed by immunohistochemical staining procedures with standard microscopy count.

Our study on 24 gastric carcinomas showed that our

cases were differently distributed compared with those of Yonemura et al. (1990b); in this latter study, the median (22%) was nearly the same as the mean value, whereas our median (15.4%) was much lower than the mean value (23%); these results, in the present study, probably corresponded to a more heterogeneous tumour cell population.

We did not observe any correlation between histological type and degree of differentiation as Yonemura et al. (1990b) did. These differences between the distribution of Pi grades according to the presence or absence of serosa invasion were not significant too. Moreover, no correlation was observed between the Pi level and carcinomatous vessel invasion, lymph node and distant metastases: these findings are not in agreement with the previous published results (Yonemura et al., 1990b) which showed a higher Ki-67 labelling from tumours with lymph node extension and vessel invasion.

Our other results were similar to those obtained in multiple parameter studies (Yonemura et al., 1988; Kamata et al., 1989): there was no correlation between DNA ploidy and histological type. The incidence of vessel and lymph node invasion and the rate of advanced cases were significantly higher in aneuploid than in euploid tumours.

When DNA ploidy and Brd-U Li were used in the same study of gastric carcinomas, there was a good correlation between these two parameters but they were

independent prognostic parameters, by analysis with the Cox proportional hazard's model, and both were associated with poor prognosis and survival (Yonemura et al., 1988; Ohyama et al., 1989). With respect to the DNA ploidy patterns, the mean Ki-67 labelling rate of aneuploid tumours was significantly higher than that of diploid tumours (Yonemura et al., 1990a,b). However, by flow cytometry analysis, used to determine the overlapping cell distribution of DNA, aneuploid clones and nuclear debris may interfere with the analysis of proliferative activity of tumour cells (Yonemura et al., 1990a,b).

In spite of the small number cases, our study emphasizes the three following points: 1) semi-automated analysis by two observers was a more precise study than that of one observer with standard microscopy count as previously reported; 2) as shown by Porschen et al. (1991), a great heterogeneity of proliferative activity within the same tumour and from one case to another is observed, as we also previously demonstrated in colorectal tumours (Hoang et al., 1989); 3) the absence of correlation between the Ki-67 labelling rates and histological type, wall invasion, vessel invasion or metastases was observed in gastric as well as in colorectal carcinomas (Hoang et al., 1989).

From these data, Ki-67 antibody labelling enables the detection of larger numbers of cycling cells than the S-phase fraction measured by Brd-U Li and does not need the external administration of mitogenic substances such as Brd-U. Moreover, by immuno-histochemistry, Ki-67 labelling offers a sensitive, simple and non-toxic method with an objective quantitative and topographical analysis of tumour sections, by opposition to cellular suspensions with flow cytometry.

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