Proliferative activity in pancreatic intraepithelial neoplasias of chronic pancreatitis resection specimens: detection of a high-risk lesion*

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Patients with chronic pancreatitis have a markedly increased risk of pancreatic cancer compared with general population. Mechanism of the increased risk is not completely known. The current progression model for pancreatic ductal adenocarcinoma proposes the progression from normal ductal epithelium through a series of lesions called pancreatic intraepithelial neoplasias (PanINs) to invasive cancer. These lesions are frequently seen in chronic pancreatitis tissue. Proliferative activity in PanINs of chronic pancreatitis tissue has not been separately studied using the current nomenclature. Our study included 36 chronic pancreatitis resection specimens. A total number of 106 PanINs found within 32 resection specimens was histologically graded and then immunolabeled using a monoclonal antibody against Ki-67 that is expressed in dividing cells. The Ki-67 labeling indices in the increasing grades of PanINs were counted with following results: PanIN-1A, 0.77%; PanIN-1B, 3.26%; PanIN-2, 14.68%; and PanIN-3, 25.4%. The difference in Ki-67 labeling indices among these types of lesions was statistically significant ($p<0.001$, t-test). These results correlate with known genetic alterations found in chronic pancreatitis, especially with p16 inactivation that was recently described in PanINs arising in patients with chronic pancreatitis. Moreover, our findings support the currently accepted pancreatic progression model and Ki-67 immunohistochemistry might represent an efficient tool for an identification of a high-risk lesion.

Key words: pancreatic intraepithelial neoplasia, proliferative acitivity, chronic pancreatitis

Chronic pancreatitis represents an inflammatory disorder that predisposes the affected patients to pancreatic ductal adenocarcinoma. Patients with chronic pancreatitis have a markedly increased risk of pancreatic cancer compared with the general population. The lifetime risk of developing pancreatic cancer in patients with sporadic chronic pancreatitis is estimated to be 1.8% after 10 years and 4% after 20 years, respectively [13, 14]. Patients suffering from hereditary pancreatitis, caused by germline mutations in the cationic trypsinogen gene (PRSS1), have an estimated 50- to 70-fold increased relative risk of pancreatic cancer [15, 21].

The mechanism by which this risk is mediated is not completely known. Infiltrating ductal adenocarcinoma is thought to arise from non-invasive intraductal dysplastic lesions called pancreatic intraepithelial neoplasias (PanINs), [6]. The progression from normal ductal epithelium to pancreatic cancer through the spectrum of PanIN lesions is characterized by the sequence of genetic alterations including activating K-ras point mutations, overexpression of HER-2/neu, and inactivation of p16, p53, DPC4, and BRCA2 tumor suppressor genes [7, 8]. Additional evidence for this currently accepted model of progression [6] brought the study of KLEIN et al that found a direct correlation between proliferative activity and dysplasia in PanINs adjacent to the structures of infiltrating ductal adenocarcinoma [10]. It has been found that PanINs are more frequently present in patients with chronic pancreatitis than in the general population [22]. The proliferative activity in PanINs of

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chronic pancreatitis tissue using the currently accepted nomenclature and diagnostic criteria has not been separately studied yet, although a demonstration of proliferative activity might represent a simple way to identify PanIN lesions at high-risk for subsequent development of invasive pancreatic cancer. The Ki-67 antigen is a nuclear protein expressed in dividing cells [5]. Proliferation rate can be easily evaluated using antibodies against Ki-67 and standard immunohistochemical approaches [3]. Therefore, we examined the expression of Ki-67 in the panel of PanIN lesions within the chronic pancreatitis resection specimens.

Material and methods

Thirty six chronic pancreatitis resection specimens from patients who had undergone pancreaticoduodenectomy at the Faculty Hospital Brno, Masaryk Memorial Cancer Institute, Surgical Hospital Delta and Bakes's Surgical Hospital between 2000–2003 were examined. Only cases of sporadic pancreatitis were included into the study. There were 33 men and 3 women with a mean age of 49 years (range, 32–63 years). Pancreatic resection specimens were fixed in 10% neutral-buffered formalin for 24 hours. Multiple hematoxylin and eosin-stained slides of pancreatic tissue from each case were examined by light microscopy to identify the samples containing PanIN lesions. Each selected PanIN lesion was graded by two observers independently and the disagreement was resolved by consensus. The grading was performed according to current accepted nomenclature and diagnostic criteria [6]. Briefly, PanIN-1A displayed flat mucinous epithelium with no dysplasia, PanIN-1B showed papillary mucinous epithelium again with no dysplasia. PanIN-2 lesions are characterized by similar architectural features to PanIN-1B but with mild to moderate nuclear dysplasia. PanIN-3 lesions demonstrate severe atypia and has in the past been called carcinoma *in situ*. Fifty one separate paraffin blocks from thirty two chronic pancreatitis resection specimens containing PanIN lesions were selected for the immunohistochemical study. Immunohistochemistry was performed on 4 μm thick sections cut from the selected formalin-fixed paraffin-embedded tissue blocks. Tissue sections were deparaffinized in xylene and rehydrated through the series of alcohols. Antigen retrieval was achieved by microwave heating in citrate buffer (pH 6.0) for 4 minutes at 120 °C. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol. Tissue sections were incubated with a mouse monoclonal antibody against Ki-67 (MIB-1, DAKO) at a dilution of 1:100. EnVision™ + detection system (DAKO) was used according to manufacturer's instructions and than developed using 3,3'-diaminobenzidine as substrate. The tissue sections were finally counterstained with hematoxylin and mounted. Sections without the primary antibody were used as negative controls. The immunolabeled cells of germinal centers of the adjacent lymph nodes served as internal positive controls. Evaluation of ductal cells labeled with Ki-67 was performed using light microscopy under a 40x objective. For each selected PanIN lesion either the entire duct was analyzed or the minimum of 200 cells was counted. The cells with a visible granular nuclear brownish immunostaining were considered to be positively labeled. For each PanIN lesion, the Ki-67 labeling index was counted (the number of positive cells/total cells x 100). Statistical analysis was performed using SYSTAT statistical package (SPSS Inc. USA).

Results

PanIN lesions were identified in 32 from 36 chronic pancreatitis resection specimens, four of the pancreata did not have any duct lesions (11.11%). Total number of 106 PanINs was identified within the 32 pancreatic resection specimens. The majority of duct lesions were low grade PanIN-1A (n=55; 51.89%) and PanIN-1B (34; 32.08%). One resection specimen contained a full spectrum of PanINs. Nine resection specimens contained only PanIN-1A lesions, 13 resection specimens PanIN-1A and/or 1B lesions. Very few ductal epithelial cells were positively labeled in PanIN-1A lesions (Fig. 1). The Ki-67 labeling index within the 55 cases of PanIN-1A lesion was in range 0–2.4 with a mean of 0.77. The Ki-67 labeling index was slightly higher in 34 PanIN-1B lesions (Fig. 1). This index was in range 1.1–7.3 with a mean of 3.26. There were 16 PanIN-2 lesions (n=16; 15.09%) identified within 9 chronic pancreatitis resection specimens. No occasional PanIN-2 lesions were found, all specimens with an identified PanIN-2 lesion displayed also some low grade lesions. The labeling index was in range 9.3–19.1 with a mean of 14.68 in PanIN-2 lesions (Fig. 1). The increase in the labeling indices was statistically significant between PanIN-1A and PanIN-1B lesions, and between PanIN-1B and PanIN-2 lesions, respectively ($p<0.001$ in t-test). There was only one PanIN-3 lesion (Fig. 1) identified within all studied chronic pancreatitis resection specimens (n=1; 0.94%). Additional PanIN-2 lesions and the spectrum of low grade PanIN lesions were identified in this case. The Ki-67 labeling index in the PanIN-3 lesion was 25.4. The

Table 1. Ki-67 labeling indices in different types of PanIN lesions

Type of the lesion	Number of cases	Ki-67 labeling index range	Ki-67 labeling index mean ± SD	Statistic significance (t-test)
PanIN-1A	55	0–2.4	0.77 ± 0.62	
PanIN-1B	34	1.1–7.3	3.26 ± 1.41	p<0.001
PanIN-2	16	9.3–19.1	14.68 ± 2.67	p<0.001
PanIN-3	1	25.4	25.40	

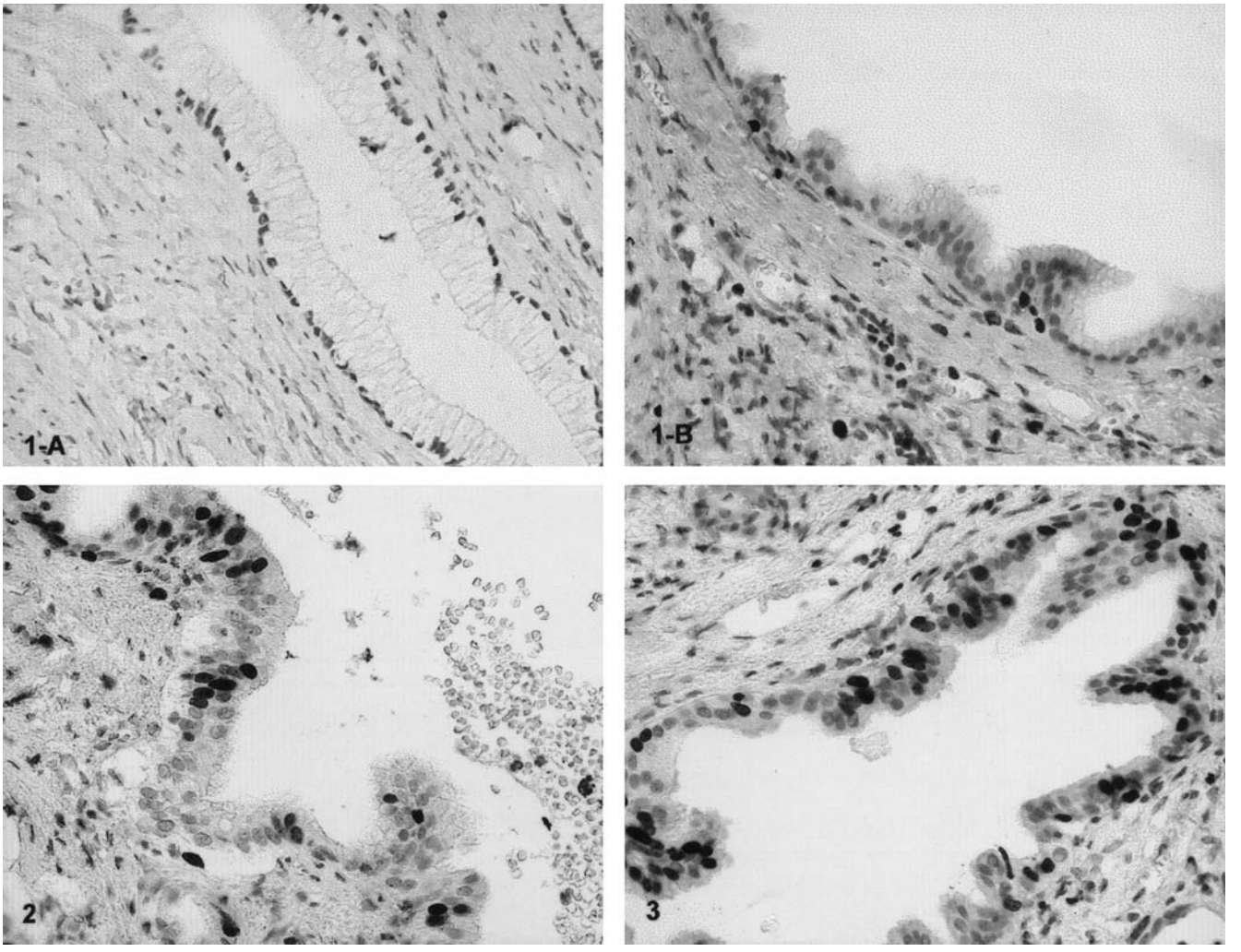

Figure 1. Ki-67 immunohistochemistry in chronic pancreatitis associated PanINs (original magnification x400): the figures demonstrate a growing nuclear expression of Ki-67 with increasing grade of dysplasia in PanINs. PanIN-2 and PanIN3 show significantly higher expression of Ki-67 as compared with low grade PanINs (1A and 1B).

results are summarized in Table 1. The graph (Fig. 2) demonstrates the growing proliferative activity in parallel with the grade of dysplasia.

Discussion

The Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G_1, S, G_2 and M-phase), but it is absent in resting cells (G_0 phase), [5]. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cells enter the non-proliferative state [17], and there appears to be no expression of Ki-67 during DNA repair processes [11]. The clone MIB-1, used in our study, is intended for use in immunohistochemistry. With hundreds of citations, the MIB-1 antibody has now been established as a reference monoclonal mouse antibody for the demonstration of Ki-67 antigen in formalin-fixed, paraffin-embedded specimens [3]. The expression of MIB-1 epitope of Ki-67 has been extensively studied in many human malignancies [1, 9], but also in precursors of these invasive lesions, for example, in cervical intraepithelial neoplasias [2], in precursors of adenocarcinomas of the human colon [18] or in prostatic intraepithelial neoplasias [19]. In pancreas, the proliferative acitivity in intraductal papillary-mucinous neoplasms, which can give rise to infiltrating adenocarcinoma in a minority of cases, has been studied [20]. However, the majority of infiltrating ductal adenocarcinomas seem to develop from noninvasive precursor lesions in the pancreatic ducts called PanINs. KLEIN et al studied the Ki-67 expression in PanINs adjacent to the structures of invasive ductal adenocarcinomas and found

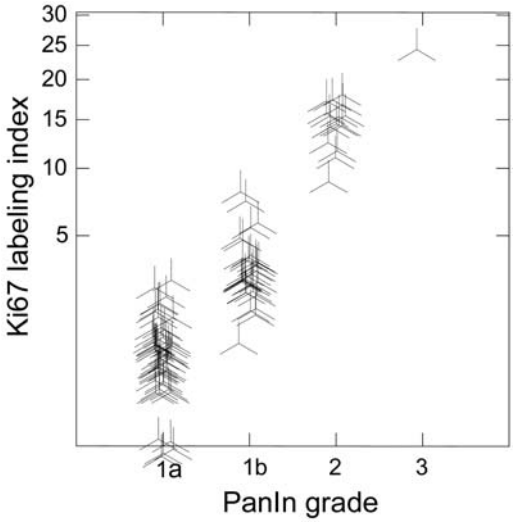

Figure 2. The graph demonstrating the growing values of the Ki-67 indices in different grades of PanINs.

direct correlation between the proliferative activity and the grade of dysplasia in PanINs [10]. Even if the precise mechanism of increased risk of chronic pancreatitis for pancreatic cancer is not explained, it was suggested that PanINs are present more often in patients with chronic pancreatitis than in general population [22] and the higher risk of the development of pancreatic cancer in patients with chronic pancreatitis was also repeatedly demonstrated [13, 14]. Our study included the chronic pancreatitis resection specimens to describe the frequency of PanINs within chronic pancreatitis tissue and to evaluate the proliferative activity in PanIN lesions of this tissue to be able to demonstrate the correlation between proliferative activity and the grade of dysplasia in PanINs of chronic pancreatitis. In agreement with previously published studies [16], the majority of duct lesions in chronic pancreatitis is represented by low grade PanIN lesions. Of 106 duct lesions identified within chronic pancreatitis resection specimen, 51.89% were PanIN-1A, 32.08% were PanIN-1B, 15.09% were PanIN-2 and 0.94% were PanIN-3 lesions. The only one PanIN-3 lesion was associated with PanIN-2 and PanIN-1 lesions within the particular resection specimen. Direct correlation between the proliferative activity and the grade of dysplasia in pancreatic intraepithelial neoplasias of chronic pancreatitis tissues revealed in our study is in agreement with the studies suggesting the p16 inactivation to be an event that may contribute to the predisposition of patients with chronic pancreatitis to develop pancreatic ductal adenocarcinoma. The tumor suppressor gene INK4A encodes cyclin-dependent kinase inhibitor $p16^{INK4A}$ – a key regulator of cell cycle progression at the G1/S phase transition point. Thus, inactivation of p16 contributes to increased proliferative activity [12]. ROSTY et al described the growing incidence of the loss of p16 expression parallel with the grade of PanIN lesions in chronic pancreatitis tissues [16]. Previously, the study of GERDES et al revealed the p16 alterations in a considerable number of low grade PanIN lesions in chronic pancreatitis tissues and suggested this abnormality as an indicator for high-risk precursor for pancreatic cancer [4].

In summary, we demonstrated direct correlation between the proliferative activity and the grade of dysplasia in PanIN lesions of chronic pancreatitis resection specimens. This finding supports the recently accepted pancreatic progression model [6]. Moreover, the finding of this relationship between the level of Ki-67 index and the grade of dysplasia might be used in an easy immunohistochemical identification and diagnostics of high-risk precursor that might progress to cancer.

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