Relationship between K-ras mutation and the expression of p21$^{WAF1/CIP1}$ and p53 in chronic pancreatitis and pancreatic adenocarcinoma*

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Overexpression of p21$^{WAF1/CIP1}$ was recently described as an early event in the development of pancreatic intraepithelial neoplasia. Since activating K-ras mutations are described in more than 80% of pancreatic cancers and are known to increase intracellular levels of p21$^{WAF1/CIP1}$ in experimental models, the possible role of activating K-ras mutations in an induction of the p21$^{WAF1/CIP1}$ expression was investigated in our study. We examined 71 surgical specimens, 29 of chronic pancreatitis and 42 of invasive ductal adenocarcinoma both having a large spectrum of PanIN (pancreatic intraepithelial neoplasia) lesions. Expression of p53 and p21$^{WAF1/CIP1}$ was examined immunohistochemically and codon 12 K-ras mutational analysis was performed using the very sensitive mutant-enriched PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis. Our study demonstrated the overexpression of p21$^{WAF1/CIP1}$ as an early event in the development of pancreatic intraepithelial neoplasia in the group of chronic pancreatitis and invasive adenocarcinoma as well. Overexpression of p21$^{WAF1/CIP1}$ increased progressively from normal ducts through the spectrum of PanIN lesions to invasive carcinomas. The p53 overexpression increased again progressively according to the severity of the lesion and seems to be a later event in the development of pancreatic intraepithelial neoplasia if compared to p21$^{WAF1/CIP1}$ expression. Our results confirmed also the possible p53 independent p21$^{WAF1/CIP1}$ expression in some PanIN2, PanIN3 lesions and invasive carcinomas. K-ras mutations were not revealed in samples with only low grade PanIN lesions (PanIN1a and PanIN1b). K-ras mutations were detected in 69,4% adenocarcinomas and in only one case of chronic pancreatitis. Two codon 12 K-ras positive pancreatic carcinomas showed K-ras mutations in the surrounding normal pancreatic tissue. In adenocarcinomas, no statistically significant correlation was found between K-ras mutational status and p21$^{WAF1/CIP1}$ and p53 expression, respectively. The possible role of activating K-ras mutations in an induction of p21$^{WAF1/CIP1}$ expression was not confirmed in this study.

Key words: Pancreatic intraepithelial neoplasia, K-ras, p21$^{WAF1/CIP1}$, p53.

Pancreatic cancer is the fifth leading cause of cancer-related death in Western countries, second only to colon cancer among malignancies of the digestive tract. Despite improved diagnostic and therapeutic modalities, pancreatic cancer still has a very poor prognosis with a 5-year survival less than 10% [22]. Etiology of pancreatic cancer is uncertain, but several factors including alcohol, cigarette smoking, high-fat diet and nitrosamines exposure are thought to increase the risk of pancreatic malignancy [16, 43]. The risk of pancreatic cancer in patient suffering from chronic pancreatitis has been demonstrated in several reports [24, 33]. Pancreatic cancer is usually advanced at the time of presentation [27] and the detection of premalignant pancreatic lesions and earlier detection of malignant pancreatic lesions may improve the clinical outcome of the disease using current treatment modalitites.

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The current accepted model of pancreatic cancer progression from normal ductal and ductular epithelium to cancer includes a series of lesions termed pancreatic intraepithelial neoplasia (PanIN), [19, 21]. Recognition of molecular pathology and natural history of early pancreatic neoplastic ductal lesions could provide an effective scope for new diagnostic and treatment approaches.

It has become clear that the activation of oncogenes is critical for the transformation of normal cells to tumor cells. One of the most frequent type of proto-oncogene activation is the ras proto-oncogene activation. The family of ras oncogenes has three well-characterized members (H-ras, K-ras and N-ras). The K-ras proto-oncogene located at chromosome 12p codes for a 21-kDa GTP binding protein ($p21^{ras}$) that is involved in the signal transduction of activated tyrosine kinase cell membrane receptors to downstream signal cascades [5]. Activation of this gene usually results from point mutations at codons 12, 13 or 61, causing the oncogenic ras protein to be in a permanently active GTP-bound state. Mutations of the K-ras proto-oncogene at codon 12 were reported in 70 to 100% of cases of pancreatic adenocarcinomas [1, 31, 42] and in 0 to nearly 40% of patients with chronic pancreatitis [13, 14, 38]. In cases of pancreatic neoplasia, other types of ras point mutations are rare [1, 41], while codon 61 point mutations in K-ras and point mutations in N-ras occur with some frequency in colonic adenocarcinomas [6, 44].

The p53 tumor suppressor gene plays a crucial role in cell cycle regulation. The p53 gene product has been implicated both in the G_1 cell cycle arrest [25, 26] and in programmed cell death in response to a variety of cellular stress signals such as DNA damage, hypoxia, heat shock, oncogene activation or metabolic changes [32]. This protein is inactivated in many human neoplasms by point mutation in the encoding p53 gene followed by a disruption of its ability to function at DNA damage checkpoint [18]. 50–75% pancreatic adenocarcinomas were reported to carry mutations in p53 gene [3, 9, 22].

$p21^{WAF1/CIP1}$ is known as an inhibitor of cyclin dependent kinase and acts to prevent pRb phosphorylation by inhibiting activation of cyclinE/cdk2 complexes that are required for Rb phosphorylation [29, 40]. Expression of $p21^{WAF1/CIP1}$ is regulated by number of signaling molecules, including p53 [12, 15]. $p21^{WAF1/CIP1}$ is induced by wild-type but not mutant p53 and is considered to be a downstream mediator of the growth suppressing and apoptosis promoting function of the wild type of p53 [11]. Tumors lacking wild-type p53 are expected not to express $p21^{WAF1/CIP1}$ if only functional wild-type p53 is able to induce $p21^{WAF1/CIP1}$ expression but data obtained from some reports suggest that $p21^{WAF1/CIP1}$ expression in pancreatic adenocarcinoma may also be induced by a p53-independent pathway [4, 10]. Biankin et al (2001), having studied the spectrum of PanIN lesions adjacent to the structures of invasive ductal adenocarcinoma suggested the overexpression of $p21^{WAF1/CIP1}$ as an early event in the development of pancreatic intraepithelial neoplasia and discussed the possible role of activating K-ras mutation in the induction of $p21^{WAF1/CIP1}$ expression [4]. There are no published data documentating the $p21^{WAF1/CIP1}$ expression in chronic pancreatitis. Our study included also surgical specimens of chronic pancreatitis both with and without the spectrum of PanIN lesions to study $p21^{WAF1/CIP1}$ expression, and its relationship to the p53 expression and to the mutational status of K-ras.

Material and methods

The studied population consisted of surgical specimens from 71 patients who underwent pancreaticoduodenectomy at the Faculty Hospital Brno, Masaryk Memorial Cancer Institute and Surgical Hospital Delta between 2000 and 2002. Twenty nine specimens with the diagnosis of chronic pancreatitis (22 alcoholic and 7 idiopathic) both with and without PanIN lesions and 42 specimens with the diagnosis of invasive ductal adenocarcinoma were included into the study. These specimens were fixed in 10% neutral-buffered formalin for 24 hours. Hematoxylin-eosin (HE) staining and immunohistochemistry (IH) were performed on 4 μm thick sections of formalin-fixed paraffin embedded tissue. HE sections were examined to identify the samples containing normal ducts and/or PanIN lesions and/or invasive adenocarcinoma, and these were selected for the immunohistochemical study. For immunohistochemistry, sections were deparaffinized in xylen and rehydrated through a series of alcohol. Antigen retrieval was performed in the microwave (Milestone) by heating in citrate buffer at pH 6.0 for 4 minutes at 120 ˚C. Endogenous peroxidase activity was quenched in 1.5% hydrogene peroxide in methanol. The two specific monoclonal antibodies against $p21^{WAF1/CIP1}$ (clone SX 118, DAKO, dilution 1:50) and p53 (clone DO-1, against both wild and mutant p53, Novocatra, dilution 1:2000) were used for immunohistochemical staining. Because of using very sensitive visualization system, the dilution of primary antibody, in which the immunostaining in the cells of cytotrophoblast is negative, was used for p53 immunohistochemistry (see explanation in the discussion). Streptavidin-biotin peroxidase detection system was used in accordance with manufacturer's instructions (Vectastain Elite Kit, Vector Laboratories) and than visualized using 3,3'-diaminobenzidine as a substrate (Sigma). The slides were counterstained with hematoxylin. Tissue sections with a known p53 status were used as positive and negative controls.

Slides were evaluated by two separate observers and the discrepancies were resolved by consensus. Up to 6 areas of the particular lesions were examined per resection specimen. The lesions were scored as a percentage of nuclei

positive in the lesion and the average score of the particular lesion was counted per case. More than 5% nuclei positive both in p53 and p21$^{WAF1/CIP1}$ immunohistochemistry were scored as 1+, more than 15% as 2+. Less than 5% nuclei positive were scored as 0.

PCR was performed on DNA isolated from the serial sections of the same paraffin embedded tissue blocks as for the immunohistochemical analysis in patients with the diagnosis of chronic pancreatitis. The mutational analysis of the tissue from patients with invasive ductal adenocarcinoma was performed on the sections from areas with histologically verified invasive adenocarcinoma and we also examined the tissue adjacent to the tumorous tissue with the histological signs of chronic pancreatitis only and with the spectrum of PanIN lesions. After deparaffinization, the DNA was isolated from five 5 μm thick tissue sections from the selected tissue blocks using DNAeasy^{TM} Tissue Kit (Qiagene) following the manufacture's instructions. After cutting the tissue sections for PCR analysis one more serial HE tissue section was prepared to check the presence of diagnostic structures and to exclude the possibility of structures of invasive carcinomas. Concentration and quality of isolated DNA was determined using standard spectrophotometry and agarose gel electrophoresis.

Point mutations in codon 12 of the K-ras oncogene were detected by a combination of mutant-enriched polymerase chain reaction and restriction fragment length polymorphism analysis according to HRUBAN et al [20], with a minor modifications. A restriction cleavage (MvaI, Takara) of wild-type codon 12 K-ras allele was performed between two rounds of nested PCR. The second amplification PCR reaction runs preferentially in samples with a mutated variant of this oncogene. The result of the second restriction analysis was compared with an unrestricted product of the second PCR using electrophoresis on 3% agarose gel containig ethidium bromide. The size of the PCR fragments remaining unchanged before and after the second restriction indicates the presence of mutation in the codon 12 K-ras oncogene (a mutation at MvaI recognition site).

Statistical analysis was performed using the analysis of contingency tables (Pearson's chi-square) in SYSTAT statistical package (SPSS Inc., USA).

Twenty nine specimens with chronic pancreatitis both with and without PanIN lesions were examined (3 with no signs of PanIN lesions, 5 with PanIN1a lesions, 8 with PanIN1b lesions, 11 with PanIN2 lesions and 2 PanIN3 lesions). The p53 and p21$^{WAF1/CIP1}$ expressions were evaluated in normal ducts of 24 pancreatic surgical specimens, in PanIN1a lesions of 23 specimens, in PanIN1b lesions of 21 specimens, in PanIN2 lesions of 13 specimens and in PanIN3 lesions of 2 specimens in the group without invasive carcinoma.

We also examined 42 specimens with histological diagnosis of invasive ductal adenocarcinoma. We evaluated not only the areas of invasive adenocarcinoma but also the spectrum of PanIN lesions adjancent to the structures of invasive adenocarcinoma. Not every surgical specimen contained the full spectrum of PanIN lesions. Some lesions were lost by serial sectioning of the tissue due to repeated immunostaining. We examined normal ducts in 33 cases, PanIN1a lesions in 30 cases, PanIN1b lesions in 31 cases (28 in p53 IH), PanIN2 lesions in 29 (28 in p53 IH) cases, PanIN3 lesions in 33 (35 in p53 IH) cases and 42 cases of invasive adenocarcinoma.

Totally, normal ducts, PanIN1a, PanIN1b, PanIN2, PanIN3 and adenocarcinomas in both groups with or without invasive adenocarcinoma were examined in 57, 53 (54 in p53 IH), 52 (49 in p53 IH), 42 (41 in p53 IH), 35 (37 in p53 IH) and 42 cases, respectively.

Results

Expression of p21$^{WAF1/CIP1}$ (results summarized in Tab. 1): The proportion of p21$^{WAF1/CIP1}$ expression in normal ducts, PanIN1a, 1b, 2, 3 lesions and carcinomas increased progressively with the severity of the lesion with staining not exceeding 1+ in normal ducts and staining 2+ in the most cases of PanIN3 lesions and carcinomas (Fig. 1).

Expression of p53 (results summarized in Tab. 2): The proportion of p53 expression in normal ducts, PanIN1a, 1b, 2, 3 lesions and carcinomas (Fig. 2) increased again progres-

Table 1. Expression of p21$^{WAF1/CYP1}$ in pancreatic ducts, PanIN lesions and pancreatic adenocarcinomas

	Negative	Staining 1+	Staining 2+
Normal ducts	51	6	0
PanIN1a	36	15	2
PanIN1b	22	25	5
PanIN2	3	23	16
PanIN3	1	7	27
Ca	1	9	32

p<0.001 (Pearson's chi-square)

Table 2. Expression of p53 in pancreatic ducts, PanIN lesions and pancreatic adenocarcinomas

	Negative	Staining 1+	Staining 2+
Normal ducts	57	0	0
PanIN1a	53	1	0
PanIN1b	46	3	0
PanIN2	26	9	6
PanIN3	13	8	16
Ca	12	7	23

p<0.001 (Pearson's chi-square)

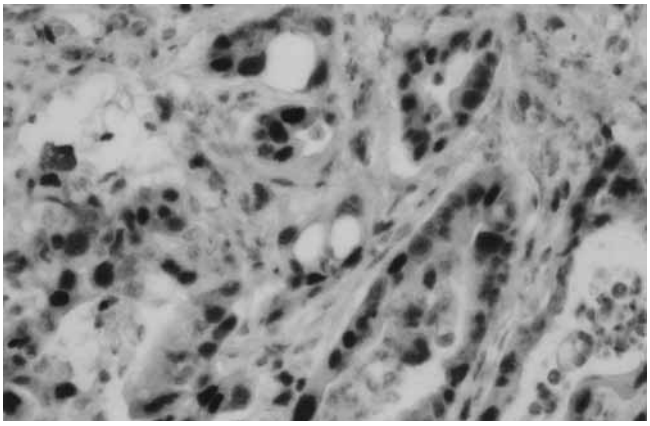

Figure 1. Overexpression of p21$^{WAF1/CIP1}$ in an invasive ductal adenocarcinoma.

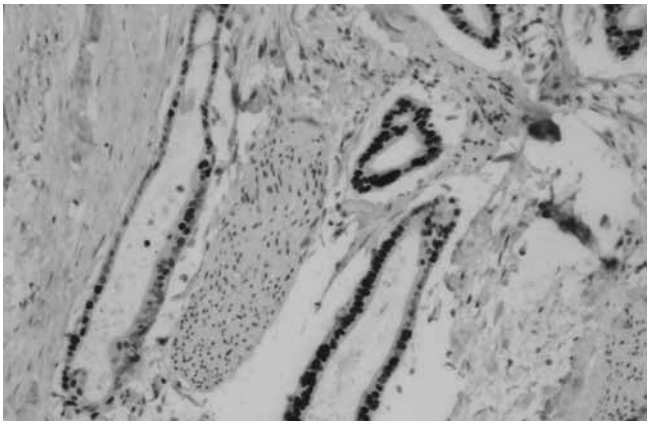

Figure 2. Overexpression of p53 in an invasive ductal adenocarcinoma with perineural spreading.

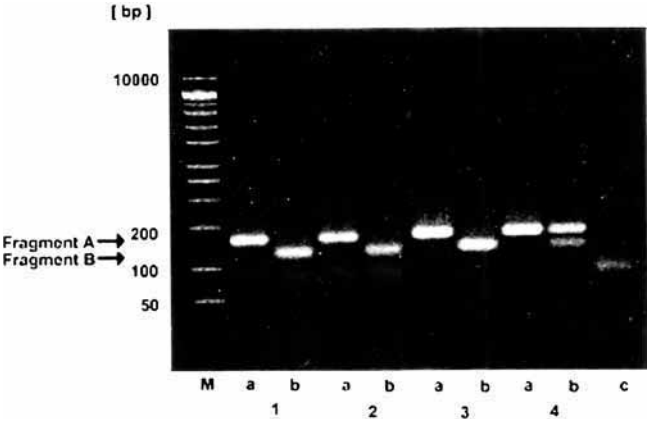

Figure 3. Electrophoresis of RFLP analysis of K-Ras PCR amplicon on 3% agarose gel (1xTAE) containig ethidium bromide (1 μg/ml). Lines: 1,2,3 – PCR samples without mutation in codon 12 of K-ras; 4 – PCR sample with point mutation in codon 12 of K-ras; a – Unrestricted PCR product after second PCR reaction; b – Result of MvaI restriction of PCR product after second PCR reaction; M – DNA size standard; c – PCR negative control (without DNA); Fragment A – MvaI unrestricted PCR fragment bearing codon 12 K-ras mutation (135 bp); Fragment B – MvaI restriction of PCR fragment with wild type K-ras alleles only (107bp).

sively with no staining in normal ducts and staining in PanIN1 lesions not exceeding 1+.

Codon 12 K-ras mutational analysis (results summarized in Tab. 3): K-ras mutational analysis revealed codon 12 K-ras mutation in one case from the chronic pancreatitis group (1/28; 3.6%). Histological examination revealed an area of PanIN3 lesion within the PCR examined specimen in this only one positive case. Mutational analysis was not performed in one case from the group of chronic pancreatitis due to DNA isolation failure.

Mutational analysis was performed in 36 invasive adenocarcinomas. Codon 12 K-ras mutation was detected in 25/36 (69.4%) cases (Fig. 3). Mutational analysis of non-tumorous tissue adjacent to the structures of invasive adenocarcinomas was performed in 33 cases and in 2/33 (6.1%) cases the codon 12 K-ras mutation was detected. Histological evaluation of serial sections from the examined block revealed high grade PanIN lesions (PanIN2 or PanIN3) in both positive cases and the absence of invasive component was excluded by histological examination of serial HE tissue sections. DNA isolation failed in six specimens in the group of invasive carcinomas.

Cumulative results. The proportion of pancreas specimens that overexpressed p21$^{WAF1/CIP1}$ in normal ducts, PanIN1a, 1b, 2, 3 and carcinomas increased progressively ($p<0.001$). Accumulation of p53 was not detected in normal ducts. In PanIN1a and PanIN1b lesions, the accumulation of p53 was not detected on 2+ level at all. Only one PanIN1a lesion and three PanIN1b lesions accumulated p53 on 1+ level. There was a significant increase in accumulation of p53 in PanIN2, PanIN3 lesions and in adenocarcinomas ($p<0.001$). Five cases accumulated both p21$^{WAF1/CIP1}$ and p53 on 2+ levels in PanIN2 lesions, 14 cases in PanIN3 lesions and 18 cases in invasive adenocarcinoma. p53 independent expression of p21$^{WAF1/CIP1}$ is suspected in these cases (relationship between expression of p21$^{WAF1/CIP1}$ and p53 in pancreatic adenocarcinoma is summarized in Tab. 4).

We did not find codon 12 K-ras mutation in samples with normal ducts, PanIN1a and Pan1b lesions. Codon 12 K-ras mutational analysis revealed mutations in 25/36 (69.4%) of invasive adenocarcinomas. Mutational analysis of non-tumorous tissue showing the spectrum of PanIN lesions adjacent to the structures of carcinomas revealed codon 12 K-ras mutation in 2/33 (6%) cases. The only one codone 12 K-ras mutation was found in a tissue from sample with histologically confirmed PanIN3 lesion in a chronic pancreatitis group. No statistically significant correlation between K-ras mutational status and p21$^{WAF1/CIP1}$ expression in adenocarcinomas was revealed (Tab. 5), $p=0.53$. The expression of p53 is also not related to the K-ras mutational status in pancreatic adenocarcinomas according to our results (Tab. 6), $p=0.97$.

Table 3. Results of K-ras mutational analysis in chronic pancreatitis cases, in invasive adenocarcinomas and in tissues adjacent to the structures of invasive adenocarcinoma (non-tumorous tissue)

	Mutated	Non-mutated	% of mutated
Chronic pancreatitis	1	27	3.6
Adenocarcinoma	25	11	69.4
Non-tumorous tissue	2	31	6.1

Table 4. Correlation of p53 and p21$^{WAF1/CYP1}$ expression in pancreatic adenocarcinoma

p21/p53	0	1+	2+
0	0	0	1
1+	3	2	4
2+	9	5	18

p=0.77 (Pearson's chi-square)

Table 5. Correlation of p21$^{WAF1/CIP1}$ expression and K-ras mutational status in pancreatic adenocarcinoma

p21/K-ras mutation	Negative	Mutated
0	0	1
1+	1	6
2+	10	18

p=0.53 (Pearson's chi-square)

Table 6. Correlation of p53 expression and K-ras mutational status in pancreatic adenocarcinoma

p53/K-ras mutation	Negative	Mutated
0	4	8
1+	2	5
2+	5	12

p=0.97 (Pearson's chi-square)

Discussion

Data obtained from immunohistochemical analysis of normal ducts, PanIN1a, PanIN1b, PanIN2, PanIN3 lesions and invasive adenocarcinomas suggest p21$^{WAF1/CIP1}$ overexpression as an early event in the development of pancreatic intraepithelial neoplasia. Our results are in agreement with the only published report that studied p21$^{WAF1/CIP1}$ expression in PanIN lesions [4]. Study of Biankin et al [4] evaluated the p21$^{WAF1/CIP1}$ expression in PanIN lesions adjacent to the structures of invasive pancreatic carcinoma in 60 resection specimens. Our study included also pancreatic resection specimens of chronic pancreatitis, both with and without the spectrum of PanIN lesions. Overexpression of p21$^{WAF1/CIP1}$ increased progressively from normal ducts through the spectrum of PanIN lesions in resection specimens of chronic pancreatitis and invasive carcinoma as well.

Expression of p53 at low levels represents an inherent part of the cell cycle in normal human tissues [39]. This protein may become stabilised under genotoxic or another stress or by a mutation of p53 gene involving the protein degradation pathway [32]. Low levels of p53 expression could be detected by sensitive immunohistochemistry under normal conditions in both embryonal (cytotrophoblast) and adult (proliferative endometrium, B cells of germinal centres, etc.) tissues [36]. To distinguish the higher expression of p53 potentially stabilized by a mutation or genotoxic stress from the expression of wild-type p53 in rapidly proliferating tissue under normal conditions, we used the dilution of primary antibody in which the cells of cytotrophoblast were completely negative. With the concentration of primary antibody (p53, DO-1, DAKO) 1:2000, the cells of cytotrophoblast were not stained at all while the proportion of immunohistochemically p53 positive cells in PanIN lesions and invasive adenocarcinomas was found to be in agreement with results of other studies [3, 9, 22, 46]. Our results suggest the overexpression of p53 as a later event in the development of pancreatic intraepithelial neoplasia if compared to p21$^{WAF1/CIP1}$ expression in agreement with Biankin et al [4]. Overexpression of p53 on 2+ level was found in 55% of invasive carcinomas. Our data also confirmed the possible p53 independent p21$^{WAF1/CIP1}$ expression in some PanIN2, PanIN3 lesions and invasive carcinomas in agreement with studies already published [4, 10, 17]. If p53 stabilised by a mutation is not able to induce the p21$^{WAF1/CIP1}$ expression [11], the co-overexpression of p53 and p21$^{WAF1/CIP1}$ could be explained by different mechanisms. The p21$^{WAF1/CIP1}$ expression may be regulated in a p53 independent pathway by a number of signaling molecules including ras [28], growth factors (EGF, FGF), vitamins D3 and E, interleukin 6 and others [15] however the overexpression of p21$^{WAF1/CIP1}$ could be also a result of wild-type p53 stabilization under genotoxic stress as described before [32]. DPC4/Smad4 dependent p21$^{WAF1/CIP1}$ expression was also considered because p21$^{WAF1/CIP1}$ expression induced by DPC4/Smad4 was already described [23] but it was recently found that overexpression of p21$^{WAF1/CIP1}$ occurs independently of DPC4/Smad4 in pancreatic intraepithelial neoplasia [4].

Previously unreported mechanism may be responsible for p21$^{WAF1/CIP1}$ expression in above stated PanIN lesions and pancreatic carcinomas. Activating mutations of K-ras are known to increase intracellular levels of p21$^{WAF1/CIP1}$ in experimental models (28). There is also an evidence of relatioship between activation of the Ras/Raf/MEK/ERK pathway and elevated p21$^{WAF1/CIP1}$ expression that may result in an activation of cyclin D/cdk4 complexes and cell

proliferation in hemopoietic cells [7]. Since activating mutations of K-ras oncogene are reported in more than 70% of pancreatic malignancies, the possible role of activating K-ras mutations in early $p21^{WAF1/CIP1}$ overexpression in pancreatic intraepithelial neoplasia was investigated in our study. We did not find activating K-ras mutations in samples containing normal ducts, PanIN1a and PanIN1b lesions that overexpressed $p21^{WAF1/CIP1}$ in some cases. These data do not support the possible role of activating K-ras mutations in the induction of $p21^{WAF1/CIP1}$ expression in the development of pancreatic intraepithelial neoplasia and do not confirm the findings of some studies presenting the $p21^{ras}$ overexpression or activating K-ras mutations in low grade PanIN lesions [2, 34]. The study of Apple et al [2] was based on immunohistochemical evaluation of $p21^{ras}$ expression but the mutational analysis of K-ras was not performed. Luttges et al [34] performed the PCR, constant denaturing gel electrophoresis and sequencing of 429 microdissected ductal lesions and demonstrated K-ras mutations in 4.4% ductal lesions (including low grade PanIN lesions) in 8 of 30 patients with chronic pancreatitis but not a single instance of p53 immunopositivity. On the other hand, the study of Gansauge et al [14] presented the results of p53 and codon 12 K-ras mutational analysis of 80 cases of chronic pancreatitis giving evidence for early occurance of p53 but not K-ras mutations which is in agreement even with our results. Using very sensitive mutant enriched PCR-RFLP analysis which is able to detect one K-ras mutation in 5000 wild type K-ras templates [20], the K-ras mutations were detected in 1 of 28 samples from patients with chronic pancreatitis and in 2 samples of tissues adjacent to the structures of invasive adenocarcinoma. Histological examination of serial tissue sections revealed high grade ductal lesions (PanIN2 or PanIN3) in these three positive cases and the role of K-ras mutation as an early event in oncogenesis of pancreatic adenocarcinoma was not confirmed in our study. The real role of K-ras in the development of pancreatic intraepithelial neoplasia has to be further studied and requires additional investigations. K-ras does not offer the general tool for detection of pancreatic neoplasia and needs to be supplemented by the demonstration of additional genetic alterations.

In the absence of activating K-ras mutation, the overexpression of HER-2neu which is detected in a significant proportion of PanIN lesions and pancreatic carcinomas [8, 30, 37, 45] may also result in $p21^{WAF1/CIP1}$ overexpression because of its signaling through ras [35]. Relationship between HER-2/neu and $p21^{WAF1/CIP1}$ overexpression has to be investigated in the spectrum of PanIN lesions and invasive carcinomas and will be a part of our additional investigation.

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