Amplification and overexpression of HER-2/neu in invasive ductal carcinomas of the pancreas and pancreatic intraepithelial neoplasms and the relationship to the expression of p21$^{WAF1/CIP1}$ *

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Overexpression of HER-2/neu was described in pancreatic intraepithelial neoplasia (PanIN) and in invasive ductal adenocarcinoma of pancreas in a variable proportion of cases. The effects of HER-2/neu overexpression on mitogenic signalling and cell cycle progression were studied in breast luminal epithelial cells and mitogen activated protein kinase-dependent induction of p21$^{WAF1/CIP1}$ was found to be necessary for G1 phase progression. Overexpression of p21$^{WAF1/CIP1}$ was described as an early event in the development of PanIN by Biankin et al. (2001) and this finding was supported by our previous study that, moreover, did not confirm the possible role of activating K-ras mutations in the induction of p21$^{WAF1/CIP1}$ overexpression.

Relationship between p21$^{WAF1/CIP1}$expression and HER-2/neu status in PanIN lesions and ductal adenocarcinoma of the pancreas was investigated in our study. Expression levels of p21$^{WAF1/CIP1}$ and HER-2/neu were examined imunohistochemically and the amplification of HER-2/neu gene was evaluated by fluorescence *in situ* hybridisation in HER-2/neu overexpressing adenocarcinomas. Fourty nine pancreatic resection specimens from patients with invasive adenocarcinoma were included into the study. A large spectrum of PanIN lesions adjacent to the structures of infiltrating adenocarcinoma was also examined. The possible role of HER-2/neu in an induction of p21$^{WAF1/CIP1}$ overexpression was not confirmed and p21$^{WAF1/CIP1}$ overexpression seems to be HER-2/neu independent in pancreatic ductal adenocarcinoma according to our results. Increasing levels of HER-2/neu expression were demonstrated in pancreatic intraepithelial neoplasia and in 18.75% of pancreatic adenocarcinoma. The only 2 from 9 HER-2/neu overexpressing adenocarcinomas showed the amplification of HER-2/neu gene. Based on these results, the overexpression of HER-2/neu in pancreatic adenocarcinoma seems to be a result of increased transcription rather than gene amplification. Therefore HER-2/neu represents a good target for therapy of pancreatic adenocarcinoma only in isolated cases.

Key words: adenocarcinoma, pancreas, HER-2/neu, p21, immunohistochemistry, FISH

Pancreatic ductal adenocarcinoma belongs to the most aggressive human malignancies. The lethality of the pancratic cancer almost equals its incidence and the overall 5-year survival rate is less than 5% [7, 13, 21]. The oncogenesis of pancreatic ductal adenocarcinoma is a multistep process characterized by the progression from ductal and ductular epithelium through the spectrum of the PanIN (pancreatic intraepithelial neoplasia) lesions to the invasive ductal adenocarcinoma [19, 20]. Recent studies repeatedly demonstrated this process as a result of genetic changes sequence resulting in activations of different oncogenes. Activation of K-ras oncogene usually caused by codon 12 point mutations was demonstrated in up to 60–100% cases of pancreatic adenocarcinoma [1, 25, 29, 41] and was also revealed in PanIN lesions [25, 26]. 50–75% pancreatic adenocarcinomas were reported to carry the mutations in tumor suppressor gene p53 [5, 12, 21]. Loss of the function of the CDKN2A (MST1) tumor suppressor gene, which en-

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codes $p16^{INK4a}$, occurs in up to 85–98% of pancreatic adenocarcinoma [28, 38]. Another tumor suppressor gene Smad4 is inactivated in approximately 50% of pancreatic cancers, compared to <10% in other tumor types [15, 39]. C-erbB-2 protooncogene, often referred as HER-2/neu, mapped to chromosome 17q21 encodes a 185 kDa transmembrane glycoprotein, designed as $p185^{HER2}$ [2, 4]. Dimerisation with other members of HER family is responsible for the formation of active receptors being able to bind a growth factor heregulin or to be active in signal transduction even in the absence of cognate ligands [32, 33]. HER-2/neu overexpression was found to be a poor prognostic factor in breast carcinoma [36] and was also demonstrated in pancreatic intraepithelial neoplasms and adenocarcinomas. However, its prognostic significance remains unclear and the percentage of immunohistochemically detected HER-2/neu overexpression in adenocarcinomas is very variable, from 16 to 82% among the studies [3, 9, 22, 24, 29, 30, 37, 43]. The only study of SAFRAN et al [37] evaluated the HER-2/neu status using fluorescence *in situ* hybridization in pancreatic adenocarcinoma. Gene amplification of HER-2/neu was detected in 3 of 11 patients with HER-2/neu overexpression revealed by immunohistochemistry [37]. Overexpression of $p21^{WAF1/CIP1}$ was recently described as an early event in the development of pancreatic intraepithelial neoplasia [6]. Expression of $p21^{WAF1/CIP1}$ seems to be p53 independent in a significant number of high grade PanIN lesions and adenocarcinomas and increased significantly with each subsequent PanIN lesion to pancreatic carcinoma along the progression model [6, 11]. The possible role of activating codon 12 K-ras mutation in the induction of $p21^{WAF1/CIP1}$ expression has not been confirmed yet [17] and the role of HER-2/neu in relationship to the $p21^{WAF1/CIP1}$ expression is discussed in the study of BIANKIN et al [6] that also revealed Smad4/DPC4 independent expression of $p21^{WAF1/CIP1}$[6]. Effects of HER-2/neu overexpression on mitogenic signalling and cell cycle progression were studied in breast luminal epithelial cells and mitogen activated protein kinase (MAPK)-dependent induction of $p21^{WAF1/CIP1}$ expression was found to be necessary for G1 phase progression [42].

Relationship between $p21^{WAF1/CIP1}$ and HER-2/neu expressions in PanIN lesions and ductal adenocarcinoma of the pancreas was investigated in our study. In addition, the amplification of HER-2/neu in pancreatic adenocarcinomas was evaluated using fluorescence *in situ* hybridization (FISH).

Material and methods

Fourty nine pancreatic resection specimens from patients who had undergone pancreaticoduodenectomy at the Faculty Hospital Brno, Masaryk Memorial Cancer Institute and Surgical Hospital Delta between 2000–2003 with the diagnosis of invasive ductal adenocarcinoma were included into the study and non-tumorous tissues adjacent to the structures of invasive carcinoma with the spectrum of PanIN lesions were also examined. Resection specimens were fixed in 10% neutral-buffered formalin for 24 hours. Slides stained by hematoxylin-eosin (HE) were examined to identify the samples containing the structures of invasive carcinoma and/or the spectrum of PanIN lesions in the tissue adjacent to invasive component. Immunohistochemistry (IH) and fluorescence *in situ* hybridization (FISH) were performed on 4 μm thick sections of formalin fixed paraffin embedded tissue applied to positively charged slides. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated through a series of alcohol. For $p21^{WAF1/CIP1}$ IH, the antigen retrieval was performed in the microwave (Milestone) by heating in citrate buffer at pH 6.0 for 4 minutes at 120 °C. The water bath containing EDTA buffer at pH 8.0 preheated to 93–95 °C was used for antigen retrieval in HER-2/neu IH. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in methanol. Tissue sections were incubated with mouse monoclonal antibodies to either $p21^{WAF1/CIP1}$ (clone SX 118, DAKO, dilution 1:50) or HER-2/neu (1:1 mixture of two monoclonal antibodies: NCL-c-erbB-2-316 in dilution 1:500 directed against internal domain of HER-2/neu and NCL-c-erbB-2-356 in dilution 1:1000 directed against external domain of HER-2/neu). 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). Slides were counterstained with hematoxylin. Tissue sections with a known HER-2/neu status were used as positive and negative controls. Slides were evaluated by two obserevers independently and the discrepancies were solved by consensus.

In $p21^{WAF1/CIP1}$ immunohistochemistry 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 were scored as 1+, more than 15% as 2+. Less than 5% nuclei positive were scored as 0.

In HER-2/neu immunohistochemistry, "0" negative cases have shown no membrane staining at all or membrane staining in less than 10% of the cells. "1+" negative cases have shown a barely perceptible membrane staining detected in more than 10% of the cells. The cells are stained only in part of their membrane. A weak to moderate immunostaining of the entire membrane observed in more than 10% of the cells was interpreted as 2+ weak positivity. 3+ strongly positive cases have shown a strong immunostaining of the entire membrane in more than 10% of the cells. Cytoplasmic labelling alone was scored as negative.

The HER-2/neu amplification was evaluated on 4 μm thick formalin fixed paraffin-embedded invasive cancer tis-

sue sections mounted on positively charged slides using Benchmark automate working with HER-2/neu probe (Ventana). Assays were strictly performed according to Benchmark user guide. Nuclear DNA was counterstained by 0.1 μg/ml DAPI in Antifade (Oncor). Scoring of amplification was performed by counting the number of all FITC signals present in 20 randomly selected, intact, non-overlapping cancer nuclei in two distinct areas within the same tissue section (i.e., a total of 40 nuclei per case). Fluorescence microscope eqquipped with DAPI and FITC filters and 100x immerse objective and LUCIA^{R} software were used for image analysis and scoring. Tumors with the mean number of signals per nucleus ≤4 were interpreted as non-amplified, whereas cases exhibiting >4 as a mean were classified as amplified.

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

Results

Normal ducts were examined in 40 cases (39 in p21$^{WAF1/CIP1}$ IH), PanIN1a lesions in 39 cases (36 in p21$^{WAF1/CIP1}$ IH), PanIN1b lesions in 36 cases (34 in p21$^{WAF1/CIP1}$ IH), PanIN2 lesions in 39 cases (33 in in p21$^{WAF1/CIP1}$ IH), PanIN3 lesions in 45 cases (37 in p21$^{WAF1/CIP1}$ IH) and invasive ductal adenocarcinomas in 48 cases (49 in p21$^{WAF1/CIP1}$). Not every resection specimen contained the full spectrum of Pan IN lesions and some of them were also lost by serial sectioning due to repeated immunohistochemical staining in some cases. Among the infiltrating adenocarcinomas, 27 were moderately differentiated, 9 well differentiated, 11 were poorly differentiated and 2 contained foci of both moderately and poorly differentiated carcinoma.

The results of immunohistochemically evaluated HER-2/neu expression are summarized in Table 1. Statistically significant correlation was revealed (p=0.002) between the level of HER-2/neu expression and the severity of a ductal lesion. Immunostaining for HER-2/neu was observed in normal ducts of 2 cases, in PanIN1a lesions of 6 cases and in PanIN1b lesions of 7 cases on 1+ level, which is reproduced as no overexpression according to conventional criteria. Only one PanIN1b lesion expressed HER-2/neu on 2+ level. Overexpression of HER-2/neu (2+ or 3+ immunostaining) was observed in PanIN2 lesions of 4 cases, in PanIN3 lesions of 9 cases and in 9 ductal adenocarcinomas (8 of them were moderately and one of them poorly differentiated). Overexpression of HER-2/neu was detected in 18.75% adenocarcinomas (Fig. 1). Among the infiltrating carcinomas, only one case expressed HER-2/neu on 3+ level. The three 2+ positive adenocarcinomas overexpressed HER-2/neu only focally. To determine the correlation between the overexpression and amplification of HER-2/neu, these 9 cases with HER-2/neu overexpression revealed by immunohistochemistry were examined using fluorescence *in situ* hybridization. A high amplification of HER-2/neu was detected in only 1 adenocarcinoma (Fig. 2) with the

Table 1. Expression of HER-2/neu in normal ducts, PanIN lesions and adenocarcinomas

	0	1+	2+	3+
Normal ducts	38(95%)	2(5%)	0(0%)	0(0%)
PanIN1a	33(85%)	6(15%)	0(0%)	0(0%)
PanIN1b	28(78%)	7(19%)	1(3%)	0(0%)
PanIN2	27(69%)	8(21%)	4(10%)	0(0%)
PanIN3	25(56%)	11(24%)	8(18%)	1(2%)
Carcinoma	25(52%)	14(29%)	8(17%)	1(2%)

p=0.002

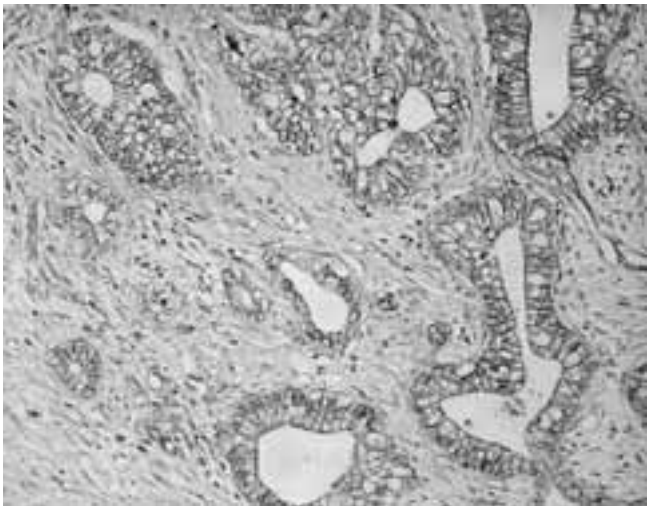

Figure 1. Overexpression of HER-2/neu in invasive adenocarcinoma: moderate membrane staining on 2+ level.

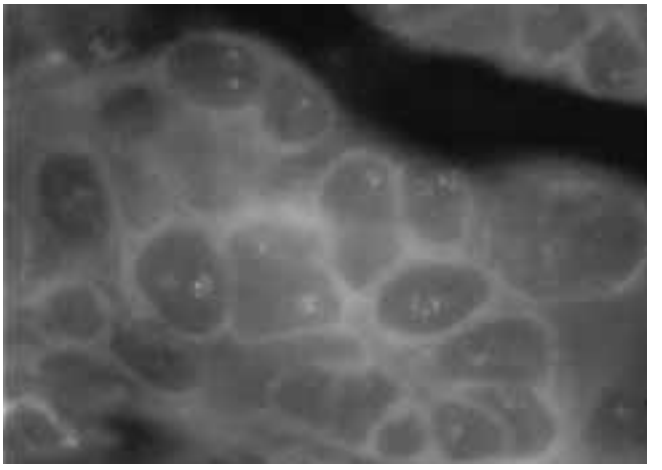

Figure 2. Amplification of HER-2/neu gene in an invasive adenocarcinoma with immunohistochemically detected HER-2/neu overexpression.

Table 2. Expression of $p21^{WAF1/CIP1}$ in normal ducts, PanIN lesions and adenocarcinomas

	0	1+	2+
Normal ducts	37(95%)	2(5%)	0(0%)
PanIN1a	29(81%)	7(19%)	0(0%)
PanIN1b	21(62%)	12(35%)	1(3%)
PanIN2	3(9%)	20(61%)	10(30%)
PanIN3	2(6%)	9(24%)	26(70%)
Carcinoma	2(4%)	12(25%)	35(71%)

p<0.000

Table 3. Relationship of HER-2/neu expression to $p21^{WAF1/CIP1}$ expression independently of the type of lesion

p21/HER-2	0	1+	2+	3+
0	77(46%)	13(32%)	3(19%)	0(0%)
1+	37(22%)	16(40%)	7(44%)	0(0%)
2+	53(32%)	11(28%)	6(37%)	2(100%)

p=0.030

Table 4. Relationship of HER-2/neu expression to $p21^{WAF1/CIP1}$ expression in adenocarcinomas

p21/Her-2	0	1+	2+	3+
0	0(0%)	1(7%)	1(12%)	0(0%)
1+	3(12%)	5(36%)	3(38%)	0(0%)
2+	22(88%)	8(57%)	4(50%)	1(100%)

p=0.244

mean gene copy number 10.8 per nucleus. This positive case expressed HER-2/neu in PanIN3 lesion and in the structures of invasive carcinoma on 3+ level. Clonally heterogenous amplification of HER-2/neu was revealed in one more case with imunohistochemically detected focal HER-2/neu overexpression. An area with a borderline amplification was identified within this tumor section. The mean gene copy number in this part of tumor was 5.3; the overall gene copy number in this case was 3.4 per nucleus.

Results of immunohistochemically evaluated $p21^{WAF1/CIP1}$ expression are summarized in Table 2. 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 along the progression model with staining not exceeding 1+ in normal ducts and staining 2+ in the most cases of PanIN3 lesions and carcinomas.

Total analysis of the relationship between HER-2/neu and $p21^{WAF1/CIP1}$ expressions in all types of ductal lesions (summarized in Tab. 3) revealed statistically significant correlation (p=0.030). There was no statistically significant correlation (p=0.244) between HER-2/neu and $p21^{WAF1/CIP1}$ expressions in adenocarcinomas (summarized in Tab. 4).

Discussion

$p21^{WAF1/CIP1}$ (CDKN1A), originally identified together with $p27^{KIP1}$ and $p57^{KIP2}$ as general inhibitors of Cdks, prevents Rb phosphorylation by inhibiting activation of cdk2/cyclin E complexes that is required for Rb phosphorylation. In this point of view, it is difficult to interpret the growing expression of $p21^{WAF1/CIP1}$ according to the severity of the ductal lesion found in the study of BIANKIN et al [6] and in our study as well. But it was found that $p21^{WAF1/CIP1}$ does not necessarily inhibit the activity of cdk/cyclin complexes since $p21^{WAF1/CIP1}$ is present in both active and inactive forms of cdk/cyclin complexes [44]. They function as cdk inhibitors when complexed with cdk2/cyclinE and as activators when complexed with cdk4/cyclin D1. The only study dealing with immunohistochemically detected expression of cyclin D1 in PanIN lesions failed to show the expression of cyclin D1 in low grade ductal lesions and the functional role, if any, of $p21^{WAF1/CIP1}$ overexpression in the development of pancreatic intraepithelial neoplasia remains unclear [6]. The levels of cyclin E expression in PanIN lesions have not been studied yet. Additionally, $p21^{WAF1/CIP1}$ was described as an assembly factor for cdk4/cyclin D1 complexes which is in low concentration required for the cdk4/cyclin D1 enzymatic activity necessary for Rb phosphorylation [8, 23, 40] and also acts as a down stream mediator of many mitogenic stimuli including the above mentioned HER-2/neu protein [27, 42]. HER-2/neu is able to signal through ras protein and activation of Ras/Raf/MEK/ERK pathway was found to be associated with increasing $p21^{WAF1/CIP1}$ expression, activation of cdk4/cyclin D1 complexes and proliferation in hemopoietic cells [8]. But $p21^{WAF1/CIP1}$ expression seems to be HER-2/neu independent in pancreatic ductal adenocarcinoma according to our results. We have revealed a statistically significant correlation between $p21^{WAF1/CIP1}$ and HER-2/neu expressions only when analysing all ductal lesions independently of their type. We suppose that this relationship represents statistically secondary correlation due to strongly significant dependency of $p21^{WAF1/CIP1}$ and HER-2/neu expressions on the type of lesions. We conclude that the possible role of HER-2/neu in an induction of $p21^{WAF1/CIP1}$expression was not confirmed in our study.

Recently published reports [22, 30, 37] demonstrated HER-2/neu overexpression in a smaller proportion of pancreatic ductal adenocarcinoma when compared to studies previously published [3, 9, 10]. The lower rate of overexpression in recent studies is comparable with the HER-2/neu overexpression in breast carcinomas [31]. The largest study of KOKA et al [22] demonstrated the HER-2/neu over-

expression in 48 out of 308 pancreatic ductal adenocarcinoma specimens (16%) and suggested that HER-2/neu may not be an appropriate target for therapy of pancreatic adenocarcinoma. This study also revealed no statistically significant difference in survival between HER-2/neu positive and negative groups and described significant variability in the levels of HER-2/neu expression across tumor sections in individual cases, which can potentially lead to a misclassification, especially in probatory excisions from unresectable tumors [22]. Multivariate analysis did not reveal statistical difference in survival between the uniformely and variably expressing tumors [22]. Our study included only pancreatic resection specimens and revealed variable HER-2/neu overexpression across tumor sections in 3 of 9 tumors with immunohistochemically detected overexpression. In our study, the overall percentage of HER-2/neu positive carcinomas (18.75%) was in agreement with the largest study of KOKA et al [22]. All these positive carcinomas were examined using FISH and amplification of HER-2/neu gene was detected in only 2 from 9 HER-2/neu overexpressing carcinomas. Tumor cells in the first amplified case showed 3+ HER-2/neu expression and the same level of expression was detected in PanIN3 lesion adjacent to the invasive tumor structures. The other case showed the HER-2/neu amplification only clonally. The only study of SAFRAN et al [37] counted the HER-2/neu gene copy number in pancreatic adenocarcinomas using FISH and revealed the amplification in a small proportion of carcinomas with immunohistochemically detected overexpression (3 of 11; 27%). This study also revealed a lower incidence of HER-2/neu overexpression in pancreatic adenocarcinomas, which is in agreement with recently published data [22, 30, 37]. The discrepancy between the proportion of HER-2/neu overexpressing cases in different studies could be explained methodologically. The type of antibody (monoclonal and polyclonal), the way of antigen retrieval and especially strict evaluating of only membrane immunostaining play the important role in separation of immunohistochemically positive and negative carcinomas. Our study used monoclonal antibodies directed against both internal and external domain of HER-2/neu protein using methodology showing statistically significant correlation with FISH in breast carcinomas; overexpressing breast carcinomas show amplification of HER-2/neu in almost all cases using this methodological appproach [18]. Results of our immunohistochemical and FISH analysis of pancreatic adenocarcinoma suggest that the overexpression of HER-2/neu in invasive pancreatic cancer is the result of increased transcription rather than gene amplification. Similarly, in non-small cell and small cell lung carcinoma the rate of HER-2/neu overexpression revealed by IH ranges between 15–25%, while FISH detects gene amplification in less than 5% of pathological samples [35]. Mild amplification of HER-2/neu was also revealed in a subset of gastrinomas and higher levels of HER-2/neu mRNA were associated with aggressiveness of these tumors. Therefore, the evaluation of HER-2/neu status could identify a patient subset with gastrinomas who might benefit from transtuzumab treatment [14]. The recently published study of POTTI et al [34] which evaluated the HER-2/neu status only immunohistochemically concluded that HER-2/neu is not significantly overexpressed in both pancreatic carcinoma and hepatocellular carcinoma. According to their results, there appears to be no role for the use of trastuzumab treatment [34]. However, they suggest further studies in pancreatic adenocarcinoma [34].

Our study represents the only second report demonstrating the amplification of HER-2/neu in pancreatic ductal adenocarcinoma in a small proportion of HER-2/neu overexpressing carcinomas [37]. In agreement with the recent literature, we conclude that HER-2/neu gene amplification seems to be a rare event in the development of pancreatic cancer according to our results and HER-2/neu appears to represent a good target for therapy of pancreatic adenocarcinoma only in isolated cases. We suggest a confirmation of HER-2/neu status using FISH, especially in HER-2/neu overexpressing adenocarcinomas.

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