Expression of carbonic anhydrase IX in human pancreatic cancer.


Source

Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg, Germany.

Abstract

BACKGROUND:

Carbonic anhydrase IX has been linked to cancer development and progression.

AIM:

To analyse carbonic anhydrase IX expression and anhydrase inhibition in pancreatic cancer and to correlate these findings with p53 expression and microvessel density.

MATERIALS AND METHODS:

Seventy-seven pancreatic cancers were examined (43 males, 34 females; mean age, 64 years). The anti-carbonic anhydrase IX M75 antibody was used for immunohistochemistry and Western blot analysis. Microvessels were visualized using the anti-CD34 antibody, and p53 expression in cancer cells was assessed with a specific anti-p53 antibody. Quantitative polymerase chain reaction was performed in order to assess carbonic anhydrase IX mRNA levels in the pancreas. Furthermore, pancreatic cancer cell lines were treated with acetazolamide, a carbonic anhydrase inhibitor.

RESULTS:

In the normal pancreas, carbonic anhydrase IX immunoreactivity was observed at the basolateral membrane of ductal cells in 24 cases (31%). Carbonic anhydrase IX expression was found at the membrane and in the cytoplasm of pancreatic cancer cells in 16 pancreatic cancers (21%). Carbonic anhydrase IX expression was independent of the localization, stage, size, metastases and differentiation of the tumour. p53 expression was significantly more frequent in poorly differentiated cancers (P=0.0323); however, p53 expression and microvessel density were independent of carbonic anhydrase IX expression. Overall, carbonic anhydrase IX expression was not altered in pancreatic cancers vs. adjacent normal pancreatic tissue as assessed by Western blot and
quantitative polymerase chain reaction analysis. However, incubation of pancreatic cancer cell lines with acetazolamide led to a significant inhibition of cell proliferation in AsPC-1 and PANC-1 pancreatic cancer cells.

CONCLUSION:

Carbonic anhydrase IX expression is observed in both ductal epithelial and cancer cells of the pancreas. Although the expression of carbonic anhydrase IX in pancreatic cancer is not associated with angiogenesis or advanced disease, it may well be a target for carbo-anhydrase inhibitors in a subset of pancreatic cancers.

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**Introduction**

Pancreatic cancer accounts for only 2\% of all newly diagnosed cancers in the USA each year, but for 5\% of all cancer deaths.\(^1\) Pancreatic cancer is resistant to conventional treatment modalities. Most pancreatic cancers are adenocarcinomas arising from the pancreatic ductal system with a frequent presence of a desmoplastic reaction around the cancer cells. In the development of pancreatic cancer, the activation of oncogenes, such as the K-ras oncogene, and a broad range of growth factors, as well as the inactivation of tumour suppressor genes, such as p53, p16 and DPC4, play an important role.\(^2\) In particular, with a frequency of 15–70\% of pancreatic cancers, the mutation of the p53 tumour suppressor gene is a major early event in pancreatic carcinogenesis.\(^3\)

Carbonic anhydrases (CAs) are zinc-containing metallo-enzymes catalysing the conversion of carbon dioxide to bicarbonate, thereby regulating the acid–base balance and respiration.\(^4\) Moreover, CAs are involved in bone resorption, gluconeogenesis, ureagenesis and the formation of gastric acid, saliva and cerebrospinal fluid, and have been suggested to play a significant role in epithelial cell interaction and cell proliferation.\(^5\) To date, 14 human isoforms of CA have been identified.\(^4\) The CA IX protein (formerly called MN or G250), which was originally detected in HeLa cells, consists of an N-terminal proteoglycan-like domain, a CA domain, a transmembrane anchor and a C-terminal cytoplasmic tail.\(^6\) In normal human tissue, the most prominent expression of CA IX has been reported in the mucosa of gastrointestinal organs, such as the stomach, intestine and gall-bladder.\(^3\)\(^4\) The expression of CA IX is most abundant on the basolateral surfaces of the crypt enterocytes in the duodenum and jejunum.\(^5\)
In mouse 3T3 cells, neoplastic transformation can be induced by transfection of CA IX cDNA. In addition, the over-expression of CA IX has been demonstrated in renal cell cancer, cervical squamous cell carcinoma and ovarian cancer, among others. However, CA IX expression is reduced in gastric cancer.

The microvessel density reflects the overall degree of angiogenesis, which contributes significantly to the progression of cancer. The microvessel density is an independent prognostic indicator in several types of malignant tumours, including breast cancer, ovarian cancer, endometrial cancer and pancreatic cancer. CA IX is usually expressed in cancers with poor vascularization, and therefore the CA IX protein has been suggested to be induced by hypoxia. In accordance with these findings, CA IX expression has been shown to be associated with tumours with a low microvessel density. However, CA IX expression has also been found in association with high microvessel density in other cancers.

The aims of this study were to analyse CA IX expression and CA IX inhibition in pancreatic cancer and to correlate these findings with microvessel density and p53 expression.

Materials and methods

Subjects

Seventy-seven patients who underwent surgical resection of the pancreas for pancreatic carcinoma were examined retrospectively. There were 43 men and 34 women. The mean age was 64 years (range, 43–81 years).

Staging was performed according to the tumour–regional lymph nodes–distant metastases (TNM) classification system as follows: there were 11 patients with pT1 tumours, 58 patients with pT2, six patients with pT3 and two patients with pT4. In 49 patients, lymph node metastases were present and, in eight patients, distant metastases. Sixty-four carcinomas were localized in the head of the pancreas, seven carcinomas in the body and six carcinomas in the tail. Histologically, all of the carcinomas were ductal adenocarcinomas. The grading was performed in accordance with the World Health Organization classification of pancreatic tumours. It was based on the combined assessment of histological and cytological features and mitotic activity. In particular, glandular differentiation (well-differentiated vs. moderately differentiated duct-like
glands vs. pleomorphic structures and poorly differentiated glands), mucin production (intensive vs. irregular vs. abortive), mitotic count (few vs. moderate vs. high) and nuclear pleomorphisms were considered. Three grades of differentiation were distinguished. One tumour was well differentiated, 31 moderately differentiated and 45 poorly differentiated. Frozen tissues were obtained from 13 patients. Immediately after surgery, tissue samples were placed in liquid nitrogen and stored at −80 °C until molecular analysis.

CA IX immunohistochemistry

For each patient, serial sections (4 µm thick) were cut from paraffin blocks, mounted on acid-cleaned glass slides and heated at 55 °C for 60 min. Sections were deparaffinized in graded alcohol and rehydrated, and the endogenous peroxidase activity was inhibited by incubation with 3% H$_2$O$_2$ in methanol (20 min at room temperature). To reduce non-specific background staining, slides were incubated with 5% goat serum (20 min at room temperature). Slides were incubated with the appropriate primary antibodies in a moist chamber overnight at 4 °C. The primary antibody used was monoclonal mouse anti-CA IX M75 antibody, with a dilution of 1 : 100. Sections of normal human intestine were used as positive controls. For the negative control, M75 antibody was substituted with phosphate-buffered saline. The avidin–biotin peroxidase complex procedure (ABC standard; Vector Laboratoires, Burlingame, CA, USA) was then performed, and peroxidase activity was detected with diaminobenzidine as substrate. Finally, sections were counter-stained with haematoxylin and cover-slipped with a synthetic mounting medium. The regional, cellular and sub-cellular immunohistochemical localization were evaluated for each slide by an experienced pathologist (U.K.).

p53 immunohistochemistry

Sections were deparaffinized in a series of graded alcohols and microwaved in ethylenediaminetetra-acetic acid buffer for 2 × 10 min at 450 W. After the sections had cooled to room temperature and had been rinsed with Tris buffer, incubation was performed with anti-p53 (Do-1; Calbiochem, Cambridge, MA, USA). Before incubation of the primary antibodies, endogenous peroxidase was blocked by pre-incubation of the slides with 0.3% H$_2$O$_2$. The p53 antibody reacted specifically with both the wild type and the mutant type of protein p53, recognizing an epitope between the amino acids 37
Anti-p53 was diluted 1:30 and incubated at 37 °C for 26 min. Bound primary antibodies were detected by the avidin–biotin complex method (Ventana Medical Systems, Tucson, AZ, USA) labelled with horseradish peroxidase and diaminobenzidine as substrates. Assessment of p53 was made by counting 1000 cells per section at high magnification and the percentage of positive tumour cells was determined. According to Kawai et al., tumours with more than 10% immunopositive cells were considered to be positive for p53. Only positive nuclear staining was evaluated. Sections from a breast carcinoma were used as a positive control. For the negative control, the specific primary antibody was substituted with phosphate-buffered saline.

**Microvessel density**

Microvessel density was assessed according to the methods described by a consensus conference. Microvessels were visualized immunohistochemically using the monoclonal mouse immunoglobulin G anti-CD34 antibody (Biogenex, San Ramon, CA, USA), which has been shown to be a sensitive and reliable marker of microvessel density. Briefly, to measure the intra-tumoral microvessel density, the stained sections were screened without any knowledge of the patient data at 50× magnification to identify the highest vascular area within the tumour. In this hot spot, the individual microvessel count was evaluated at 200× magnification using a square ocular grid. Any cluster of cells stained by CD34 and clearly distinguishable from the background was counted as a vessel. Branching structures were counted as a single vessel. Vessels that lay obliquely to the plane of the section were excluded. Vascular counts for each case were calculated as the mean and median of 10 fields, and are expressed as the number of microvessels per square millimetre.

**Real-time quantitative analysis of CA IX mRNA levels**

Tissue specimens were homogenized with an ultrasound homogenizer (Ultra-Turrax T25 basic, IKA, Staufen, Germany). After the removal of chromosomal DNA by DNase (Invitrogen, Karlsruhe, Germany), total RNA was extracted using an RNeasy Midikit (Qiagen, Hilden, Germany), quantified by measuring the optical density at 260 nm and separated by gel electrophoresis. Total RNA (1 µg) was reverse transcribed at 37 °C.
for 1 h in a final volume of 20 µL reverse transcription buffer (50 mM Tris-HCl pH 8.3, 7 mM MgCl₂, 40 mM KCl and 10 mM dithiothreitol) containing 100 U MMLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Mannheim, Germany), 16 U RNase inhibitors (Promega), 200 pmol random primer (Promega) and 0.5 mM dNTPs (Biomol Feinchemikalien, Hamburg, Germany). The reaction was terminated by incubating the mixture at 95 °C for 5 min. Polymerase chain reaction (PCR) amplification of the cDNA was performed as described previously. Briefly, PCR primers were designed to amplify a 240-base pair cDNA fragment of the CA IX gene (sense 5′-AGGAGGATCTGCCCAGTGA-3′; anti-sense 5′-GCCAATGACTCTGGTCATC-3′). The expression level of CA IX was determined using the LightCycler technique (Roche Diagnostics GmbH, Mannheim, Germany). Standard curves were obtained by serial dilutions of at least three plasmid DNA samples with the cloned PCR fragment (TOPO TA Cloning Kit, Invitrogen, Leek, The Netherlands) in each run. The housekeeping gene β2-microglobulin was chosen as a reference: each PCR result was normalized against β2-microglobulin. PCR analyses for CA IX were performed in 20-µL volumes in glass capillaries using the LightCycler DNA Master Hybridization Probes Kit (Roche Diagnostics GmbH, Mannheim, Germany) in combination with Hybridization Probes (TIB MOLBIOL, Berlin, Germany). Hybridization Probes consists of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of PCR cycles. One probe is labelled at the 5′-end with a LightCycler Red fluorophore and the other probe is labelled at the 3′-end with fluorescein. Each primer (0.5 µM), 3 mM MgCl₂, 0.8 µM LightCycler Red 640-labelled Probe (5′-LC Red 640-TTGGACCTCTGGAGATCCTAp-3′) and 0.4 µM Donor-F Probe (5′-CTGAAGTTAGGATCTACCTACTX-3′) were used in each PCR run under the following conditions: initial denaturation at 95 °C for 10 s, followed by 45 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and elongation at 72 °C for 10 s, with a temperature transition rate of 20 °C/s. The Second Derivative Maximum Method provided by the LightCycler software was used to estimate the concentration of each sample.

**Western blot analysis**

Human pancreatic tissues were lysed in buffer containing 1 mM ethylenediaminetetra-acetic acid, 50 mMβ-glycerophosphate, 2 mM sodium orthovanadate, 1% Triton-100, 10% glycerol, 1 mM dithiothreitol and protease inhibitors (10 mg/mL benzamidine, 2 mg/mL antipain and 1 mg/mL leupeptin). The lysates were homogenized and incubated
on ice for 1 h, and then centrifuged at 10 000 × g and 4 °C for 15 min. The protein concentration of the supernatants was determined by the bicinchoninic acid (BCA) assay. Twenty-five micrograms of protein of each sample was adjusted to Laemmli buffer composition [2% sodium dodecyl sulphate, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol and 0.1% bromophenol blue], denatured by heating at 95 °C for 5 min and subsequently separated on 12% polyacrylamide gels by sodium dodecyl sulphate gel electrophoresis. After separation, proteins were transferred on to immunoblot polyvinylidene difluoride membrane. The membrane was blocked in 5% non-fat milk in 1% TBST overnight, and then incubated with 1 : 200 anti-CA IX antibody for 1 h at room temperature. The monoclonal murine antibody M75 recognizing the N-terminal domain of CA IX was applied. Membranes were then washed three times in TBS/0.1% Tween-20, incubated for 1 h with a secondary antibody (1 : 2000) diluted in blocking solution and washed again as described previously. Membrane-bound secondary antibodies were detected by enhanced chemiluminescence following the instructions of the manufacturer (Amersham, Freiburg, Germany). To ensure equal loading amounts, the blots were stripped in 200 mmol/L glycine, 1% Tween-20, 0.1% sodium dodecyl sulphate, pH 2.2, for 2 h at room temperature and re-probed using a monoclonal antibody to β-actin (clone AC-74, Sigma, Deisenhofen, Germany) at a dilution of 1 : 2000. HeLa cells were used as a positive control for Western blot analysis.

Cell culture and treatment with acetazolamide

Human pancreatic cancer cells [AsPC-1 (CRL-1682), Mia PaCa-2 (CRL-1420) and PANC-1 (CRL-1469)] were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum (Gibco Invitrogen, Eggenstein, Germany) and 50 µg/mL rifobacin. Cells were seeded in 96-well plates at a density of 30 000 cells/200 µL per well in the presence or absence of 10 µM acetazolamide. After 40 h of culture at 37 °C, 5% (v/v) CO₂, cell cultures were pulsed for an additional 8 h with ³H-methyl-thymidine (0.2 µCi per well). Cells were harvested on to glass fibre membranes, and the incorporated radioactivity was measured by scintillation counting. In each case, DNA synthesis was assessed six times in parallel and repeated once, resulting in 12 experiments per cell line in total.
Statistical analysis

The correlation between CA IX immunoreactivity and patient characteristics was analysed using Fisher's exact test. The Mann–Whitney U rank sum test was used to compare CA IX and p53 immunoreactivity with microvessel density. Kruskal–Wallis analysis was used to examine the correlation between microvessel density and various clinicopathological data. All tests were two-sided, and a P value of <0.05 was considered to be statistically significant. All analyses were performed using GraphPad InStat 3.0 (San Diego, CA, USA) software.

Results

CA IX expression in normal pancreatic tissue

CA IX immunoreactivity was observed in ductal epithelial cells in the adjacent normal pancreatic tissue in 24 cases (31%). The CA IX immunostaining was confined to the basolateral membrane of epithelial cells. The acinar cells of the adjacent normal pancreatic tissues showed CA IX positivity in only two cases. The islet cells exhibited no CA IX immunoreactivity.

CA IX expression in pancreatic cancer

The cancer cells in 16 pancreatic cancer specimens (21%) exhibited CA IX immunoreactivity. The expression of CA IX was focal and predominantly localized to the membrane of cancer cells (Figure 1a). In pancreatic cancer cells with higher CA IX expression, additional cytoplasmic staining could be observed (Figure 1b). CA IX immunoreactivity was considered to be low in nine cases and moderate in five cases. CA IX immunoreactivity in the cancer cells was independent of tumour localization, tumour stage, tumour size, lymph node metastases, distant metastases and tumour differentiation (Tables 1–3). No expression of CA IX was detected in the stromal compartment of pancreatic cancers (Figure 1B). Using Western blot analysis, both the 54-kDa and 58-kDa isoforms of CA IX could be detected in the pancreas. In four randomly selected pancreatic cancer specimens, CA IX protein expression was either increased or decreased compared with the matched non-cancerous control (Figure 2).
Overall, CA IX mRNA levels were not altered in pancreatic cancer vs. adjacent normal pancreatic tissue; however, three cancers exhibited increased mRNA levels (Figure 3).

Figure 1. Carbonic anhydrase IX (CA IX) expression in pancreatic ductal adenocarcinoma. Whilst some cancer cells (a) exhibit only weak (asterisk) or moderate (arrow) CA IX immunoreactivity, others (b) exhibit strong CA IX expression (arrow). CA IX expression is confined mainly to the plasma membrane of these carcinoma cells (a). Carcinoma cells with increased CA IX expression (b) exhibit both membranous and cytoplasmic CA IX immunostaining (arrow). The stromal compartment (S) and the mononuclear cells (arrowhead) of the desmoplastic connective tissue show no expression of CA IX (b). Scale bar: 50 µm (a), 20 µm (b).

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Table 1. Clinicopathological characteristics of patients with pancreatic cancer

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1. B, body; CA IX, carbonic anhydrase IX; F, female; H, head; M, male; T, tail; UICC, Union International Contre le Cancer.
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Table 2. Carbonic anhydrase IX (CA IX) expression: comparison with p53 expression and microvessel density (vessels/mm²)

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Table 3. Characteristics of pancreatic cancer patients with carbonic anhydrase IX (CA IX) expression in pancreatic cancer cells

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Figure 2. Carbonic anhydrase IX (CA IX) protein expression in four pancreatic cancers using Western blot analysis. Proteins from lysates of pancreatic tissues (25 µg) and from the HeLa cell line (15 µg) were loaded on to individual lanes. Both the 54-kDa and 58-kDa isoforms of CA IX were identified. N, normal tissue; TU, tumour tissue; PC, positive control (HeLa cell); P1–4, patients 1–4.

Download figure to PowerPoint
Carbonic anhydrase IX (CA IX) mRNA levels in pancreatic cancer. Using quantitative polymerase chain reaction, the levels of CA IX mRNA were assessed in 13 pancreatic cancers (dark columns) and matched adjacent non-cancerous pancreas (white columns). Overall, no alterations of CA IX mRNA levels were observed; however, three cancers exhibited increased CA IX mRNA levels.

**p53 expression**

p53 expression was found in the nuclei of cancer cells in 27 pancreatic cancer specimens (35%). The expression of p53 protein was significantly more frequent in the undifferentiated or poorly differentiated pancreatic cancers than in the moderately or well-differentiated pancreatic cancers ($P = 0.0323$). No association between p53 expression and other clinicopathological factors or patient characteristics was observed. There was no association between CA IX and p53 expression.

**Microvessel density**

The mean microvessel density in the highest vascular area of pancreatic carcinoma was 5.98 vessels/mm$^2$, with a range from 1.6 to 14 vessels/mm$^2$ (Figure 4). The median microvessel density was 5.4 vessels/mm$^2$. A distinction between pre-existing capillaries and newly formed microvessels induced by the tumour was not possible. For statistical evaluation, the degree of microvessel density was sub-classified as above or below the sample median. The microvessel density was independent of CA IX immunoreactivity, p53 expression, tumour stage, lymph node metastases, distance spread, histological grade, localization within the pancreas and the age and sex of the patient (Table 3).

**Figure 4.** CD34 expression in human pancreatic cancer. Consecutive sections of pancreatic cancers were stained with an anti-CD34 antibody. Several foci of marked CD34 expression indicate various levels of microvessel density in human pancreatic cancer.
Inhibition of pancreatic cancer cell proliferation by acetazolamide

As acetazolamide has previously been shown to inhibit various CAs, including CA IX, we incubated three well-known pancreatic cancer cell lines, i.e. AsPC-1, Mia PaCa-2 and PANC-1, with acetazolamide. The analysis of DNA synthesis in cells without acetazolamide relative to cells incubated with 100 µM acetazolamide revealed a significant inhibition of cell proliferation in AsPC-1 ($P = 0.03$) and PANC-1 ($P < 0.05$) pancreatic cancer cells (Figure 5).

Figure 5. Inhibition of cell proliferation by acetazolamide. Three pancreatic cancer cell lines (ASPC-1, Mia PaCa-2, Panc-1) were incubated with (white column) and without (black column) 100 µM acetazolamide as outlined in ‘Materials and methods’. Acetazolamide led to a significant inhibition of cell proliferation in ASPC-1 ($P = 0.03$) and Panc-1 ($P < 0.05$) cells.

Discussion

The expression of CA IX protein has been the subject of intense investigation in both gastrointestinal and extra-intestinal tumours. The expression of CA IX can be induced by hypoxia in a number of cancer cell lines, and the transcription of CA IX gene is regulated by hypoxia-inducible factor 1.25 As tumour hypoxia is associated with intense cell proliferation, an increased risk of metastasis formation and a poor prognosis, CA IX
may not only be a hypoxia marker, but also a potential prognostic factor. However, Kivelä et al. indicated only a limited value for CA IX as a diagnostic marker. Using a larger cohort of pancreatic cancer patients, we also focused on the expression of CA IX in pancreatic cancer, and extended our analysis to p53 expression and microvessel density in order to reveal potential associations between CA IX expression and tumour progression and angiogenesis.

In this study, CA IX immunoreactivity was present in approximately one-third of the normal counterparts of pancreatic cancer specimens. CA IX expression was observed predominantly in the ductal epithelium. The acinar cells showed a very infrequent and mild CA IX staining, whereas the endocrine pancreas exhibited no CA IX expression. Similarly, Kivelä et al. reported a strong CA IX expression in the ductal compartments, and a weaker CA IX expression in the acinar compartment, both in a basolateral location, whereas no expression of CA IX could be observed in Langerhans islands.

In our material, 21% of ductal adenocarcinoma specimens exhibited CA IX expression, which was confined to the membrane of carcinoma cells, with an additional cytoplasmic immunoreactivity in tumour cells with higher CA IX intensity. In the study by Kivelä et al. on CA IX expression in pancreatic cancer, 35% of adenocarcinoma cases expressed CA IX. CA IX showed a combined pattern, with positive staining in both the membrane and cytoplasm, in pancreatic cancer, non-small-cell lung cancer, head and neck squamous cell carcinoma and breast cancer. The apparent cytoplasmic localization of CA IX in cancer cells may reflect an accumulation of CA IX in transformed cells, thereby contributing to enhanced growth and proliferation. However, our quantitative analysis of CA IX protein and mRNA levels in pancreatic cancers revealed no significant change compared with the normal pancreas.

We found no CA IX expression in the stromal compartment of pancreatic cancer specimens. In other studies, fibroblasts and endothelial cells only occasionally exhibited positive CA IX immunostaining in pancreatic cancer, non-small-cell lung cancer and breast cancer. In contrast, desmoplastic tissue exhibited intense CA IX expression in colorectal cancer, indicating that the formation of the tumour stroma may underlie different biological processes in these cancers.

Comparing CA IX-positive and CA IX-negative tumours, no statistically significant difference was observed with regard to age, tumour localization, tumour stage, tumour size, lymph node and distant metastases and tumour differentiation. Interestingly, none of the tumours with distant metastases exhibited CA IX expression; however, this finding is biased by the selection of pancreatic cancer patients undergoing surgical
interventions. Nonetheless, these findings are entirely in agreement with the results of Kivelä et al. and Bartosova et al., who could not demonstrate any significant relationship between CA IX expression and clinicopathological parameters.

The mutation of the p53 tumour suppressor gene is an early event in the tumorigenesis of pancreatic cancer. Nuclear immunoreactivity for p53 protein may indicate mutant forms of the gene because of the short half-life of the wild-type protein. In order to elucidate a potential association with CA IX expression, immunostaining for p53 was performed in parallel sections. In this study, 35% of pancreatic cancer specimens stained positively for the p53 protein. This finding is in line with the data of Dong et al., who reported a frequency of p53 expression ranging from 40 to 63% in Western countries, 15–49% in Japanese and up to 70% in Chinese. We observed a statistically significant association of p53 expression with undifferentiated or poorly differentiated pancreatic cancers. The same relationship between p53 expression and tumour differentiation has been demonstrated by others. However, we could not detect any further correlation between p53 expression and clinicopathological factors or patient characteristics. We also failed to find any correlation between the expression of CA IX and p53 in pancreatic cancer. This is supported by an in vitro study by Lieskovska et al., who could not establish a direct relationship between the expression of CA IX and p53 in HeLa cells.

In histological sections, the degree of neovascularization can be determined morphometrically using antibodies to endothelial cell antigens such as CD34, which is expressed by endothelial cells in the desmoplastic tumour stroma. Recent studies by Niedergethmann et al. and Linder et al. have demonstrated that the degree of microvessel density may be associated with an advanced stage of pancreatic cancer. As CA IX expression has been shown to be induced by hypoxia, we analysed microvessel density in our specimens. In our cohort, microvessel density did not correlate with CA IX expression or any of the clinicopathological parameters. Interestingly, in head and neck squamous cell carcinoma and non-small-cell lung cancer, CA IX expression correlates with a higher microvessel density. However, Koukourakis et al. demonstrated CA IX expression occurring mainly in very poorly vascularized tumours. Thus, the role of CA IX in tumour angiogenesis remains rather inconclusive.

Recent studies have indicated that the inhibition of CAs may be a target for the treatment of cancers over-expressing CAs. Thus, various CA inhibitors have been tested in several human cancer cell lines, including colon, ovarian, renal and lung cancer cells, resulting in potent growth inhibition which has been assumed to involve the inhibition
of CAs IX, XII and XIV. Interestingly, in a study by Wingo et al., human CA IX was specifically inhibited by three classic sulphonamides, including acetazolamide. Recent studies have further established the role of one of these sulphonamides, i.e. acetazolamide, in the suppression of cancer cell growth. Using renal cancer cells, acetazolamide led to a significant suppression of invasion, indicating that this substance may exert various biological effects, including the suppression of proliferation and invasion of cancer cells in vitro. Based on these observations, we also treated three pancreatic cancer cell lines with acetazolamide, which resulted in the significant inhibition of cell proliferation in two of the three cell lines analysed.

In summary, we found CA IX expression to be present in a sub-group of pancreatic cancers. CA IX expression was not associated with advanced disease and was independent of p53 expression and microvessel density. We conclude that the role of CA IX is limited to a sub-group of pancreatic cancers, and may rather contribute to the early phases of pancreatic carcinogenesis. However, the growth inhibition of pancreatic cancer cells by CA inhibitors indicates that CAs may well represent interesting targets for carbo-anhydrase inhibitors in this malignancy.

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