Gut 1996; **38:** 403–409

p53 Tumour suppressor gene expression in pancreatic neuroendocrine tumour cells

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Abstract

Neuroendocrine pancreatic tumours grow slower and metastasise later than ductal and acinar carcinomas. The expression of the p53 tumour suppressor gene in pancreatic neuroendocrine tumour cells is unknown. Pancreatic neuroendocrine cell lines (n=5) and human tumour tissues (n=19) were studied for changed p53 coding sequence, transcription, and translation. Proliferative activity of tumour cells was determined analysing Ki-67 expression. No mutation in the p53 nucleotide sequence of neuroendocrine tumour cell was found. However, an overexpression of p53 could be detected in neuroendocrine pancreatic tumour cell lines at a protein level. As no p53 mutations were seen, it is suggested that post-translational events can also lead to an overexpression of p53.

(*Gut* 1996; **38:** 403–409)

Keywords: pancreatic tumour, tumour suppressor gene.

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Accepted for publication 24 August 1995

Carcinomas of the pancreas are common forms of cancer in the gastroenteropancreatic system. They can be classified according to their morphological and functional characteristics. The non-neuroendocrine pancreatic carcinomas are mainly of ductal origin accounting for over 90% of all pancreatic tumours. Among other rare neoplasms (for example, giant cell and epidermoid carcinomas, lymphomas, cystadenocarcinomas, etc), carcinomas with acinar cell differentiation represent a distinct entity within pancreatic neoplasms with an overall frequency of about 1% of all pancreatic carcinomas.[1–3] Recent studies showed that acinar tumours can even be subdivided whereby approximately one third of these tumours possess both neuroendocrine and exocrine features (amphicrine tumours). Neither amphicrine nor pure exocrine acinar carcinomas showed immunoreactivity for the tumour suppressor gene p53.[4] The residual 5% of all pancreatic neoplasmas are of islet cell or other neuroendocrine origin.

Neuroendocrine tumours of the pancreas (especially gastrinomas, VIPomas, and glucagonomas) are malignant in about 60% of cases. Half of these tumours are clinically functional – that is, they exhibit an endocrine activity (secreting insulin, gastrin, vasoactive intestinal polypeptide, glucagon, pancreatic polypeptide, etc) – whereas the other half of these tumours does not cause endocrine symptoms.[5]

Based on their growth and metastatic rate, pancreatic carcinomas can be clinically divided into two groups, neuroendocrine and non-neuroendocrine metastatic tumours. In contrast, non-neuroendocrine carcinomas exhibit clearly faster growth along with a higher metastatic potential.

In various neoplasms of the gastroenteropancreatic system the expression of the p53 tumour suppressor gene has been implied as an important factor for cell growth and has also been extensively investigated in a large number of other human tumours.[6] The 53 kDa nuclear phosphoprotein encoded by 393 amino acids has often been found to be functionally inactivated by single point mutations occurring in 99% of the cases within four of five evolutionarily highly conserved domains.[7] These point mutations generally lead to an extended half life caused by protein stabilisation and conformational changes inhibiting the normal function of the protein. Other inactivating mechanisms for wild type p53 include changed protein phosphorylation and complex formation with mutant p53 protein, viral oncogene proteins, the mdm2-gene product or with members of the heat shock protein family,[8–11] resulting in conformational changes and cytoplasmatic sequestration. p53 Has been implicated in the cellular response mechanisms to DNA damage and has been found to induce apoptosis in colon carcinoma cell lines.[12 13]

Similarly in ductal pancreatic carcinomas, a variety of p53 inactivating mutations have been found in cell lines and in tumour tissues.[14–16]

In contrast, acinar pancreatic carcinomas, resembling clinically and prognostically ductal carcinomas do not seem to contain mutated p53,[4] suggesting that p53 mutations may not influence cellular proliferation. So far, neuroendocrine pancreatic tumours, known to proliferate slowly, have not been studied for changed expression and synthesis of p53.

We investigated a variety of neuroendocrine pancreatic tumour cell lines for p53 point mutations, as well as the level of mRNA and protein. In parallel, tissue specimens (n=19) were tested for p53 immunoreactivity.

Methods

Cell lines and tumour samples

The following pancreatic neuroendocrine cell lines were used: Bon,[17] QGP1,[18] AR42J,[19] RIN 38,[20] and InR 1G9.[21] Pancreatic ductal cell lines Capan1 and Capan2,[22] DanG,[23] and Panc1[24] as well as the hepatoma cell lines HepG2,[25] Huh7,[26] and PLC/PRF/5[27] and the rat pheochromocytoma cell line PC 12[28] served as controls.

TABLE I *Oligodeoxynucleotides used for PCR amplification of p53 sequences of genomic and complementary DNA*

Primer number	*Primer sequence*	*Length of amplificate*	*Intron*	*Exon*	*Intron*
3696	TTC CTC TTC CTG CAG TAC TC		(4) 15	(5) 376–380	
3697	ACC CTG GGC AAC CAG CCC TGT	245			(5) 26–46*
3130	ACA GGG CTG GTT GCC AGG GT			(6) 668–672	(6) 15
3128	AGT TGC AAA CCA GAC CTC AG	183			(5) 26–46*
3488	GTG TGG TCT CCT AGG TTG GC		(6) 14	(7) 673–678	
3489	GTC AGA GGC AAG CAG AGG CT	189			(7) 45–65*
3129	TAT CCT GAG TAG TGG TAA TC		(7) 12	(8) 783–791	
3127	AAG TGA ATC TGA GGC ATA AC	213			(8) 47–67*
4165	CAG AAA ACC TAC CAG GGC AGC			(4) 298–318	
4164	TGC TCG CTT AGT GCT CCC TGG	621		(8/9) 901–921	
9585	ACC AAG GCA ACT ATG GCT TCC			(4) 302–322	
9586	GTG CTC TCT TTG CAC TCC CTG	615		(8) 896–916	

Numbers for primer pairs were arbitrarily chosen. The number in brackets given above refers to the specific intron or exon number. In case of exons, the number of coding nucleotides is given. In the case of introns the number of primer nucleotides of the primers chosen within the intron is given. With the exception of the last two sequences, which correspond to rat cDNA, all other sequences are human. Only the last four sequences given correspond to cDNA but not genomic DNA. *Shows that the primer is completely positioned within the respective intron. Numbers given refer to the exons of the last proceeding coding nucleotide. The relative position of the oligodeoxynucleotides chosen is shown in Fig 1.

Tumour tissues studied included one gastrinoma, two insulinomas, one VIPoma, and 15 non-functional islet cell carcinomas as well as three hepatomas. They were either frozen, aceton fixed or paraffin wax embedded, formalin fixed.

Antibodies

Antibody CM1 raised in rabbits with recombinant wild type p53 was purchased from Medac, Hamburg, Germany. This antibody reacts with human and rodent p53. Antibody 1618 was raised in rabbits against a peptide comprising the 12 N-terminal amino acids. This antibody reacts also with human and rodent p53. Murine, monoclonal antibody DO7 was obtained from Dianova, Hamburg, Germany. This antibody recognises amino acids 1–45 of wild type p53 and is specific for human tissues and cells. Murine, monoclonal antibody pAb240 was obtained from Medac, Hamburg, Germany. This antibody reacts with amino acids 212–217 of human mutant p53 or amino acids 206–211 of mouse mutant p53.

Murine, monoclonal antibody pAb1801 was obtained from Medac, Hamburg, Germany. This antibody reacts with wild type human p53 and recognises the N-terminal domain of p53. Antibody MiB1 was obtained from Dianova, Hamburg, Germany, and reacts with the fragment of wild type Ki67. The immunohistochemical staining was performed as previously described.[29] For immunofluorescence microscopy a Texas-Red conjugated goat-anti-mouse-IgG, Dianova, Hamburg, Germany was used. To estimate the degree of the neuroendocrine cell proliferation, Ki67 labelling indices were determined by evaluation of 300 cells. Results are given in positively stained nuclei per 100 cells.

DNA and RNA preparation and reverse transcription

Genomic DNA was prepared by proteinase K digestion followed by phenol/chloroform extraction according to standard protocols.[30]

Total RNA was obtained by the guanidinium-thiocyanate-method followed by a CsCl-gradient centrifugation step essentially as already described.[30] For cDNA analysis 5 μg of total RNA were reverse transcribed using polymerase chain reaction (PCR) – buffer (see later), 2·5 μM random hexamer primer (Boehringer Mannheim, Mannheim, Germany), 200 U M-MLV-reverse transcriptase (Gibco BRL, Berlin, Germany), and 1 mM dNTPs (Boehringer Mannheim, Mannheim, Germany). The dNTPs used for reverse transcription were sufficient for subsequent PCR.

Oligodeoxynucleotides and PCR amplification procedure

The oligodeoxynucleotides used for PCR and direct sequencing were synthesised on an Applied Biosystems (Weiterstadt, Germany) DNA synthesiser. Table I gives the sequences of the oligodeoxynucleotides used for PCR.

PCR amplification was performed on a Biomed Thermocycler 60 (Theres, Germany) and consisted of 35 cycles of one minute at 92°C denaturation, one minute 30 seconds annealing, and two minute 30 seconds at 72°C extension. The amplification was preceded initially with a five minute 92°C denaturation step and followed by a 10 minute 72°C extension step. Annealing temperatures were 61°C for cDNA, 60°C for Exon 5–7, and 58°C for Exon 8. We used 2·5 U Taq-Polymerase (Promega, Heidelberg, Germany), the supplied reaction buffer, 0·5 mM of each primer, and 150 μM dNTPs in a 100 μl reaction volume.

The amplificate was resolved on a 1% agarose-TRIS-borate-EDTA-gel, visualised by ethidium bromide staining and photographed.[30] Bands corresponding to the expected size were excised, eluted using the IQIAEX-kit (Diagen, Hilden, Germany). In general 20% of the resulting eluate was sufficient to perform direct sequencing.

Sequencing protocol

Sequencing by the method of Sanger was performed using the Sequenase 2·0 Kit (USB, Darmstadt, Germany and [^{35}S]-dATP (Amersham, Braunschweig, Germany) according to the protocol proposed by Thein.[31] The

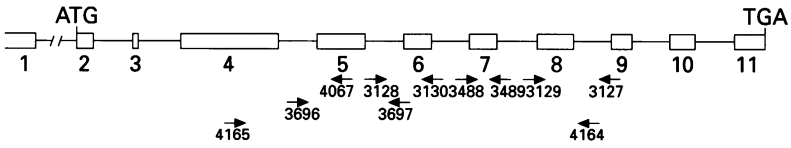

Figure 1: Position of oligodeoxynucleotides within the p53 gene used for PCR analysis on human samples; note that the oligodeoxynucleotide 4164 spans the intron between the exons 8 and 9.

sequencing primers were identical to those used for PCR amplification.

Northern blotting

mRNA was analysed by northern blotting using 20 μg of denatured total RNA separated on a formaldehyde-agarose-gel and transferred to a nylon membrane (Gene Screen, DuPoint, Bad Homburg, Germany) by SSC mediated diffusion blot. pC53SN3 containing human wild type p53 cDNA was a kind gift from A Teresky, Princeton, NJ, USA. The BamHI-fragment of pC53SN3 was used to hybridise to human mRNA and the XhoI/SstII-fragment of p11–4[32] (kind gift from J Martinez, Princeton, NJ, USA) for murine mRNA. DNA probes were radioactively end labelled with [32P]-dCTP (Amersham, Braunschweig, Germany) using the multiprime DNA labelling kit (Amersham, Braunschweig, Germany) according to the manufacturer's instructions. Hybridisation was done at 58°C following the procedure described by Bouwmester *et al.*[33]

Immunoprecipitation and western blotting

Radioactive cellular lysates were prepared by metabolic labelling of subconfluent 100 mm dishes of the described cell lines using 200 mCi α-[^{35}S]-methionine (Amersham, Braunschweig, Germany). Cells were lysed under RIPA conditions,[30] cleared by centrifugation, and comparable amounts of protein as determined by liquid scintillation were incubated at 40°C with 15 ml protein A-sepharose beads to which 3 ml of purified monoclonal antibody or 5 ml rabbit antiserum have been coupled previously. Intensive washing steps were included. The coupled protein was then separated by SDS-PAGE on a 10% gel. Subsequently the gel was dried and exposed to a film (*X* Ray, Kodak, Berlin, Germany). ^{14}C-labelled molecular weight standards were purchased from Amersham, Braunschweig, Germany. For non-radioactive protein detection, comparable amounts (matched by cell counting) of total protein from centrifugation cleared cellular lysates were subjected to immunoprecipitation as already described, separated by SDS-PAGE, transferred to nitrocellulose (Schleicher and Schüll, Dassel, Germany) by electroblotting and then treated according to the manufacturer's instructions (ECL-Western blotting kit, Amersham, Braunschweig, Germany).

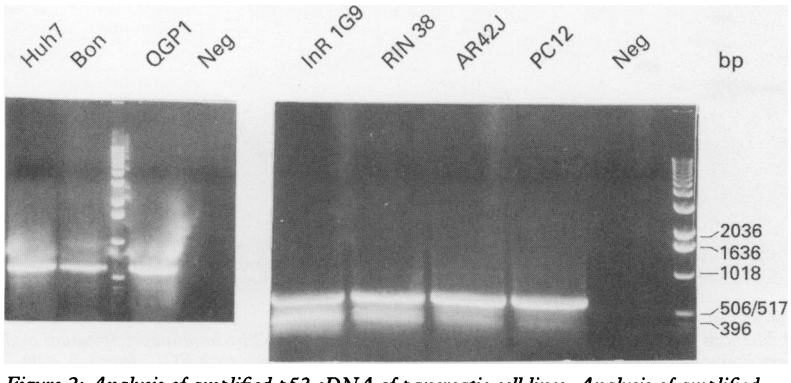

Figure 2: Analysis of amplified p53 cDNA of pancreatic cell lines. Analysis of amplified p53 cDNA on a preparative 1% agarose gel after reverse transcription of 5 μg total RNA and 35 cycles of PCR. The cell lines are given on the top. The numbers on the right correspond to base pairs (bp) of a marker. Negative control reaction does not contain any DNA except oligodeoxynucleotides. Oligodeoxynucleotides 4164 and 4165 were used to amplify human p53 cDNA, oligodeoxynucleotides 9585 and 9586 (not listed in Fig 1) for amplification of rat and hamster cDNA. The total reaction volume was loaded onto the gel (for details see Methods).

Results

In gastroenteropancreatic cancer, especially in pancreatic carcinomas, almost all the inactivating p53 mutations have been detected within four of five evolutionarily highly conserved domains corresponding to exons 4 to 8. Therefore, PCR mediated amplification of a 621 bp fragment of human cDNA (615 bp for murine cDNA) spanning these exons was chosen. In addition, primer sets for amplification of exons 5 to 8 of genomic DNA were designed and localised to the neighbouring introns (Fig 1 and Table II).

Neuroendocrine pancreatic tumour cell lines do not contain typical point mutations of p53

Amplification of cDNA and genomic DNA resulted in a reaction product of the expected size for every cell line studied. Altered sizes suggesting major sequence changes were not seen. Figure 2 shows products of reverse transcribed and amplified DNA visualised on an ultraviolet screen. All neuroendocrine cell lines and the human hepatoma cell line Huh7 displayed a band of the correct size. This was also the case for genomic DNA amplifications (data not shown). Negative control samples (Neg) did not contain any template DNA. Direct sequencing of the amplified DNA fragments showed only one point mutation in each of the ductal pancreatic cell lines Panc1 and Capan1 as well as in the hepatoma cell line PLC. The mutations were found to be present in both alleles. For Panc1 the G to A exchange resulted in an Arg to His substitution at amino acid position 273 (Fig 3). The same base exchange was discovered in Capan1 leading to Ala to Val substitution to codon 159. In PLC, a G to T base substitution replaced an Arg by a Ser at codon 249 (data not shown). This was not surprising as this codon is commonly mutated in human hepatocellular carcinoma. In contrast, none of the neuroendocrine cell lines displayed point mutations within the examined DNA sequences.

Wild type protein p53 varies in neuroendocrine tumour cell lines

By northern blot analysis, a mRNA of an expected length of 2·8 Kb was found in all neuroendocrine cell lines (QGP1, InR 1G9,

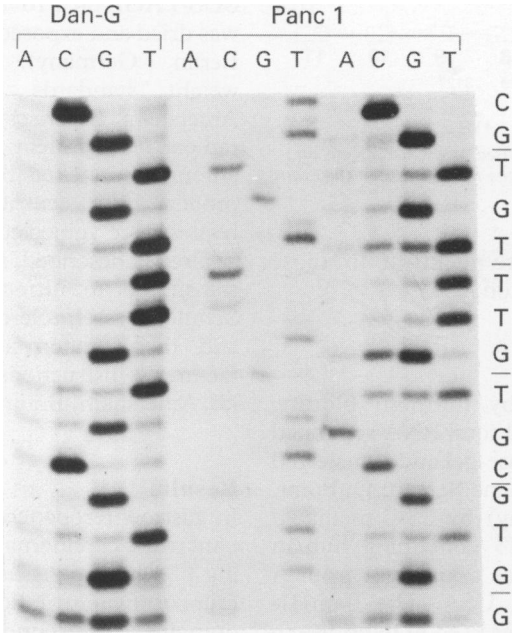

Figure 3: Identification of point mutations in pancreatic cell lines. Autoradiograph of a direct sequencing reaction of PCR amplified exon 8 (oligodeoxynucleotides 3127 and 3129) from genomic DNA of cell lines Dan-G and Panc1 (both strands). The p53 mutation of Panc1 showed a G to A mutation corresponding to mutation from Arg to His at amino acid position 273. For comparison, the Dan-G wild type sequence is shown.

RIN 38, AR42J, PC12). For the cell line Bon, a transcript was only detected by PCR amplification, but not by northern blotting. Transcripts of 2·8 Kb were also detected by northern blotting in the ductal pancreatic carcinoma cell lines Capan 1 and 2. Transcripts of DanG and Panc1 were slightly larger in size (roughly 3·2 Kb, data not shown).

To investigate whether the observed changes found so far had an influence on p53 protein expression, we performed metabolic labelling with [α^{35}S]-methionine followed by immunoprecipitation (Fig 4). All cell lines showed an immunoprecipitate of M_r about 50 kDa except for the cell line Bon. The p53 protein precipitated from the murine cell lines InR 1G9 and RIN 38 had a slightly lower apparent molecular weight (M_r about 50 kDa) whereas Capan1 showed a protein of a larger molecular weight of about 56 kDa. Panc1 displayed an additional signal of an apparent molecular weight of 60 kDa. Interestingly, the monoclonal antibody pAb 240 specific for the mutant p53 phenotype, failed to precipitate p53 from Huh7, whereas all other antibodies tested did (data not shown). This suggests that overexpression of p53 in this cell line is probably not due to mutations leading to conformational changes recognised by pAb 240. Furthermore, we examined the cell lines by immunoprecipitation combined with western blotting using an enhanced chemoluminescence detection system (Fig 5). The neuroendocrine cell line QGP1 as well as the control cell lines Huh7 and PLC showed a detectable protein of the correct molecular weight using the polyclonal antiserum 1618 for precipitation and the monoclonal antibody pAb 1801 for detection (Fig 5A). In contrast, Bon as well as AR42J and PC12 gave no signal. Capan2 and DanG were also negative (data not shown). Using pAB 240 for precipitation and detection we were able to show a signal of the appropriate size for all

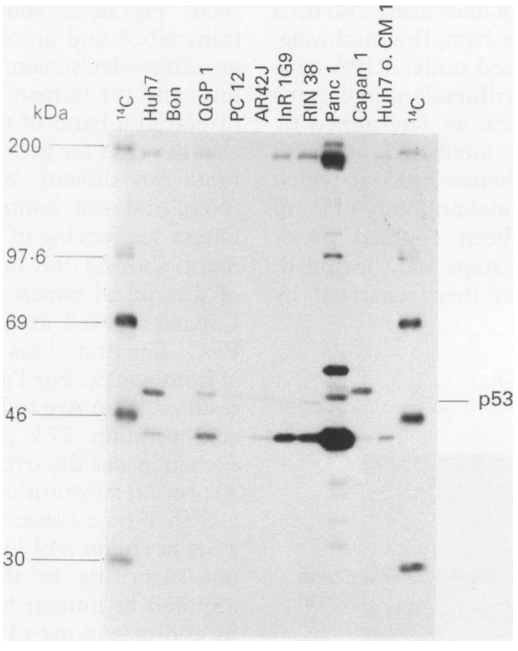

Figure 4: Immunoprecipitation of p53 in various cell lines. Lysates prepared as described in Methods from [^{35}S]methionine-labelled cells were immunoprecipitated with antibody CM1, directed against recombinant human p53, and separated on a 10% SDS-PAGE. Comparable amounts of protein were used as determined by scintillation counting. In lane 11, Huh7 lysate has been incubated without antibody CM1 as a control. p53 Which varies in size around the indicated p53 marker is detectable in both neuroendocrine and ductal cell lines. Standard ^{14}C marker proteins were used (Amersham).

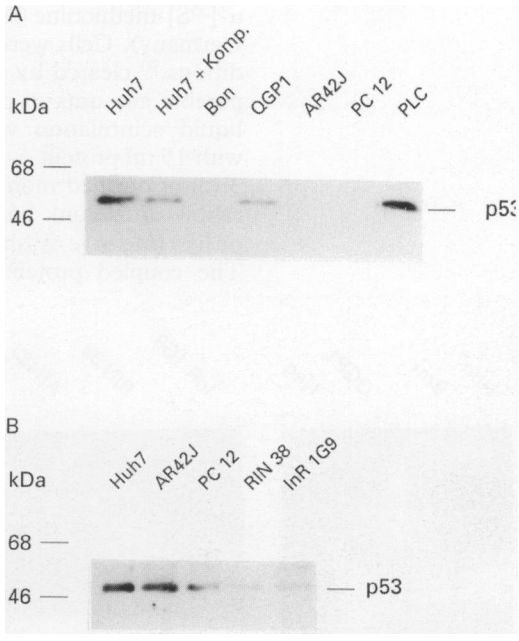

Figure 5: Identification of p53 by immunoprecipitation and subsequent western blot analysis with ECL-detection. (A) For precipitation, antiserum 1618 directed against the 12-carboxyterminal amino acids of human p53 was used and for western blotting monoclonal antibody pAb 1801, recognising amino acids 45 to 91 of the human p53 amino terminus, was used. (B) The mutation specific antibody pAb240, recognising an epitope located between amino acid 161 and 220 present in human as well as rodent p53, was used for both immunoprecipitation and detection.

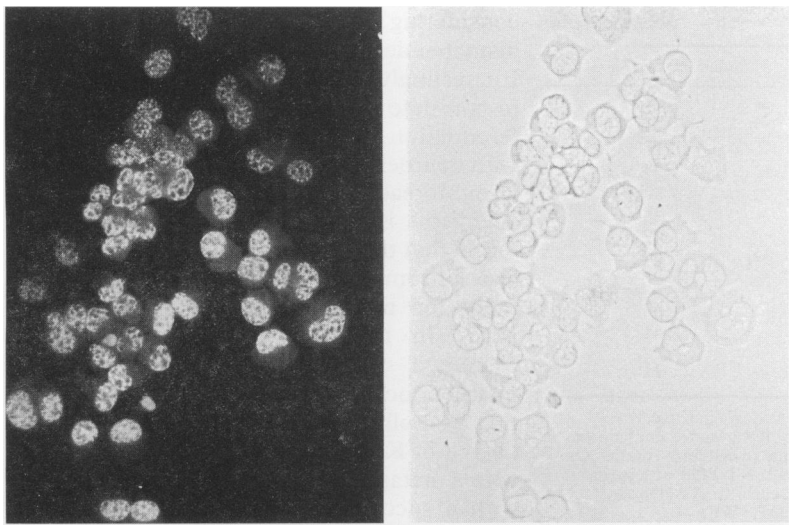

Figure 6: Detection of p53 by immunochemistry. Immunohistochemistry of insulinoma cells (RIN 38) using antibody CM1 (see Fig 4) leading to positive staining of almost all nuclei. Immunofluorescence (left); phase contrast (right), magnification ×400.

murine neuroendocrine cell lines (InR 1G9, RIN 38, AR42J, PC12; Fig 5B and data not shown). Surprisingly, Huh7 taken as a control cell line showed a signal in this immunoprecipitation western blot assay whereas pAb 249 was unable to detect the protein in the radioimmunoprecipitation assay.

Immunohistochemical analysis showed an exclusively nuclear staining[34 35] for seven of 12 cell lines tested with the antiserum CM1 against p53 (Fig 6). To test whether the degree of proliferation would correlate with the p53 staining, we performed Ki-67 staining as a marker for cellular proliferation[36] in parallel. Figure 7 shows two typical immunohistochemical staining results for Ki-67.

Neuroendocrine cell lines InR 1G9 and RIN 38 as well as the ductal cell lines Capan1, Capan2, and Panc1 and the hepatoma cell lines Huh7 and PLC showed a clear nuclear staining for p53 with CM1. In contrast, the human neuroendocrine cell lines Bon and QGP1 as well as the murine AR42J, the ductal DanG and the pheochromocytoma PC12 showed no nuclear staining signals. The immunohistochemical results for Bon, InR 1G9, RIN 38, Capan1, Panc1, Huh7, and PLC corresponded with the results obtained by radioimmunoprecipitation. QGP1, AR42J and PC12 showed no immunohistochemical signal despite the precipitable p53 protein. Interestingly, the positive immunohistochemical staining for Capan2 was in contrast with the negative western blot results using pAb 240. DanG was negative both in the western blot analysis and in the immunohistochemical staining. The ductal cell lines as well as the human hepatoma cell lines displayed a Ki-67 LI varying from 35–83%. Three pancreatic neuroendocrine cell lines (Bon, QGP1, and RIN 38) showed a positive nuclear staining with Ki-67 in almost all cells. This is in contrast with the neuroendocrine pancreatic tumours where only a Ki-67 LI of below 5% is seen. Microwave treatment of paraffin wax embedded tissue sections did not increase the immunoreactivity of antibody Mib1. Thus, no correlation between the immunoreactivity for p53 and Ki-67 was seen in neuroendocrine cell lines. Table II summarises the results obtained for the various cell lines studied.

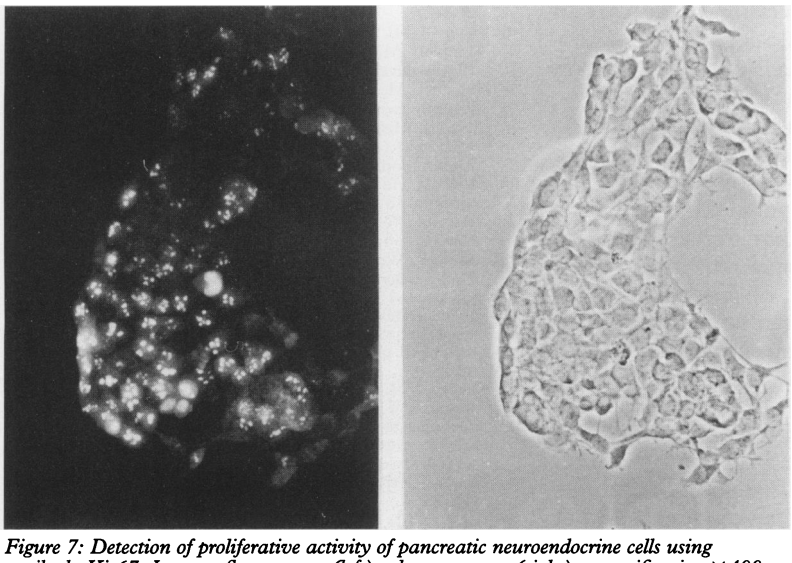

Figure 7: Detection of proliferative activity of pancreatic neuroendocrine cells using antibody Ki-67. Immunofluorescence (left); phase contrast (right); magnification ×400.

In 19 neuroendocrine tumour tissue specimens no immunoreactivity for p53 using the polyclonal antiserum CM1 was found. However, human hepatomas (n=3), used as positive controls, showed a strong nuclear immunostaining (data not shown). Sequence analysis was not performed because of the lack of sufficient amounts of human tumour tissue.

Discussion

This is the first comprehensive study on the expression of the tumour suppressor gene p53 in pancreatic neuroendocrine cancer. Almost all neuroendocrine cell lines examined, contained increased protein and mRNA levels of wild type protein p53. In contrast with ductal pancreatic cancer cells, known to express mutated p53, deletions or point mutations within the evolutionary highly conserved parts of the coding sequence were not detected in pancreatic neuroendocrine cells. These findings are based on the examination of both pancreatic neuroendocrine tumour cell lines (n=5) and pancreatic neuroendocrine tumour tissues (n=19). At a protein level, increased amounts of p53 were detected in almost all pancreatic neuroendocrine cell lines by western blot analysis, radioimmunoprecipitation and, only in part, by immunohistochemistry. In neuroendocrine tumour tissues, no immunoreactivity for p53 was seen. At the mRNA level, almost all neuroendocrine tumour cell lines had detectable amounts of p53 specific mRNA. Nucleotide sequence analysis of the evolutionary highly conserved stretch encompassing 5 exons of the coding sequence did not show any deletions or point mutations for pancreatic neuroendocrine cells. In contrast and as already previously shown, several pancreatic ductal[14–16] and hepatoma[37 38] cell lines and carcinomas contained point mutations within the evolutionary conserved part of the coding sequence (Fig 1). Until now, detectable p53 at a protein and

TABLE II *Summary of results obtained for various cell lines*

Cell line	*Northern*	*cDNA*	*IF (CM1)*	*WB*	*RIP*	*Mutation*	*IF (Ki-67 LI)*
Hep G2	+	+	nt	nt	nt	nt	nt
Huh7	+++	++	+++	+	+	−	57
PLC	+	+	+++	+	+	+	83
Bon	−	+	−	−	−	−	34
QGP1	++	++	−	−	+	−	5
Capan1	++	++	++	+	+2	+	35
Capan2	(+)	(+)	++	−	nt	−	47
DanG	+1	+	−	−	nt	−	64
Panc1	(+)1	++	+++	+	+	+	nt
AR42J*	+	+	−	+	+	−	−
PC 12*	+	+	−	+	+	−	−
InR 1G9†	+	+	+++	+	+3	−	−
RIN 38*	+	+	+++	+	+3	−	11

Signal intensity: −: negative; (+): weakly positive; +: positive; ++: strongly positive; +++: very strongly positive. Northern: northern blot analysis of 20 μg total RNA loaded per lane was done with the BamHI-fragment of cDNA clone pC53SN3 for the human cell lines and the Xho I/Sst II-fragment from cDNA clone p11-4 for murine cell lines; cDNA: RT-PCR with species-specific cDNA-oligodeoxynucleotides (exon 5–8) of 5 μg total RNA each; IF (CM1): immunofluorescence staining of acetone fixed cells with antiserum CM1; WB: immunoprecipitation with the C-terminal peptide antiserum 1618 (pAb 240 for murine cell lines respectively) with subsequent ECL detection in combination with western blot; RIP: radioimmunoprecipitation of [35S]-metabolically labelled cells with CM1; Mutation: sequence analysis of genomic or complementary DNA; IF (Ki-67 LI); immunofluorescence microscopy of acetone fixed cells with Ki-67 or Mib-1; the percentage of positively stained cells nuclei is given; −: no nuclear stained cells; nt: not tested; *: rat cell lines; †: hamster cell line; 1: slightly larger transcript; 2: higher; 3: lower apparent molecular weight in SDS-PAGE.

mRNA level was considered pathognomonic for a deletion or mutation of p53. Our study shows that an overexpression of p53 can also result, at least in some pancreatic neuroendocrine cancer cells, from post-translational modifications of the protein, as no deletions or mutations were found. This could lead to a changed state of p53 phosphorylation or to a protein stabilisation similar to a switch between the wild type and mutant conformation of p53 with wild type sequence.[39] An alternative explanation for our findings could be that the mutation or deletion was missed based on incomplete sequencing. This seems unlikely, however, as all mutations described so far have been only seen in the evolutionary highly conserved region. In addition, several studies on the human hepatoma cell line Huh7 showed that the well known overexpression of p53 could not be accounted for by a deletion or mutation despite sequence analysis of the whole cDNA. Similar to our findings, it was postulated, that post-translational modifications could be responsible for overexpression of p53. Thus, variable degrees of p53 overexpression could lead, especially in the case of post-translational modifications, to less pronounced effects on the cell cycle resulting in different forms of pancreatic cancer.

Using immunohistochemistry in combination with antibodies reacting with wild type and mutated p53[40 41] we show that only a small portion of the pancreatic neuroendocrine cancer cells contain increased protein levels of p53. This is particularly relevant for immunohistodiagnostics of human tumour tissue, where tissue is often only available for histochemistry but it escapes biochemical and molecular biological analysis because of insufficient tissue amounts. In addition, PCR analysis of small amounts of tumour tissue is of limited value, because an exact attribution of overexpressed p53 in tissue homogenates is not possible. Thus, our study suggests that lack of p53 immunoreactivity by immunohistochemistry has to be interpreted with care, considering also the negative immunohistochemical results obtained so far.[42] Interestingly, an overexpression of p53 seems to correlate with neuroendocrine cellular differentiation – that is, small cell lung cancer with neuroendocrine histology are found to be immunoreactive for p53 by immunohistochemistry in most cases.[43 44] Similarly, we found that the expression of p53 correlated, at least in some cell lines, with an immunoreactivity for the cellular proliferation marker Ki67. In this context, Chaudhry *et al*[45] examined a large number of metastatic neuroendocrine tumours of the small intestine as well as pancreas and found that the degree of Ki67 expression corresponded well with a metastatic tumour spread and inversely with a success of an antitumour treatment. Thus, it is tempting to speculate that p53 expression in neuroendocrine pancreatic tumour disease correlates, at least in part, with the state of the neuroendocrine tumour cell differentiation (see also Barbareschi *et al*[43]). Future studies will have to deal with the identification of components responsible for post-translational modifications during cellular differentiation of neuroendocrine tumour disease.

We wish to thank L Schmitt for expert technical assistance and M E Hearne for synthesising the DNA oligodeoxynucleotides. We are grateful to H Stein, Department of Pathology, Klinikum Benjamin Franklin, FU Berlin, Germany, for the gift of Ki-67 monoclonal antibody and to A Teresky and J Martinez from the Department of Molecular Biology, University of Princeton, NJ, USA, for supplying the plasmids pC53SN3 and p11-4 respectively. We thank Dr Avril Smith, Klinikum Benjamin Franklin, FU Berlin, Germany, for critically reading the manuscript.

This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Dr Mildred Scheel Stiftung to KM and BW as well as the Vaillant-Stiftung, to KM.

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