American Journal of Pathology, Vol. 147, No. 4, October 1995

Short Communication

p53-Independent Expression of the Cyclin-Dependent Kinase Inhibitor p21 in Pancreatic Carcinoma

Joseph A. DiGiuseppe,* Mark S. Redston,* Charles J. Yeo,† Scott E. Kern,*‡ and Ralph H. Hruban*‡

From the Departments of Pathology, * *Surgery,*† *and Oncology,*‡ *The Johns Hopkins Medical Institutions, Baltimore, Maryland*

The p53 tumor suppressor gene is mutated in the majority of pancreatic adenocarcinomas, and several studies have suggested that loss of p53 function may contribute to the aggressive clinical behavior of pancreas cancer. Although immunocytochemical accumulation of the p53 gene product has previously been assessed as a marker for p53 mutations in cancers of the pancreas and other organ systems, the relationship between p53 mutations and p53 protein accumulation is variable. The cyclin-dependent kinase inhibitor, p21 (also known as WAF1 and CIP1), is induced by wild-type but not mutant p53, and recent work has implicated p21 as a downstream mediator of the growth-suppressing and apoptosis-promoting functions of wild-type p53. In the present work, we sought to determine whether loss of p21 expression could more precisely identify those tumors with p53 mutations and/or loss, compared with immunocytochemical assessment of p53 protein accumulation. We evaluated p53 and p21 expression immunohistochemically in a series of 21 ductal adenocarcinomas of the pancreas with known p53 mutational status. Diffuse overexpression of p53 was found in 3 of 8 cases (38%) with wild-type p53 and 7 of 13 cases (54%) with p53 mutations with or without loss of heterozygosity at 17p. Surprisingly, expression of p21 correlated neither with p53 mutational status nor with p53 protein expression. In particular, strong p21 expression was seen even in carcinomas in which molecular analysis revealed a frameshift mutation in one allele of p53 and loss of the second. These data suggest that p21 expression in pancreatic adenocarcinoma may also be induced by a p53-independent pathway and that p21 expression, as assessed immunocytochemically, does not reflect the functional status of p53 in these carcinomas. (Am J Pathol 1995, 147:884–888)

The tumor suppressor gene, p53, has assumed major significance as the single most commonly mutated gene yet identified in human neoplasms.[1] In the last several years, our understanding of the normal function of the p53 gene product has improved, yielding insights into the mechanisms of p53-mediated tumor suppression. The p53 gene product has been implicated both in the G_1 cell cycle arrest seen after DNA damage[2–4] and in apoptosis triggered under certain conditions.[5–7] p21 (also known as WAF1/CIP1) is an inhibitor of cyclin-dependent kinases, the activation of which is required for cell cycle progression.[8,9] The discovery that p21 is induced by wild-type p53, but not mutant p53, led to the suggestion that the tumor suppressor function of p53 might be mediated through induction of p21, an hypothesis that has been supported experimentally.[10]

Supported in part by NIH P50-CA62924 and a Resident Research Project Award from the Department of Pathology.

Accepted for publication June 26, 1995.

Address reprint requests to Dr. Ralph H. Hruban, Department of Pathology, Meyer 7–181, The Johns Hopkins Hospital, 600 North Wolfe St., Baltimore, Maryland 21287-6417.

Several studies have examined the status of p53 in pancreas cancer, either by sequencing the gene or by immunohistochemical assessment of accumulation of the p53 protein[11–21](reviewed in Ref. 22). Wild-type p53 is a short-lived protein, which is degraded by the ubiquitin system.[23,24] In contrast, some tumors harboring mutant p53 accumulate nuclear p53 protein, and this overexpression has been used as an immunocytochemical marker for p53 mutations. However, the relationship between p53 mutations and p53 accumulation is imprecise,[25] and this lack of correlation has recently been demonstrated in pancreas cancer.[13]

We have recently reported the frequent accumulation of the p53 gene product in a series of pancreatic cancers.[19] Of note, diffuse p53 accumulation appeared to correlate with poor prognosis in our study, although the relationship did not reach statistical significance.[19] One potential explanation for lack of a stronger correlation between p53 accumulation and patient outcome is that some of these cases may have been misclassified with respect to functional p53 status because of the imperfect relationship between p53 mutations and protein accumulation. In an effort to improve the sensitivity and specificity of immunohistochemical staining for the p53 gene product in identifying p53 mutations, we assessed both p53 and p21 expression in a series of pancreas cancers with known p53 mutational status. If functional p53 is required to induce p21 expression, as suggested above, then p21 expression should be lost in tumors lacking wild-type p53 activity. Surprisingly, we found that p21 expression correlates neither with p53 mutational status nor with p53 protein overexpression, suggesting that, in carcinomas of the pancreas, p21 expression may also be induced by a p53-independent pathway.

Materials and Methods

Patients and Specimens

Specimens from 21 patients with pancreatic adenocarcinoma who had undergone pancreaticoduodenectomy at The Johns Hopkins Hospital were selected on the basis of tissue availability.[20] All of these patients had preoperative and intraoperative evaluation to exclude extrapancreatic spread of these tumors before resection. Data regarding patient demographics, pathological grade, and stage of these tumors have been reported elsewhere.[20]

p53 Gene Mutations and 17p Allelic Loss

DNA was prepared from tumors and normal tissue after microdissection, and p53 exons 2 to 4 and 5 to 9 were amplified separately by the polymerase chain reaction (PCR).[20] Sequencing of PCR products was carried out either directly, or from pooled clones of PCR products ligated into pBluescript II (Stratagene, La Jolla, CA).[20] When pooled DNA specimens yielded an ambiguous band, individual clones were sequenced; a mutation was considered to be present only when it was found in at least five separate clones.[20] Allelic loss at 17p, the locus of the p53 gene, was determined either by Southern blot with probes p14406, pTNH37.3, pYNZ22.1, and pMCT35.1 or from sequencing gels in cases with adequate cellularity.[20]

Immunohistochemistry

Immunohistochemical staining for p53 and p21 was performed on formalin-fixed, paraffin-embedded material with the Bio-Tek Techmate 1000 (Santa Barbara, CA) and a heat-based antigen enhancement system that utilizes citrate buffer. The monoclonal antibody D-07 (Dako, Carpinteria, CA) was used in studies of p53 protein expression. Antibody to p21 was obtained from Oncogene Science (Cambridge, MA). The dilution of primary antibody was titrated on the basis of initial studies so as to yield a maximal signal. Staining for p53 was scored as absent, focal, or diffuse, as described previously.[19] p21 staining was nuclear, as expected, and was reproducibly strong in normal pancreatic islets and vascular smooth muscle cells, which served as positive internal controls. When present in tumor cells, staining for p21 was scored with respect to both pattern (patchy or extensive) and intensity (weak or strong).

Results

p53 and p21 immunoreactivity data are summarized with the corresponding p53 mutational status for each of the 21 cases of pancreatic carcinoma studied in Table 1. Accumulation of the p53 gene product to immunocytochemically detectable levels is neither a sensitive nor a specific marker for p53 mutations (Table 1); only 7 of 13 cancers (54%) with p53 mutations demonstrated diffuse immunohistochemical accumulation of p53, compared with 3 of 8 cancers (38%) without demonstrated mutations in the gene. Focal p53 accumulation was seen in only 2 cancers, both of which were wild type for p53. p21 expression showed similar heterogeneity among tumors both

Table 1. *Summary of Molecular and Immunophenotypic Data*

Case	p53 mutation	17p LOH	Immunostaining p53	Immunostaining p21
1	WT		2	1–1
2	WT		0	2–2
3	WT		0	2–2
4	WT		1	1–2
5	WT		1	2–2
6	WT		2	2–2
7	WT		2	1–2
8	WT		0	2–1
9	FS	+	0	2–2
10	FS	+	0	1–1
11	FS	+	0	1–2
12	FS	+	0	1–1
13	FS	+	2	0
14	FS		0	2–2
15	PM		2	2–2
16	PM		2	1–1
17	PM	–	2	1–2
18	PM	+	2	2–2
19	PM		2	0
20	PM	+	2	2–2
21	PM		0	1–1

WT, wild type; FS, frameshift; PM, point mutation. For 17p LOH, a blank indicates either not tested or an uninformative probe. For p53: 0, absent; 1, focal/rare staining; 2, diffuse staining. For p21: 0, absent; 1–1, patchy, weak; 1–2, patchy, strong; 2–1, extensive, weak; 2–2, extensive, strong.

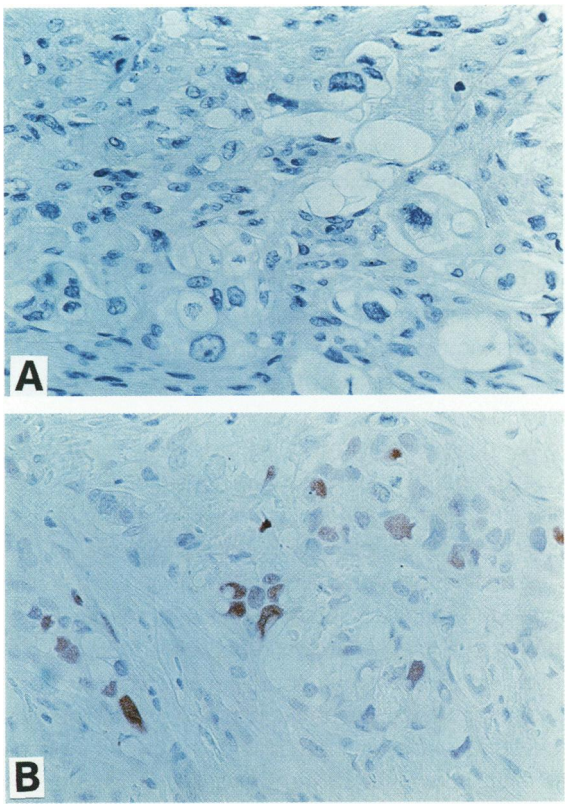

Figure 1. *Immunohistochemical staining for p53* (**A**) *and p21* (**B**) *in a pancreatic carcinoma harboring a frameshift mutation in p53 and 17p LOH (case 9 in Table 1). Extensive, strong p21 immunoreactivity is observed in the tumor cells, despite the absence of functional p53. Magnification, ×400.*

with and without p53 mutations. Of 8 cancers that were wild type for p53, 4 (50%) displayed extensive, strong immunoreactivity for p21, and 5 of 13 cancers (38%) with demonstrated p53 mutations stained similarly for p21. Only 2 of the 21 cancers studied were completely negative for p21 staining; both of these cancers harbored p53 mutations. Likewise, when compared with p53 protein accumulation, p21 immunoreactivity did not show a consistent pattern of expression. Extensive, strong p21 expression was seen in 4 of 9 (44%) tumors that did not stain for p53 and 4 of 10 (40%) cancers with diffuse p53 accumulation. In only 2 of the 13 p53-mutated cancers (15%) was there complete concordance among p53 mutational status, p53 overexpression, and lack of p21 expression.

This lack of correlation among p53 mutational status, p53 accumulation, and p21 immunoreactivity suggests that p21 expression can be independent of p53 in these tumors. Consistent with this proposal, four of five (80%) cancers with a frameshift mutation in p53 and documented 17p loss of heterozygosity (LOH), which would thus be predicted to have no functional p53, nonetheless displayed some p21 immunoreactivity (an example is shown in Figure 1). Similarly, two of the cancers with strong, extensive immunoreactivity for p21 were shown by molecular analysis to have point mutations in one allele of the p53 gene and LOH at 17p (Table 1). Because mutant p53 does not transactivate p21,[10] and only the mutant p53 allele remained in these two cancers, p21 expression would appear to be independent of p53 in these tumors.

Discussion

Several groups have investigated the status of the p53 tumor suppressor gene in pancreas cancers,[11–21] and there is general agreement that p53 alterations (mutation and/or protein accumulation) are detectable in most of these cancers (reviewed in Ref. 22). In some of these reports, accumulation of the p53 gene product has also been observed in a significant proportion of cases of intraductal lesions, which are thought to represent precursors to invasive cancer.[11,17,19] Taken together, these data suggest that p53 mutations are common and early pathogenetic events in pancreatic neoplasia.

In the present study, we sought to determine whether immunohistochemical staining for p21 could be used to identify pancreas cancers harboring p53

mutations. p21 was discovered independently as a gene specifically activated by wild-type p53 (WAF1)[8] and as an inhibitor of cyclin-dependent kinases (CIP1),[9] the activation of which is required for cell cycle progression. These findings led to the suggestion that p21 may be a downstream effector of the growth-regulatory properties of wild-type p53. Consistent with this proposal, p21 is induced in p53-dependent G_1 arrest and apoptosis.[10] Because p21 appears to mediate several of the growth-regulatory functions of p53, its expression would be predicted to reflect the functional status of p53 more precisely than p53 protein accumulation. For example, a tumor harboring a nonsense mutation of one p53 allele with deletion of the other would not be expected to accumulate immunoreactive p53. In such a case, no immunoreactive p53 would be detected, leading to the incorrect conclusion that it lacked a p53 mutation. However, assuming that p53-dependent p21 expression would be lost in such a tumor, immunostaining for p21 might help to identify some of the false negative results obtained with p53 staining alone. Likewise, physiological upregulation of wild-type p53 expression might yield protein levels that are detectable immunohistochemically, leading to the incorrect interpretation that p53 was mutated. In such a case, concomitant p21 expression would suggest intact function of p53.

The data presented here, however, suggest that p21 expression is not specifically linked to functional p53 status in pancreas cancers. Expression of p21 did not correlate either with p53 mutational status or with p53 accumulation detected by immunohistochemistry. Furthermore, p21 expression was seen in four cases known to harbor both a frameshift mutation in p53 and 17p LOH, a combination predicted to result in absence of functional p53. The absence of immunoreactive p53 seen in these four cases is consistent with this prediction. Finally, strong p21 expression was also seen in cases with 17p LOH in conjunction with p53 point mutations, a combination expected to abrogate the p21-transactivating potential of p53.

These data suggest that p21 expression in pancreatic carcinoma can be independent of p53. This finding is of particular interest in light of the very recent discovery of p53-independent induction of p21 expression during terminal differentiation in skeletal muscle and other cell lineages.[26,27] Likewise, in differentiating leukemic cells studied *in vitro*, p53-independent induction of p21 expression has been demonstrated and shown to result from stabilization of p21 mRNA.[28] If, as these results suggest, p21 may mediate growth arrest and differentiation in the absence of functional p53, it is still possible that p21 expression may correlate with clinical behavior in pancreas cancers. The number and relatively short follow-up duration of cases in the present series are insufficient to test this hypothesis.

The data presented here, however, do not exclude a component of p53-dependent p21 expression. For example, both cases without detectable p21 immunoreactivity were p53 mutant, as were four of five cases with focal, weak p21 staining. In addition, it is possible that p21 immunoreactivity may be dependent on p21 mutational status. Although there are no published data on the prevalence of p21 mutations in pancreas cancer, the available data in other malignancies suggest that such mutations are extremely rare. For instance, no p21 mutations were found in one study of 351 cases of 14 different types of human malignancies.[29] In any event, our data are compatible with the existence of both p53-dependent and p53-independent pathways of p21 expression.

In summary, we did not find a consistent relationship between functional p53 status and p21 expression, implying a p53-independent pathway of p21 expression in this series of tumors. On a practical level, immunohistochemical detection of p21 is not a useful marker for p53 functional status in pancreas cancers. However, if, as in differentiating cells, p53-independent p21 expression modulates cell cycle withdrawal, then p21 may yet be a significant biological marker in pancreas cancer.

Acknowledgments

The authors thank Amanda Lietman for her outstanding assistance and expertise in preparing this manuscript and Bill Woodcock for his expert performance of the immunohistochemistry.

References

1. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 1991, 253:49–53
2. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci USA 1992, 89:7491–7495
3. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW: Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991, 51:6304–6311
4. Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ Jr: A mammalian cell cycle checkpoint pathway utilizing p53

and GADD45 is defective in ataxia-telangiectasia. Cell 1992, 71:587–597
5. Lowe SW, Ruley HE, Jacks T, Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993, 74:957–967
6. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993, 362:847–849
7. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH: Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993, 362:849–852
8. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell 1993, 75:817–825
9. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: The p21 cdK-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell 1993, 75:805–816
10. El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, Vogelstein B: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis: Cancer Res 1994, 54:1169–1174
11. Barton CM, Staddon SL, Hughes CM, Hall PA, O'Sullivan C, Klöppel G, Theis B, Russell RCG, Neoptolemos J, Williamson RCN, Lane DP, Lemoine NR: Abnormalities of the p53 tumor suppressor gene in human pancreatic cancer. Br J Cancer 1991, 64:1076–1082
12. Ruggeri B, Zhang S-Y, Caamano J, DiRado M, Flynn SD, Klein-Szanto AJP: Human pancreatic carcinomas and cell lines reveal frequent and multiple alterations in the p53 and Rb-1 tumor-suppressor genes. Oncogene 1992, 7:1503–1511
13. Scarpa A, Capelli P, Mukai K, Zamboni G, Oda T, Iacono C, Hirohashi S: Pancreatic adenocarcinomas frequently show p53 gene mutations. Am J Pathol 1993, 142:1534–1543
14. Kalthoff H, Schmiegel W, Roeder C, Kasche D, Schmidt A, Lauer G, Thiele H-G, Honold G, Pantel K, Riethmüller G, Scherer E, Maurer J, Maacke H, Deppert W: p53 and K-*ras* alterations in pancreatic epithelial cell lesions. Oncogene 1993, 8:289–298
15. Lee CS, Rush M, Charalambous D, Rode J: Alimentary tract and pancreas: immunohistochemical demonstration of the p53 tumor suppressor gene product in cancer of the pancreas and chronic pancreatitis. J Gastroenterol Hepatol 1993, 8:465–469
16. Casey G, Yamanaka Y, Friess H, Kobrin MS, Lopez ME, Buchler M, Beger HG, Korc M: p53 mutations are common in pancreatic cancer and are absent in chronic pancreatitis. Cancer Lett 1993, 69:151–160
17. Zhang SY, Ruggeri B, Agarwal P, Sorling AF, Obara T, Ura H, Namiki M, Klein-Szanto AJP: Immunohistochemical analysis of p53 expression in human pancreatic carcinomas. Arch Pathol Lab Med 1994, 118:150–154
18. Pellegata NS, Sessa F, Renault B, Bonato M, Leone BE, Solcia E, Ranzani GN. K-*ras* and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. Cancer Res 1994, 54:1556–1560
19. DiGiuseppe JA, Hruban RH, Goodman SN, Polak M, van den Berg FM, Allison DC, Cameron JL, Offerhaus GJA: Overexpression of p53 protein in adenocarcinoma of the pancreas. Am J Clin Pathol 1994, 101: 684–688
20. Redston MS, Caldas C, Seymour AB, Hruban RH, da Costa L, Yeo CJ, Kern SE: p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. Cancer Res 1994, 54:3025–3033
21. Simon B, Weinel R, Höhne M, Watz J, Schmidt J, Körtner G, Arnold R: Frequent alterations of the tumor suppressor genes p53 and DCC in human pancreatic carcinoma. Gastroenterology 1994, 106:1645–1651
22. DiGiuseppe JA, Hruban RH: Pathobiology of cancer of the pancreas. Semin Surg Oncol 1995, 11:87–96
23. Ciechanover A, DiGiuseppe JA, Bercovich B, Orian A, Richter JD, Schwartz AL, Brodeur GM: Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. Proc Natl Acad Sci USA 1991, 88:139–143
24. Scheffner M, Huibregste JM, Vierstra RD, Howley PM: The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 1993, 75:495–505
25. Hall PA, Lane DP: Editorial. p53 in tumour pathology: can we trust immunohistochemistry? - revisited! J Pathol 1994, 172:1–4
26. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar AB: Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 1995, 267:1018–1024
27. Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledge SJ: p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science 1995, 267: 1024–1027
28. Schwaller J, Koeffler HP, Niklaus G, Loetscher P, Nagel S, Fey MF, Tobler A: Posttranscriptional stabilization underlies p53-independent induction of $p21^{WAF1/CIP1/SDI1}$ in differentiating human leukemic cells. J Clin Invest 1995, 95:973–979
29. Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen D-L, Vogelstein B, Koeffler HP: Absence of WAF1 mutations in a variety of human malignancies. Blood 1994, 84:3781–3784