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Tumor-suppressive Pathways in Pancreatic Carcinoma[1]

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ABSTRACT

During tumorigenesis, positive selection is exerted upon those tumor cells that alter rate-limiting regulatory pathways. A corollary of this principle is that mutation of one gene abrogates the need for alteration of another gene in the same pathway and also that the coexistence in a single tumor of mutations in different genes implies their involvement in distinct tumor-suppressive pathways.

We studied 42 pancreatic adenocarcinomas for genetic alterations in the K-*ras* oncogene and the *p16, p53,* and *DPC4* tumor suppressor genes. All of them had the K-*ras* gene mutated. Thirty-eight % of the tumors had four altered genes, another 38% had three altered genes, 15% had two altered genes, and 8% of the tumors had one altered gene. Interestingly, we noted a high concordance of *DPC4* and *p16* inactivations ($P = 0.007$), suggesting that the genetic inactivation of *p16* increases the selective advantage of subsequent mutation in *DPC4*. No statistically significant association was identified between the alteration of these cancer genes and pathological or clinical parameters.

This type of multigenic analysis in human tumors may serve to substantiate experimental tumor models and thus increase our understanding of the truly physiologically relevant tumor-suppressive pathways that are abrogated during human tumorigenesis.

INTRODUCTION

Cancer is a genetic disease where alterations in several genes accumulate during the development of the tumor (1). Genetic alterations will be selected in a carcinoma only if the mutations had provided the tumor cell with a selective growth advantage over its neighboring cells and thus had allowed that particular cell to evolve into a separate clonal population of tumor cells. This growth advantage is the phenotypic reflection of changes in the biological pathways in which the protein products of the mutated genes normally participate. A corollary of this theory is that the mutation of a second gene in such a tumor-suppressive pathway will not provide a tumor cell with an additional growth advantage and will, therefore, not give rise to a clonal outgrowth of that particular tumor cell. Similarly, the coexistence in a tumor of mutations in different genes indicates that these genes function in distinct tumor-suppressive pathways; otherwise, their mutations would not have been selected in the tumorigenic clone. The identification of genetic alterations in human tumors, therefore, will aid our understanding of the biological processes underlying tumorigenesis, because these tumors are the uniquely relevant integrators of the biological events that have occurred over the generally many decades of tumor development in humans.

Some reports have evaluated the mutational status of multiple genes in individual tumors, and such analyses have indeed confirmed some putative tumor-suppressive pathways that had been suggested by experimental models (2, 3). For example, in sarcomas and gliomas, mutations in the *p53* gene and amplification of its inactivator *MDM2* were found to be mutually exclusive, supporting the notion that these genes function in the same suppressive pathway (4, 5). Similarly, in lung cancer and melanoma, genetic alterations in the *RB1, p16,* and *CDK4* genes are mutually exclusive (6–11). Conversely, evidence supporting the distinctness of the *p16* and *p53* pathways has been provided by their coexistent mutations in melanoma, bladder, and pancreatic cancer (12, 13).

We previously reported genetic changes involving the K-*ras, p16, p53, DPC4,*[3] and *BRCA2* genes in pancreatic adenocarcinomas (12, 14–16), but the accumulative genetic changes in these tumors had not been addressed. We here report an in-depth mutational analysis of these cancer genes in an extended sample set of 42 pancreatic carcinoma xenografts. Because of the known involvement of several cancer genes in pancreatic carcinoma, this tumor type offers a unique opportunity to establish the coexistence of genetic alterations in individual tumors and thus the evaluation of the interactions among the various tumor-suppressive pathways under the natural selective pressures during human tumorigenesis.

MATERIALS AND METHODS

Patient Population and Tumor Specimens. First-passage xenografts from 42 unrelated pancreatic exocrine adenocarcinomas surgically resected at The Johns Hopkins Hospital and normal tissues from the patients were obtained as described (12). Patient information was obtained from the Johns Hopkins Tumor Registry and from the data base of the Clinical Correlates of Pancreatic Cancer Study. The average age of the patients was 63 years; 64% were male and 90% were white, reflecting the referral biases of the hospital. Fifty % had a history of 10 pack-years or more of cigarette smoking. Fifty-seven % had a history of cancer in a first-degree relative.

All histological slides were reviewed, and tumors of the duodenum, ampulla/papilla, and common bile duct were excluded. One tumor was a mucinous cystadenocarcinoma. Of the remaining invasive ductal adenocarcinomas, 4 were from the tail of the pancreas, and 37 were from the head. The average tumor diameter was 4 cm. Lymph node metastases were identified in 81% of cases. Histological differentiation was graded as well-differentiated in 2%, moderate in 72%, and poor in 26%.

Mutational Analyses. DNA was prepared and analyzed for alterations in the K-*ras, p53, p16,* and *DPC4* genes as described (12, 14, 15). Briefly, tumor DNA was amplified and sequenced with primers specific for these genes. All alterations were confirmed by a second independent amplification and subsequent sequencing of the amplified product. The somatic nature of the mutations was confirmed by amplifying and sequencing normal DNA from the same patient. The *p16, p53,* and *DPC4* alterations include data reported previously (12, 14, 15). The *BRCA2* data have been published elsewhere (16).

Statistical Analyses. All statistical computations were performed using the SAS (Statistical Analysis System) or EGRET (Statistics and Epidemiological Research Corp.) PC packages.

RESULTS

Mutational Analyses. Forty-two unrelated pancreatic adenocarcinoma xenografts were analyzed for alterations in the K-*ras* oncogene and the *p16, p53,* and *DPC4* tumor suppressor genes (Table 1). All 42 carcinomas had activating point mutations in the K-*ras* gene. Individual mutation frequencies for the tumor suppressor genes *p16, p53,* and

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[3] The abbreviations used are: DPC, deleted in pancreatic carcinoma; LOH, loss of heterozygosity.

Table 1 *Mutational analysis of pancreatic adenocarcinoma*[a]

		K-ras		*p16*[b]			*p53*[b]			*DPC4*[b]			*BRCA2*[c]		
Known alterations	Unique no.[d]	Alteration[e]	Predicted product[f]	Allelic loss[g]	Alteration[e]	Predicted product[f]	Allelic loss[g]	Alteration[e]	Predicted product[f]	Allelic loss[g]	Alteration[e]	Predicted product[f]	Allelic loss[g]	Alteration[e]	Predicted product[f]
Four genes															
	PX16	12 GGT to GTT	Gly to Val	LOH	72 CGA to TGA	Arg to stop	LOH	191 del: GCCCCTC-CTCAGCATCTTA to GCCCCTCTA	Frameshift	LOH	Homoz. del	Absence	Ret	ND	ND
	PX19	12 GGT to GTT	Gly to Val	LOH	Homoz. del.	Absence	LOH	273 CGT to TGT	Arg to Cys	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX21	12 GGT to GAT	Gly to Asp	LOH	102 TGG to TAG	Trp to stop	LOH	213 CGA to CTA	Arg to Leu	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX23	12 GGT to GTT	Gly to Val	LOH	Homoz. del.	Absence	LOH	238 del: 12 bp	In-frame del	LOH	358 GGA to TGA	Gly to stop	Ret	ND	ND
	PX28	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	196 CGA to TGA	Arg to stop	LOH	483 splice Agt to Aat	Truncation	LOH	Wt	–
	PX74	12 GGT to CGT	Gly to Arg	LOH	Homoz. del.	Absence	LOH	Exon 7 ins: 11 bp	Frameshift	LOH	412 TAC to TAG	Tyr to stop	LOH	Wt	–
	PX76	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	245 GGC to GAC	Gly to Asp	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX86	12 GGT to GTT	Gly to Val	LOH	Homoz. del.	Absence	LOH	157 del: CGCGTCCGC TO CGCC	Frameshift	LOH	493 GAT to CAT	Asp to His	ND	ND	ND
	PX88	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	134 TTT to CTT	Phe to Leu	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX91	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	195 ATC to ACC	Ile to Thr	LOH	Homoz. del.	Absence	ND	ND	ND
	PX92	12 GGT to GAT	Gly to Asp	LOH	5'UTR GG-39 bp-GC to GGCAGC	Absence	LOH	206 TTG to TAG	Leu to stop	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX94	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	273 CGT to CAT	Arg to His	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX101	12 GGT to GTT	Gly to Val	LOH	26 GCG-18 bp-CCG to GCTGCG	In-frame del	LOH	220 TAT to TGT	Tyr to Cys	LOH	515 AGA to TGA	Arg to stop	LOH	751 ins: ACT to ACTT	Frameshift
	PX102	12 GGT to CGT	Gly to Arg	LOH	Homoz. del.	Absence	LOH	273 CGT to GGT	Arg to Gly	LOH	516 del. CAGAGCTCC to C	Frameshift	Ret	ND	ND
	PX122	12 GGT to GAT	Gly to Asp	LOH	70 del: CTCACCCGACCC to CTCCC	Frameshift	LOH	Intron 8 splice del: tcact-45 bp-CAGCT to tcaGCT	Truncation	LOH	Homoz. del.	Absence	ND	ND	ND
Three genes															
	PX13	12 GGT to GTT	Gly to Val	LOH	6 del: CTGGCTGGC to CTGGC	Frameshift	LOH	158 CGC to CCC	Arg to Pro	LOH	Wt	–	Ret	ND	ND
	PX20	12 GGT to GAT	Gly to Asp	LOH	102 TGG to TAG	Trp to stop	LOH	273 CGT to TGT	Arg to Cys	Ret	Wt	–	Ret	ND	ND
	PX65	12 GGT to GCT	Gly to Ala	LOH	72 CGA to TGA	Arg to stop	LOH	271 GAG to AAG	Glu to Lys	Ret	Wt	–	Ret	ND	ND
	PX68	12 GGT to GTT	Gly to Val	LOH	82 del: TTCCT to TTCT	Frameshift	LOH	220 TAT to TGT	Tyr to Cys	LOH	Wt	–	Ret	ND	ND
	PX72	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	157 GTC to GGC	Val to Gly	LOH	Wt	–	ND	ND	ND
	PX75	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	228 ins: GAC to GACTGATCCACCC	Frameshift	LOH	Wt	–	Ret	ND	ND
	PX90	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	Ret	Wt	–	LOH	ND	ND	ND	ND	ND
	PX93	12 GGT to GAT	Gly to Asp	LOH	Wt	–	LOH	245 GGC to AGC	Gly to Ser	LOH	ND	ND	ND	ND	ND
	PX105	13 GGT to GTT	Gly to Val	LOH	72 CGA to TGA	Arg to stop	LOH	151 CCC to ACC	Pro to Thr	LOH	Wt	–	ND	ND	ND
	PX107	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	238 TGT to TAT	Cys to Tyr	LOH	Wt	–	Ret	ND	ND
	PX117	12 GGT to CGT	Gly to Arg	LOH	Homoz. del.	Absence	LOH	306 CGA to TGA	Arg to stop	LOH	Wt	–	ND	ND	ND
	PX120	12 GGT to GTT	Gly to Val	LOH	Homoz. del.	Absence	LOH	194 CTT to CGT	Leu to Arg	LOH	Wt	–	ND	ND	ND
	PX24	12 GGT to GTT	Gly to Val	LOH	97 del: GG-20 bp-CG to GGCG	Frameshift	LOH	Wt	–	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX27	12 GGT to GTT	Gly to Val	LOH	145 splice AGgt to AGgc	Truncation	LOH	Wt	–	LOH	Homoz. del.	Absence	LOH	Homoz. del.	Absence
	PX30	12 GGT to GCT	Gly to Ala	LOH	75 CAC to TAC	His to Tyr	LOH	Wt	–	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX61	12 GGT to GCT	Gly to Ala	LOH	15 GGT to GAT	Gly to Asp	Ret	Wt	–	LOH	Homoz. del.	Absence	Ret	ND	ND
	PX64	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	Wt	–	LOH	Homoz. del.	Absence	LOH	Wt	–

Two genes													
	PL45	12 GGT to GAT	Gly to Asp	Ret	Wt	–	LOH	255 ATC to AAC	Ile to Asn	LOH	Wt	–	LOH	Wt	–
	PX9	12 GGT to GAT	Gly to Asp	Ret	ND	ND	LOH	273 CGT to TGT	Arg to Cys	LOH	ND	ND	ND	ND	ND
	PX17	12 GGT to GAT	Gly to Asp	LOH	Wt	–	LOH	51 del: ATTGAAC-27 bp-ATGAAGCTCCCA to ATCCCA	Frameshift	LOH	Wt	–	Ret	ND	ND
	PX55	12 GGT to GAT	Gly to Asp	LOH	Homoz. del.	Absence	LOH	Wt	–	LOH	Wt	–	LOH	Wt	–
	PX56	12 GGT to GTT	Gly to Val	LOH	Homoz. del.	Absence	LOH	Wt	–	LOH	Wt	–	Ret	ND	ND
	PX66	12 GGT to GAT	Gly to Asp	LOH	Wt	–	LOH	248 CGG to CAG	Arg to Gln	LOH	Wt	–	LOH	1982 del: AGT GG to AGGG	Frameshift
	PX67	12 GGT to GAT	Gly to Asp	LOH	Wt	–	LOH	306 CGA to TGA	Arg to stop	LOH	Wt	–	Ret	ND	ND
One gene															
	PX26	12 GGT to GCT	Gly to Ala	Ret	Wt	–	Ret	Wt	–	Ret	Wt	–	Ret	ND	ND
	PX29	12 GGT to GAT	Gly to Asp	LOH	Wt	–	Ret	Wt	–	Ret	Wt	–	ND	ND	ND
Summary of data		100% altered (41/41)			82% altered (33/40)			76% altered (31/41)			53% altered (20/38)			10% altered (3/30)	

[a] The only cystadenocarcinoma (PX104) was not included in this table.
[b] Includes data reported previously (12, 14, 15).
[c] *BRCA2* data were reported elsewhere (16). Grouping by the number of alterations excludes BRCA2 status because whole gene sequencing was not performed.
[d] The PX series comprises pancreatic adenocarcinoma xenografts, and PL45 is a cell line, derived from unrelated patients. PX26 is the only tumor with multiple microsatellite shifts characteristic of the mutator phenotype.
[e] Codon number and alteration. Homoz. del., homozygous deletion; del, deletion; ins, insertion; Wt, wild-type sequence; 5'-UTR, 5' untranslated region; ND, not determined. The codon 1982 mutation in *BRCA2* in tumor PX66 represents the Ashkenazi-linked mutation referred to by nucleotide position as 6174delT.
[f] Del, deletion; –, wild-type product expected; ND, not determined.
[g] Allelic loss assay using dinucleotide repeat polymorphisms. Scored markers are: for *p16*, *IFNA* and *D9S171*; for *p53*, *D17520*, *D17578*, and *D17799*; for *DPC4*, *D18S46*, *D18S363*, and *D18S547*; for *BRCA2*, *D13S260*, *D13S171*, and *D13S267*. Ret, both alleles retained; ND, not determined.

Table 2 *Coexistence of tumor suppressor gene inactivations in pancreatic adenocarcinoma*

Inactivation patterns	No. of tumors
Cystadenocarcinoma	
Wild-type *p53*, *p16*, *DPC4*	1
Ductal adenocarcinoma	
p53, *p16*, and *DPC4* inactivated	15[a]
p53 and *p16* inactivated	10
p53 and *DPC4* inactivated	0
p16 and *DPC4* inactivated	5[b]
p53 inactivated	4[a]
p16 inactivated	2
DPC4 inactivated	0
Wild-type *p53*, *p16*, *DPC4*	2[c]
Total	39[d]

[a] Includes one tumor with a germ-line *BRCA2* mutation and a somatic loss of the wild-type *BRCA2* allele.
[b] Includes one tumor with a somatic homozygous deletion of *BRCA2*.
[c] Includes one tumor known to have a mismatch repair defect.
[d] Three additional tumors are described in Table 1 but are excluded from this table because of incomplete sequence data for *p53* and/or *p16*.

DPC4 were 82% (33 of 40), 76% (31 of 41), and 53% (20 of 38) of the tumors, respectively. All mutations in the tumor suppressor genes were accompanied by LOH and could be predicted to inactivate the protein function. All mutations were shown to be of somatic origin through analysis of DNA of normal tissue of the patients. No silent mutations, indicative of random unselected mutations, were identified. One tumor (PX26) had multiple microsatellite shifts characteristic of the mutator phenotype (Table 1).

Overall, the most frequent type of nucleotide change among the tumor suppressor genes was a G-to-A transition. Twelve of 16 (75%) of the transitions in the *p53* gene and 7 of 8 (87%) of those in the *p16* gene involved CpG sites. Despite these general tendencies toward nucleotide transitions, five of six (83%) *DPC4* point mutations were transversions.

The concurrent alterations of the three tumor suppressor genes in each tumor is depicted in Table 2. Inactivation of all three tumor suppressors, as well as activation of K-*ras*, occurred in 15 of 39 (38%) of the tumors. Another 15 of 39 (38%) had coexistent alterations in two of the three tumor suppressor genes. Six of 39 (15%) of the tumors had inactivated a single tumor suppressor gene (*p16* or *p53*), together with the K-*ras*-activating mutation. Three of 39 tumors (8%) had no tumor suppressor mutations but had the K-*ras* activation. One of these (PX104) was the sole cystadenocarcinoma of the collection, and one (PX26) was the sole tumor with evidence for a mismatched repair defect. The remaining tumor (PX29) was from a patient who had a family history of unspecified cancers in one parent and in three siblings.

Association Analyses. The only cystadenocarcinoma (PX104) was excluded from these analyses. Associations were evaluated between the genetic status of the *p16*, *p53*, and *DPC4* tumor suppressor genes and pathological and clinical parameters, including tumor diameter and differentiation, nodal status, age at surgical resection, survival and death of disease, family history of cancer, sex, race, and smoking history. Associations were tested for statistical significance using χ^2 or Fisher's exact tests where appropriate. Cox proportional-hazard regression was used to assess the effects of multiple factors on survival times.

DPC4 inactivation was always (20 of 20) accompanied by inactivation of the *p16* gene ($P = 0.007$; Table 3). The converse was not true, because *p16* inactivation did not require *DPC4* inactivation

Table 3 *Association of p16 and DPC4 alterations in pancreatic adenocarcinoma*[a]

	DPC4		
	Inactivated	Wild-type	Prevalence of inactivation
p16 inactivated	20	12	62.5%
p16 wild-type	0	6	0%

[a] $P = 0.007$; Fisher Exact test on all 38 tumors having complete sequence analysis.

(Tables 1 and 2). *p53* inactivation was not associated with *p16* or *DPC4* mutations ($P = 1.0$). A previously observed trend for *p53* mutation as a prognostic factor (14) was confirmed (hazard ratio, 1.9; 95% confidence interval, 0.8–4.4; $P = 0.15$). Another trend for inactivation of all three suppressor genes as a risk factor for death from disease was also suggested (hazard ratio, 1.7; 95% confidence interval, 0.8–3.5; $P = 0.16$). Although these trends were not statistically significant, they may suggest further evaluation in studies encompassing more tumor specimens. No other associations were identified among any of the tested parameters. A previously reported association between smoking history and risk for pancreatic cancer (17) was not confirmed in our series. Similarly, a multivariate analysis of 201 patients from the Johns Hopkins Hospital that indicated tumor diameter and nodal status to be significant adverse prognostic factors (18) was not confirmed in our series. The reason for these discrepancies is not clear but may be related to the relatively small number of specimens (41 tumors) that were analyzed in our study.

DISCUSSION

We have analyzed the mutational status of the K-*ras* oncogene and the *p16, p53,* and *DPC4* tumor suppressor genes in a collection of 42 pancreatic adenocarcinomas. The vast majority of these tumors were found to have accumulated alterations in three or four of these genes (38% for each group). Mutational analysis in some of these tumors revealed the coexistence of *BRCA2* mutations with three of these genes and in one tumor (PX101) with all four genes (16). Our findings underscore the multigenic nature of cancer, and tumor PX101, having alterations identified in five genes, exemplifies the extent of accumulation of genetic alterations. It has been hypothesized that up to seven genes might be altered in most human carcinomas (19), and allelotype analysis of pancreatic adenocarcinoma had indeed suggested the involvement of several additional tumor suppressor genes in this tumor type (20). The high prevalence of alterations in several genes appears unusual among human adenocarcinomas. It is as yet unclear whether this is a feature restricted to pancreatic carcinoma or whether the high prevalence events have not yet been identified in other tumor types.

The vast majority (83%) of pancreatic carcinomas had a distinctive genetic fingerprint, comprising activation of the K-*ras* oncogene and inactivation of the *p16* gene, generally also accompanied by alterations in the *p53* gene (in 76% of the tumors). The activation of K-*ras* appears nearly to be a prerequisite for the development of pancreatic carcinoma. Also, the binary alteration of K-*ras* and *p16* is an extremely uncommon combination among other human tumor types. This particular genetic imprint of pancreatic carcinomas could have diagnostic utility in the evaluation of patients with metastatic adenocarcinoma of unknown primary origin.

The evaluation of genetic alterations as they naturally occur in human tumors allows the formulation of hypotheses concerning the biological processes that involve human tumorigenesis. A central tenet of tumorigenesis, that positive selection is exerted upon those tumor cells that alter rate-limiting regulatory pathways, implies that mutation of one gene abrogates the need for inactivation of another gene in the same tumor-suppressive pathway. It follows, therefore, from the genetic profile of pancreatic carcinoma that K-*ras, p53, p16, DPC4,* and *BRCA2* each belong to a distinct tumor-suppressive pathway. The concept of distinct tumor-suppressive pathways, however, should not exclude the possibility of cooperative interactions between the pathways. In this series, for example, *DPC4* gene inactivation was limited to tumors with genetic inactivation of the *p16* gene. Perhaps inactivation of the *DPC4* pathway does not provide the tumor cell with a strong selective growth advantage unless the *p16* pathway had been inactivated earlier during tumorigenesis.

The involvement of the *p53, p16,* and *DPC4* pathways in pancreatic carcinoma is likely to be even greater than suggested by our current mutational analysis. For example, we have not yet addressed the mutational status of the *RB1* gene, which participates in the same pathway as *p16* and has been reported to be mutated in about 7% of pancreatic carcinomas (21). Also, methylation abnormalities that might abrogate the expression of the p16 protein (22) have yet to be evaluated in our collection of pancreatic carcinomas. Additional genes that are identified as participants of one of these tumor-suppressive pathways would in turn become candidate genes for alterations in subsets of pancreatic carcinomas that have not yet had mutations identified within the particular pathway. The identification of such additional genetic targets would improve the description of the genetic profile of pancreatic carcinoma and thus enhance our understanding of the tumor-suppressive pathways that are involved in the development of this tumor type.

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