TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Genome-Wide Somatic Copy Number Alterations and Mutations in High-Grade Pancreatic Intraepithelial Neoplasia

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To obtain a better understanding of the genetic alterations of high-grade pancreatic intraepithelial neoplasia (HG-PanIN), we performed whole-genome copy number analysis by using single nucleotide polymorphism microarrays and targeted next-generation sequencing of 11 microdissected HG-PanIN and two low-grade PanIN lesions associated with HG-PanIN. HG-PanIN mutation profiles were compared with those of their associated invasive pancreatic ductal adenocarcinoma. All PanIN lesions harbored somatic *KRAS* mutations. The most common copy number losses in the HG-PanIN were at the *CDKN2A* (9p21), *TP53* (17p13), and *SMAD4* (18q21) loci. Chromosomal losses in HG-PanIN were also found at 6p25–p24, 6q11–q27, 12q24, and 17q23–q24. Biallelic inactivation of *CDKN2A* and *TP53* was detected in five of eight and in three of eight evaluable PanIN lesions, respectively. None of the HG-PanIN lesions had *SMAD4* mutations or homozygous deletion. Copy number gains were noted at the *MYC* (8q24) and *CCNE1* (19q12) loci and at 1q25–q31. Four HG-PanINs and one low-grade PanIN harbored chromothripsis-like regions. Five of seven pancreatic ductal adenocarcinomas evaluated had additional mutations that were not found in their associated HG-PanIN. HG-PanIN harbors widespread copy number alterations and commonly shows evidence of biallelic inactivation of *CDKN2A* and *TP53* but not *SMAD4*. Chromothripsis events contribute to the copy number alterations of HG-PanIN. (*Am J Pathol* 2018; 188: 1723–1733; <https://doi.org/10.1016/j.ajpath.2018.03.012>)

Pancreatic cancer is one of the most lethal cancers with a 5-year survival rate of only approximately 8%.¹ The early detection of pancreatic cancer enables curable resection and better long-term survival.² However, because most patients with early-stage invasive pancreatic cancer will die of their disease, there is considerable interest in understanding the molecular alterations of noninvasive precursor lesions because their detection and treatment offers an opportunity to prevent the development of invasive pancreatic cancer.

Pancreatic intraepithelial neoplasia (PanIN) is the most common precursor to invasive pancreatic cancer.^{3,4} PanINs are noninvasive epithelial proliferations within pancreatic ductules and can be flat or papillary, are graded histologically as low-grade PanIN (LG-PanIN; previously termed PanIN-1 and PanIN-2) and high-grade PanIN (HG-PanIN;

previously termed PanIN-3) on the basis of the degree of their architectural and cellular atypia.⁵ Most PanINs are LG-PanIN, particularly in pancreata not involved by pancreatic ductal adenocarcinoma (PDAC).^{6–10} Paralleling this histologic progression is a genetic progression. LG-PanINs almost always harbor mutated *KRAS* but do not typically harbor other gene mutations.¹¹ Limited genetic characterization of HG-PanIN has been reported in part because these lesions are much less common than LG-PanIN.⁷ HG-PanINs are most often detected in the pancreata of patients with PDAC and patients with a familial/inherited susceptibility to pancreatic cancer with screen-detected

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precursor lesions.^{8,12} To understand the genetic progression of PanIN to invasive pancreatic cancer, early studies used immunohistochemistry to detect loss of tumor suppressor gene protein expression.^{4,13} These studies found that loss of *CDKN2A* expression in a minority of LG-PanINs and in more than one-half of HG-PanINs, whereas overexpression of *p53* (which is a consequence of most *TP53* missense mutations) is not evident in LG-PanIN but is present in some HG-PanINs. More recent genetic studies of PanIN found a low prevalence of *TP53* mutations in HG-PanIN lesions isolated from pancreata without an invasive carcinoma.¹⁴ Initial studies also found that loss of *SMAD4* expression, consistent with mutational inactivation, occurred in some HG-PanINs,¹⁵ but genetic analysis of PanIN lesions has not found evidence of *SMAD4* inactivation in HG-PanIN.^{14,16} Although immunohistochemistry is a valuable tool to detect evidence of *TP53* mutations or *SMAD4* gene inactivation, these initial reports that used immunohistochemistry to evaluate molecular alterations of PanIN may have sometimes misclassified invasive cancer growing into a preexisting duct (so called cancerization of the ducts) as HG-PanINs.

Chromosomal instability is a characteristic feature of most human cancers, including PDAC.¹⁷ Tumor suppressor gene inactivation usually involves a combination of intragenic mutational inactivation with chromosomal loss; focal chromosomal amplification drives oncogenic signaling. Although the chromosome alterations of PDACs have been extensively characterized,^{18–23} only one study has reported on genome-wide copy number analysis of LG-PanIN, which found that, unlike invasive PDACs, these lesions harbor few if any somatic copy number alterations.²⁴ Recent studies have also identified chromothripsis events (chromosomal shattering with chaotic reassembly) in some pancreatic cancers.^{25–27} Notta et al²⁵ recently asserted that their observations of chromothripsis events in invasive pancreatic cancers (one or more such events were identified in 65% of 107 pancreatic cancers) were a challenge to the existing PanIN progression model. Their study did not examine the chromosomal alterations of PanIN lesions. Genome-wide chromosomal copy number analysis of HG-PanIN would provide a better understanding of chromosomal alterations that arise during PanIN progression, but such a study has not been reported.

The goal of this study is to obtain a more detailed understanding of the genetic alterations of HG-PanINs by performing genome-wide single nucleotide polymorphism (SNP) array analysis and targeted next-generation sequencing (NGS).

Materials and Methods

Patients and Tissue Samples

Pancreatic tissues were obtained from patients undergoing pancreatic resection at the Johns Hopkins Hospital from 2009 to 2014. Frozen sections of primary resected pancreas that contained areas of PanIN were obtained from optimal

controlled temperature blocks created in the surgical pathology suite shortly after the resection specimen was received and mounted onto membrane slides for subsequent laser capture microdissection. Available invasive PDAC tissue samples from patients were also obtained to compare PanIN and invasive pancreatic cancer mutation profiles. All elements of this study were approved by the Johns Hopkins Institutional Review Board, and written informed consent was obtained from all patients.

Histologic Assessment

HG-PanIN lesions were identified by a pathologist specializing in pancreas pathology (R.H.H.). Pathologic diagnosis and staging of PDACs were determined by using the *AJCC Cancer Staging Manual*, eighth edition.²⁸ PanIN from one patient was obtained from pancreata resected for ampullary carcinoma (diagnosed and staged using the *AJCC Cancer Staging Manual*, seventh edition²⁹).

It is rare to identify HG-PanIN in fresh-frozen sections of pancreas that did not have an associated PDAC, but we were able to identify two patients with HG-PanIN who did not have an associated PDAC with sufficient numbers of frozen sections for analysis. The remaining PanINs were obtained from pancreata that also had an infiltrating PDAC. Two LG-PanINs that were immediately adjacent to two of the HG-PanIN lesions were also characterized. Because HG-PanIN lesions can sometimes be difficult to distinguish definitively from intraductal spread of infiltrative PDAC,⁵ deeper sections from the block with the PanIN lesions were reviewed to ensure that there was no invasive cancer in the immediate vicinity of the PanIN lesions.⁴ Mutation analysis of the adjacent invasive cancer was also performed to identify genetic differences between the PanIN and the invasive cancer.

Laser Capture Microdissection

Laser capture microdissection was performed to isolate near pure PanIN cells and adjacent normal pancreas and to obtain invasive PDAC cells. Frozen tissue sections were placed on ultraviolet-irradiated, membrane-coated slides (Carl Zeiss Microimaging, Munich, Germany) and stored at -80°C until needed. Slides were briefly stained with hematoxylin and eosin and were microdissected by using a laser capture microdissection system (Leica LMD7000; Leica, Buffalo Grove, IL). One frozen section slide was stained with hematoxylin and eosin as a guide. For each PanIN lesion, dissections were performed on five to eight adjacent slide sections from the same contiguous PanIN ductule. Dissections from multiple scattered PanINs were not pooled even when they were on the same slide because PanIN in different ductile cross sections may represent different PanIN lesions. For all patients, paired normal pancreatic tissues from the same patient were also microdissected and analyzed for SNP microarray analysis. Cancer cells were

Table 1 Clinicopathologic Features of Included Patients

Patient ID	Age, years	Sex	Family history of pancreatic cancer	Sample ID	Dissected PanIN lesion	Diagnosis	Stage*	Histologic differentiation
37T	74	F	None	TH12	HG-PanIN	PDAC	T3N0M0	G2
49T	66	M	None	TH13	LG-PanIN (PanIN-2)	PDAC	T3N1M0	G2
				TH14	HG-PanIN			
39T	56	M	None	TH15	HG-PanIN	PDAC	T1N2M0	G3
70T	75	F	None	TH16	HG-PanIN	PDAC	T2N0M0	G1
19T	74	M	None	TH17	HG-PanIN	PDAC	T3N2M0	G2
69T	61	M	None	TH18	HG-PanIN	PDAC	T2N1M0	G3
22T	63	F	None	TH19	HG-PanIN	PDAC	T3N2M0	G3
45T	58	F	None	TH20	LG-PanIN (PanIN-2)	Ampullary carcinoma	T3N0M0	G2
				TH21	HG-PanIN			
29T	63	F	None	TH22	HG-PanIN	Extensive PanIN	TisN0M0	N/A
78T	54	F	None	TH23	HG-PanIN	PDAC	T2N0M0	G3
780T	70	F	None	TH24	HG-PanIN	PDAC	T3N1M0	G2

*Pathologic diagnosis determined by *AJCC Cancer Staging Manual*, eighth edition²⁸ (pancreatic ductal adenocarcinoma, PDAC) and *AJCC Cancer Staging Manual*, seventh edition²⁹ (ampullary carcinoma).

F, female; M, male; HG, high-grade; LG, low-grade; PanIN, pancreatic intraepithelial neoplasia.

similarly microdissected from five to eight slides. Representative examples of PanIN tissue images before and after laser capture microdissection are provided in [Supplemental Figure S1](#).

Genomic DNA Extraction and WGA

Genomic DNA from microdissected tissues was extracted by using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA). Whole-genome amplification (WGA) was conducted by using the multiple annealing and looping-based amplification cycles (MALBAC) method. MALBAC was used because this method has the unique feature of quasi-linear amplification and a lower allele dropout rate compared with other conventional WGA methods.^{30,31} One nanogram of extracted DNA samples was amplified by using the

MALBAC Single Cell WGA Kit according to the manufacturer's protocol (Yikon Genomics, Beijing, China). DNA was quantified by using the Quantifiler Human DNA Quantification kit (Applied Biosystems, Foster City, CA) before and after WGA. The uniformity of MALBAC amplification was checked at six randomly selected chromosomal loci ([Supplemental Figure S2](#)). MALBAC applied to microdissected DNA yielded uniform distributions of amplicons, ranging from 300 to 2000 bp in all samples ([Supplemental Figure S3](#)).

SNP Array

SNP array analysis was performed on the HumanCytoSNP-12 version 2.1 BeadChip platform (Illumina, San Diego, CA) according to the manufacturer's instructions. The

Table 2 Copy Number Altered Chromosomal Arms in PanIN Lesions

Patient ID	Sample ID	PanIN lesion	Gain	Loss
37T	TH12	HG-PanIN	17q	14p, 14q, 17p, 17q
49T	TH13	LG-PanIN	No CNA	No CNA
49T	TH14	HG-PanIN	3p, 3q, 7p, 7q, 8p, 8q, 9p, 9q, 10p, 10q	No CNA
39T	TH15	HG-PanIN	17q	6p, 6q, 9p, 9q, 17p, 17q, 19p, 19q, 21p, 21q, 22q
70T	TH16	HG-PanIN	3q, 8q, 18p, 19q	8p, 9p, 11p, 12q, 18q, 19p
19T	TH17	HG-PanIN	18p, 19q	13q, 18q, 19p
69T	TH18	HG-PanIN	19q	22q
22T	TH19	HG-PanIN	1q, 8q, 18p, 19q	6p, 6q, 13p, 13q, 18q, 19q
45T	TH20	LG-PanIN	1q, 19q	9p, 17p, 18p, 18q, 19p, 19q
45T	TH21	HG-PanIN	19q	9p, 17p, 19q
29T	TH22	HG-PanIN	1p, 1q, 18p	1p, 1q, 14p, 14q
78T	TH23	HG-PanIN	1q, 15p, 15q	6p, 6q, 9p, 9q, 12q, 17p, 17q, 18p, 18q, 19p, 19q
780T	TH24	HG-PanIN	17p	17p, 19p

CNA, copy number alteration; HG, high-grade; LG, low-grade; PanIN, pancreatic intraepithelial neoplasia.

Figure 1 **A:** Scatter plot shows correlation between log R ratio (LRR) of *CDKN2A* copy number alterations (CNAs) from single nucleotide polymorphism array and copy number ratio of *CDKN2A/RNaseP* from droplet digital PCR analysis. Spearman's correlation test was used to compare the correlation. R^2 means coefficient of determination. **B:** Comparison of *CDKN2A/RNaseP* ratio between high-grade pancreatic intraepithelial neoplasia (HG-PanIN) samples with or without copy number loss. Horizontal bar represents the median value. Nonparametric U-test was used to compare ratio variables. $**P < 0.01$. CNV, copy number variation.

platform provides information on approximately 300,000 SNP markers. Microarray hybridizations were performed in the Microarray Core Facility at Johns Hopkins University School of Medicine.

Array Data Analysis

GenomeStudio software version 2011.1 (Illumina) was used to analyze the SNP array data. The software determines the log R ratio (LRR) and B allele frequency for each sample, and acquired original LRRs were normalized by performing a GC-content correction (Supplemental Figure S4).³² With the use of the Bioconductor R package, CGHcall, LRRs were then segmented by using the Circular Binary Segmentation algorithm with the default parameters to calculate the mean log fold copy change estimate for each such segment.³³ This probability-based calling allowed us to estimate the probability of a given segment being classified as a gain or a loss. Sex chromosomes were not included in this analysis. Amplification was defined when LRR values were >0.8 and homozygous deletion when LRR values were <-1.2 . Segments were aligned to reference human genome assembly, hg19 (GRCh37). Genes overlapped by copy number variations were identified through the University of California, Santa Cruz, genome browser. Recurrent short segment copy number alterations (approximately 20 probes) observed in normal pancreas tissues were manually excluded as potentially representing areas of incomplete WGA rather than true germline alteration. Cancer-related genes were annotated by using multiple databases, including COSMIC (<http://cancer.sanger.ac.uk/cosmic>, last accessed January 2018), CancerGenetics Web (<http://www.cancer-genetics.org>, last accessed January 2018), TAG

database (<http://www.binfo.ncku.edu.tw/TAG>, last accessed January 2018), and Atlas of Genetics and Cytogenetics in Oncology and Hematology (<http://atlasgeneticsoncology.org/index.html>, last accessed January 2018) and PubMed.

To check for chromothripsis a web-based server, CTLPScanner, was used with the following parameters: copy number aberration status change events of ≥ 11 and Log10 of likelihood ratio of ≥ 8 . To exclude artifacts, only chromosomal segments of size ≥ 50 kb were considered.³⁴

Targeted NGS

Targeted NGS was performed by using the Ion Torrent semiconductor sequencing platform. An Ion AmpliSeq Custom Panel was designed to target selected regions of 11 genes commonly mutated in pancreatic cancer (*KRAS*, *GNAS*, *CDKN2A*, *TP53*, *SMAD4*, *RNF43*, *ARID1A*, *BRAF*, *FBXW7*, *PIK3CA*, and *TGFBR2*).^{35–37} The quality of template DNA from microdissected tissues was evaluated by the Agilent 2100 Bioanalyser on-chip electrophoresis (Agilent Technologies, Santa Clara, CA). Duplicate AmpliSeq libraries from each sample were prepared by using 2 ng of genomic DNA. The libraries were cleaned, quantified, loaded into a OneTouch2 for emulsion PCR, enriched, loaded, and sequenced onto a 318v2 chip by using an Ion Torrent Personal Genome Machine (Life Technologies) according to the manufacturer's protocols. Postsequencing data analyses, including alignment to the hg19 human reference genome and variant calling, were performed by using NextGENe software version 2.4 (SoftGenetics, Chicago, IL). Alignments and putative mutations were visually verified by using Integrative

Table 3 Genetic Gain and Loss Regions in Isolated HG-PanIN Lesions

CNA	Cytoband*	Start*	Stop*	Gene symbols	Patient ID	Sample ID	Lesion	LRR
Gain	1q25.2–q31.1	180022343	187135612	<u>LAMC2</u> , <u>PLA2G4A</u> , <u>PTGS2</u> , <u>TPR</u>	45T	TH20	HG-PanIN	0.4864
					29T	TH22	HG-PanIN	0.2516
					78T	TH23	HG-PanIN	0.5393
Gain	8q24.21–q24.3	128213233	141524484	<u>MYC</u> , <u>KCNK9</u> , <u>WISP1</u> , <u>NDRG1</u>	49T	TH14	HG-PanIN	0.4835
					70T	TH16	HG-PanIN	0.4516
					22T	TH19	HG-PanIN	0.6603
Gain	19q12	28771117	31050122	<u>CCNE1</u>	70T	TH16	HG-PanIN	0.3467
					19T	TH17	HG-PanIN	0.8888
					69T	TH18	HG-PanIN	0.3405
					45T	TH20	LG-PanIN	0.8365
					45T	TH21	HG-PanIN	0.8362
Gain	19q13.2–q13.32	42676481	47465366	<u>BCL3</u> , <u>CADM</u> , <u>CBL</u> , <u>CEACAM1</u> , <u>CIC</u> , <u>ERCC1</u> , <u>ERCC2</u> , <u>PPP1R13L</u> , <u>RELB</u>	70T	TH16	HG-PanIN	0.3467
					19T	TH17	HG-PanIN	0.5762
					69T	TH18	HG-PanIN	0.9417
Loss	6p25.3–p24.3	204909	7374207	<u>DUSP22</u> , <u>IRF4</u> , <u>FOXC1</u> , <u>RREB1</u>	39T	TH15	HG-PanIN	–0.2883
					22T	TH19	HG-PanIN	–0.3551
					78T	TH23	HG-PanIN	–0.4501
Loss	6q11.1–q27	62351428	166070424	<u>BACH2</u> , <u>HACE1</u> , <u>IGF2R</u> , <u>LATS1</u> , <u>PLAGL1</u> , <u>PRDM1</u> , <u>CTGF</u> , <u>MYB</u> , <u>PTP4A1</u> , <u>TIAM2</u>	39T	TH15	HG-PanIN	–0.5373
					22T	TH19	HG-PanIN	–0.4003
					78T	TH23	HG-PanIN	–0.6227
Loss	9p24.3–p21.3	46587	21600424	<u>PTPRD</u> , <u>MLLT3</u> , <u>JAK2</u> , <u>KDM4C</u> , <u>TYRP1</u>	39T	TH15	HG-PanIN	–0.9140
					70T	TH16	HG-PanIN	–0.5040
					45T	TH20	LG-PanIN	–0.4940
					45T	TH21	HG-PanIN	–0.5439
					78T	TH23	HG-PanIN	–0.5533
Loss	9p21.3	21600424	22811336	<u>CDKN2A</u> , <u>CDKN2B</u>	39T	TH15	HG-PanIN	–0.4654
					70T	TH16	HG-PanIN	–1.5687
					45T	TH20	LG-PanIN	–1.2731
					45T	TH21	HG-PanIN	–1.3433
					29T	TH22	HG-PanIN	–1.8574
					78T	TH23	HG-PanIN	–0.5535
					70T	TH16	HG-PanIN	–0.3665
					45T	TH21	HG-PanIN	–0.2766
Loss	12q24.23–q24.31	120104173	124845413	<u>CDK2AP1</u> , <u>DIABLO</u> , <u>RSRC2</u>	78T	TH23	HG-PanIN	–0.6194
					37T	TH12	HG-PanIN	–0.6747
					39T	TH15	HG-PanIN	–0.5327
					45T	TH20	LG-PanIN	–1.0724
					45T	TH21	HG-PanIN	–1.1674
Loss	17p13.2–p11.2	18901	19703855	<u>TP53</u> , <u>ALOX15</u> , <u>CRK</u> , <u>HIC1</u> , <u>USP6</u> , <u>XAF1</u> , <u>MYBBP1A</u> , <u>PER1</u>	78T	TH23	HG-PanIN	–0.5619
					780T	TH24	HG-PanIN	–0.1895
					37T	TH12	HG-PanIN	–0.6213
					39T	TH15	HG-PanIN	–0.4583
					78T	TH23	HG-PanIN	–0.5323
Loss	17q23–q24.3	60981969	68416552	<u>AXIN2</u> , <u>PRKAR1A</u> , <u>CD79B</u> , <u>KPNA2</u> , <u>MAP2K6</u> , <u>STRADA</u> , <u>GNA13</u>	70T	TH16	HG-PanIN	–0.4227
					19T	TH17	HG-PanIN	–0.5008
					22T	TH19	HG-PanIN	–0.3881
					45T	TH20	LG-PanIN	–0.4278
					78T	TH23	HG-PanIN	–0.5228

Underlined genes indicate known oncogenes in regions of gain or tumor suppressor genes in regions of loss.

*Based on hg19 assembly.

CNA, copy number alteration; HG-PanIN, high-grade pancreatic intraepithelial neoplasia; LG-PanIN, low-grade pancreatic intraepithelial neoplasia; LRR, log R ratio.

Genomics Viewer version 2.3 (Broad Institute, Cambridge, MA) and NextGENeViewer. The potential pathogenicity of somatic mutations identified by digital NGS was evaluated by using ClinVar (<https://www.ncbi.nlm.nih.gov/>

clinvar, last accessed January 2018) and COSMIC (<http://grch37-cancer.sanger.ac.uk/cosmic>, last accessed January 2018), with the likelihood of pathogenicity determined by the FATHMM (Functional Analysis

Table 4 Chromothripsis-Like Regions Identified in PanIN Lesions

Patient ID	Sample ID	PanIN lesion	Chr	Start	Stop	Cytoband	Region, Mb	CNA switching times	Likelihood ratio, Log10
37T	TH12	HG-PanIN	17	20000001	69691432	17p11.2–17q24.3	49.69	11	7
39T	TH15	HG-PanIN	17	15000001	77435964	17p12–17q25.3	62.44	12	8
45T	TH20	LG-PanIN	17	1	40000000	17p13.3–q21.2	40.00	14	10
	TH21	HG-PanIN	17	1	46944323	17p13.3–q21.32	46.94	11	7
29T	TH22	HG-PanIN	1	1	40000000	1p36.33–p34.3	40.00	11	7

Chr, chromosome; CNA, copy number alteration; HG, high-grade; LG, low-grade; PanIN, low-grade pancreatic intraepithelial neoplasia.

Through Hidden Markov Models) score (0.9 or higher being considered likely pathogenic).

Results

Clinicopathologic Features

A summary of the individuals whose PanIN lesions were included in this study is provided in Table 1. One patient (49T) had undergone preoperative chemotherapy (gemcitabine with a hedgehog inhibitor as part of a clinical trial); none of the patients had undergone preoperative radiotherapy. Two LG-PanIN (PanIN-2) lesions from patients 45T and 49T, respectively, that were immediately adjacent to the HG-PanINs were also included (Table 1).

Copy Number Variation in PanIN Lesions

Somatic copy number alterations were observed in all HG-PanIN lesions, and one of the two LG-PanINs (patient 45T). On average, there were 2.7 regions of somatic copy number gain and 4.3 regions of copy number loss per PanIN (Table 2). The most common regions of copy number alteration were 9p21 (*CDKN2A* locus), 17p13 (*TP53*), and 18q21 (*SMAD4*). Other regions of chromosomal loss included 6p25–p24, 6q11–q27, 9p24–p21, 12q24, and 17q23–q24 (Figure 1 and Table 3). Each of these loci showed evidence of loss in at least 3 of 11 HG-PanIN lesions. Other chromosomal arms were also lost in one or two HG-PanINs (Table 3). Some loci that have been identified as commonly lost in PDACs, such as the *FHIT* locus on 3p, were not lost in any HG-PanINs, consistent with evidence that loss of fragile histidine triad (FHIT; which occurs in approximately 50% of PDACs)²³ is a late event that most commonly occurs with the progression of HG-PanIN to invasive cancer. Somatic copy number gain regions were detected at 8q24 (*MYC*) and 19q12 region (*CCNE1*) as well as 1q25–q31 (Table 3). There were regions of low-level focal amplification at 19q12 in the 19T HG-PanINs (LRR: 0.8888), 45T (0.8365 in LG-PanIN and 0.8362 in HG-PanIN), and at 19q13 in patient 69T HG-PanIN (0.9417) (Table 3). Homozygous deletions that involved the *CDKN2A* locus at 9p21.3 in the HG-PanIN from patient 70T (LRR: –1.567), the PanIN (–1.2731 in the LG-PanIN and –1.3433 in the HG-PanIN) from patient 45T, and the 29T HG-PanIN (–1.8574) were also identified (Table 3).

Copy number losses identified by the SNP array at the *CDKN2A* locus were validated by PCR. The copy number ratio determined by PCR (*CDKN2A*/reference gene *RNA-seP*) was concordant with the copy number ratio determined by the SNP array (Figure 1).

Detection of Chromothripsis-Like Events in PanIN Lesions

Chromothripsis-like events were identified in five PanIN lesions (Table 4). A similar but not identical chromothripsis pattern that included the *TP53* locus was identified in two related PanINs from patient 45T, one a LG-PanIN and the other a HG-PanIN (Table 4, Supplemental Table S1). This pair of PanIN lesions was also notable for having similar but not identical copy number alterations. In addition to multiple shared chromosomal gains and losses, the LG-PanIN had loss of one allele of 18q and 19p not detected in the HG-PanIN, and the HG-PanIN had 12q loss not detected in the LG-PanIN. The genetic alterations responsible for the progression of the LG-PanIN to HG-PanIN were not evident but this comparison was limited by the lack of available DNA for mutational analysis of the LG-PanIN DNA. The chromothripsis-like pattern in patient 29T involved the *ARID1A* region of 1p36 (Supplemental Table S1). In another PanIN, the chromothripsis pattern included copy number gains of *ERBB2*. The chromothripsis-like alterations are shown in Figure 2.

Gene Mutations in PanIN Lesions

Because MALBAC WGA can induce artifactual reads that mimic mutations,³¹ the original PanIN DNA was used for mutation analysis. Sufficient DNA was available from eight of the PanINs. *KRAS* mutations were detected in all evaluable HG-PanIN lesions (Table 5). Intragenic *CDKN2A* mutations were identified in two patients (19T and 69T). Intragenic *TP53* mutations were detected in four of eight HG-PanINs (50%), one patient had two *TP53* intragenic mutations. All seven HG-PanINs showed concordance with the associated cancer for their mutations, and several HG-PanINs had the same signature mutation(s) that were present in the cancer, indicating that it was these HG-PanIN lesions that subsequently evolved into the invasive cancer

Figure 2 Chromothripsis and copy number aberrations plot from CTLPScanner. Highlighted chromothripsis-like regions are shown in the left side of the graph. The x and y axes represent genomic positions and log R ratio of segments, respectively. The **orange lines** represent areas of copy number gain; **blue lines**, areas of copy number loss; **zero lines**, no gain/loss; **orange rectangles**, identified chromothripsis regions. Histograms on the right side show the number of copy number segment change events. Chr, chromosome; HG-PanIN, high-grade pancreatic intraepithelial neoplasia; LG-PanIN, low-grade pancreatic intraepithelial neoplasia.

(Table 5). This concordance may not be surprising, but PanIN lesions are often multifocal,¹² so it would not be surprising if HG-PanIN lesions were sometimes genetically distinct from nearby PDACs. Although many HG-PanIN lesions had copy number losses that involved the *SMAD4* locus, no *SMAD4* mutations or homozygous deletions were identified in the HG-PanIN. In contrast, DNA isolated from the microdissected invasive pancreatic cancers associated with seven of the PanINs found that two cancers had a *SMAD4* mutation and one had a *SMAD4* homozygous deletion. Overall five of the seven patients with paired PanIN and cancer mutation profiles had additional mutations detected in the cancer that were not seen in their HG-PanINs (in *SMAD4*, *TP53*, *TGFBR2*, *RNF43*). No deleterious mutations were detected in the PanIN or cancer samples that involved *GNAS*, *ARID1A*, *BRAF*, *FBXW7*, or *PIK3CA* (Table 5).

Evidence of biallelic inactivation was found (because of either inactivating mutation with copy number loss, homozygous deletion, or evidence of biallelic mutation) of *CDKN2A* in five of eight evaluable PanINs and for *TP53* in three of eight evaluable PanIN lesions. One HG-PanIN had a single *TP53* mutation with an allele frequency of approximately 50%, suggesting there was no biallelic inactivation. Although 3 of the 11 HG-PanINs had loss of one allele at the *SMAD4* locus, no homozygous deletions or intragenic mutations were found to indicate biallelic inactivation of *SMAD4* in these PanIN lesions (Supplemental Table S2).

Discussion

The most notable findings of our genetic analysis of HG-PanIN are that there was no evidence for *SMAD4* mutations or homozygous deletion, many HG-PanINs have *TP53* mutations, and some have chromothripsis-like changes. These results help refine our understanding of the PanIN progression model. With respect to genetic inactivation of *SMAD4*, the original PanIN model found loss of *Smad4* expression in some PanIN-3 lesions, but the results of this study are consistent with the results of recent studies.^{14,16} Loss of one copy of the *SMAD4* locus was commonly observed in HG-PanIN in this study and has been reported previously in PanIN-2 and PanIN-3 lesions,¹⁶ but it is biallelic inactivation of *SMAD4* that is thought to be necessary to promote pancreatic cancer development.³⁸ Yet, in some settings *SMAD4* haploinsufficiency promotes neoplasia, such as in the pathogenesis of juvenile polyps,³⁹ and in genetic engineered mice where it promotes the development of mucinous cystic neoplasia.⁴⁰ In this study, no *SMAD4* mutations or homozygous deletion events were found in the HG-PanIN, a clear difference to the presence of *SMAD4* mutations and homozygous deletions in the invasive pancreatic adenocarcinomas related to these HG-PanINs and to the approximately 50% rate of genetic inactivation of *SMAD4* in invasive PDACs in published studies.³⁸ This model of *SMAD4* inactivation during PanIN progression represents a revision of the original PanIN progression model. Overall, these results are consistent with the hypothesis that once biallelic inactivation of *SMAD4* occurs, it triggers the emergence of invasive adenocarcinoma.¹⁴

Table 5 Somatic Mutations of PanIN Lesions Identified by Next-Generation Target Sequencing

Patient ID	Sample ID	Lesion	Gene	Gene locus	Amino acid change	Mutant allele frequency, %	Mutation type	Function	CNA
19T	TH17	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12D	63.4	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	p.W110X	83.2	Substitution	Nonsense	No CNA
			<i>TP53</i>	17p13.1	p.Y126H	44.2	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.L130V	50.1	Substitution	Missense	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	34.6	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	p.W110X	72.5	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	c.375+1C>CT	9.8	Substitution-intronic	Splice site	No CNA
22T	TH19	HG-PanIN Cancer	<i>TGFB2</i>	3p24.1	p.L358delinsPTfs	72.2	Insertion	Frameshift	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	52.6	Substitution	Missense	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	25.3	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.R209Kfs	14.8	Deletion	Frameshift	No CNA
			<i>SMAD4</i>	18q21.2	p.I525V	31.7	Substitution	Missense	No CNA
37T	TH12	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12D	48.4	Substitution	Missense	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	32.9	Substitution	Missense	No CNA
45T	TH21	HG-PanIN	<i>KRAS</i>	12p12.1	p.G12V	45.2	Substitution	Missense deletion	No CNA
			<i>CDKN2A</i>	9p21.3	Homozygous deletion	NA	Deletion		CNA
49T	TH14	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12D	50.6	Substitution	Missense	No CNA
			<i>RNF43</i>	17q22	p.R404Pfs	88.0	Deletion	Frameshift	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	29.8	Substitution	Missense	No CNA
			<i>RNF43</i>	17q22	p.R404Pfs	48.7	Deletion	Frameshift	No CNA
			<i>TP53</i>	17p13.1	p.P8S	8.8	Substitution	Missense	No CNA
69T	TH18	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12D	41.6	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	p.R80X	81.9	Substitution	Nonsense	No CNA
			<i>TP53</i>	17p13.1	p.C135Y	77.9	Substitution	Missense	No CNA
			<i>KRAS</i>	12p12.1	p.G12D	31.5	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	p.R80X	60.8	Substitution	Nonsense	No CNA
			<i>RNF43</i>	17q22	p.A169T	31.1	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.C135Y	41.0	Substitution	Missense	No CNA
			<i>SMAD4</i>	18q21.2	Homozygous deletion	NA	Deletion	Deletion	Loss
70T	TH16	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12D	44.2	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.R175H	52.1	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	Homozygous deletion	NA	Deletion	Deletion	Loss
			<i>KRAS</i>	12p12.1	p.G12D	48.8	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.R175H	49.5	Substitution	Missense	No CNA
			<i>CDKN2A</i>	9p21.3	Homozygous deletion	NA	Deletion	Deletion	Loss
78T	TH24	HG-PanIN Cancer	<i>KRAS</i>	12p12.1	p.G12V	14.2	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.R306X	17.6	Substitution	Nonsense	Loss
			<i>KRAS</i>	12p12.1	p.G12V	25.7	Substitution	Missense	No CNA
			<i>RNF43</i>	17q22	p.R343H	26.8	Substitution	Missense	No CNA
			<i>TP53</i>	17p13.1	p.R306X	40.9	Substitution	Nonsense	Loss
			<i>SMAD4</i>	18q21.2	p.R361S	35.7	Substitution	Missense	No CNA

CNA, copy number alteration; HG, high-grade; PanIN, low-grade pancreatic intraepithelial neoplasia.

In this study, four of the eight evaluable HG-PanINs had mutated *TP53*. In contrast, in a recent analysis of 17 HG-PanINs from pancreata without invasive carcinoma only two had *TP53* mutations.¹⁴ The reasons for this apparent difference is not clear, but one possibility is that HG-PanIN lesions identified in patients with an invasive PDAC tend to be genetically more advanced than from patients whose HG-PanINs are resected before the development of an

invasive cancer. Given its accuracy, some studies use p53 immunohistochemistry as an additional test to identify evidence of *TP53* mutation in neoplastic tissues.⁴¹ The finding of *KRAS* mutations in all HG-PanINs and *CDKN2A* alterations in five of the eight evaluable HG-PanINs is consistent with prior studies.^{6,11}

Chromosomal regions affected by chromothripsis-like events were found in 5 of the 13 PanIN lesions evaluated.

Two of these chromothripsis-like events involved the *TP53* gene locus and were found in genetically related PanINs (one LG-PanIN and one HG-PanIN) from the same patient. Chromothripsis that involved the *TP53* locus has been reported in pancreatic and other cancers.^{25,42,43} Clonal copy number alterations are uncommon in LG-PanINs,²⁴ but this example suggests that chromothripsis can first emerge in LG-PanINs and that the underlying chromosomal instability helps promote progression to HG-PanIN and invasive cancer. One chromothripsis-like region involved chromosome 1p36, a region frequently deleted in a broad range of human cancers.⁴⁴ AT-rich interactive domain-containing protein 1A (*ARID1A*), which is known to be mutated in a small percentage of PDACs, localizes to this region.^{22,36,45}

The PanIN model predicts that clonal progression of PanIN occurs as a result of multiple independent genetic events, including oncogenic activation of *KRAS* (one event) and biallelic inactivation of multiple tumor suppressor genes (usually two genetic events for each tumor suppressor gene, except in patients with *CDKN2A* promoter methylation). The original PanIN model did not stipulate that all three major PDAC tumor suppressor genes (*CDKN2A*, *TP53*, and *SMAD4*) be inactivated for pancreatic cancer to develop; indeed, it was known that most pancreatic cancers do not inactivate all three of these genes,⁴⁶ and the model did not rule out other pathways to invasive cancer. Notta et al²⁵ performed copy number and mutation analyses of 107 pancreatic cancers to examine the extent of chromothripsis, polyploidy, and translocation events and concluded that because chromothripsis events could simultaneously involve multiple tumor suppressor loci, it discredited the existing PanIN model and was more consistent with a catastrophic model of tumor progression.^{25,47} However, the finding of chromothripsis in pancreatic cancers or in PanIN lesions is consistent with the multistep PanIN model of progression and is not a sufficient mechanism to cause catastrophic neoplastic progression. The original PanIN progression model predicted that the number of genetic events required for a PanIN to develop and progress to an invasive cancer could be as few as five to seven events (one event for *KRAS* mutation; typically two events for biallelic inactivation of *CDKN2A* by mutation, chromosomal loss, and/or promoter methylation; and two events for biallelic inactivation of *TP53* and/or *SMAD4* and/or another tumor suppressor gene by mutation and/or chromosomal loss). The model did not stipulate mechanisms of chromosomal loss or rule out other driver gene events during PanIN progression. A chromothripsis event that resulted in the simultaneous loss of two chromosomal arms on separate chromosomes (eg, simultaneous loss of 18q and 9p) would reduce by one event the number of genetic events required for PanIN progression to invasive cancer. Even in one rare example in which three tumor suppressor loci are simultaneously affected by chromothripsis,²⁵ additional genetic events are still needed for biallelic tumor suppressor gene inactivation and progression to invasive cancer.⁴⁸ Even if some PanINs

developed chromothripsis in multiple independent areas of the genome, which was not observed and is probably rare, additional genetic events would still be required. Although Notta et al²⁵ observed chromothripsis events in the majority (65%) of pancreatic cancers, most of these events did not target any of the major tumor suppressor gene loci (*CDKN2A*, *TP53*, *SMAD4*), and only a minority (14%) of the chromothripsis (or translocation) events simultaneously targeted multiple tumor suppressor loci.²⁵

Understanding the timing of PanIN progression is important for early detection efforts, particularly strategies designed to detect mutations arising from PanIN in pancreatic fluids.⁴⁹ It is also important to distinguish the growth and progression of PanIN to invasive cancer from the growth and progression of invasive PDACs. Once an invasive pancreatic cancer cell emerges, it likely takes several years for that cell to grow into a detectable tumor mass.⁵⁰ Although a time frame of several years for pancreatic cancer growth may seem inconsistent with rapid clinical progression observed in clinical practice, it is not, because for most of this period the tumor is too small to be detected by clinical tests. Once a pancreatic tumor reaches a detectable size (generally at least 5 to 10 mm in diameter), it can be expected to soon progress to advanced-stage disease.⁵¹

Conclusions

We find evidence that HG-PanINs frequently harbor *TP53* but not *SMAD4* mutations. In addition to chromosomal copy number alterations, many PanINs harbor evidence of chromothripsis. Overall, the genetic alterations identified in HG-PanINs are consistent with a multistep model of PanIN progression.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.03.012>.

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