

Widespread activation of the DNA damage response in human pancreatic intraepithelial neoplasia

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Pancreatic intraepithelial neoplasia (PanIN) lesions are the most common non-invasive precursors of pancreatic adenocarcinoma. We postulated that accumulating DNA damage within the PanIN epithelium activates checkpoint mechanisms. Tissue microarrays were constructed from 81 surgically resected primary pancreatic adenocarcinomas and an independent set of 58 PanIN lesions (31 PanIN-1, 14 PanIN-2, and 13 PanIN-3). Immunohistochemical labeling was carried out using anti- γ H2AX^{Ser139}, anti-phosphoATM^{Ser1981}, anti-phosphoChk2^{Thr68}, and anti-p53. A 'histologic score' combining area and intensity of labeling in the nuclear compartment was determined for each lesion. A progressive increase in γ H2AX^{Ser139} labeling, consistent with escalating DNA damage, was observed in the non-invasive precursor lesions (scores of 4.34, 6.21, and 7.50, respectively, for PanIN-1, -2, and -3), compared with the pancreatic ductal epithelium (score 2.36) (ANOVA, $P < 0.0001$). In conjunction, activation of the ataxia telangiectasia mutated (ATM)–Chk2 checkpoint pathway was observed in all histological grades of PanIN lesions. Specifically, pATM^{Ser1981} histologic scores for PanIN-1, PanIN-2, and PanIN-3 were 4.83, 5.14, and 7.17, respectively, versus 2.33 for the ductal epithelium (ANOVA, $P < 0.0001$); the corresponding scores for pChk2^{Thr68} were 5.43, 7.64, and 5.44 in PanINs-1, -2, and -3, respectively, versus 2.75 in the ductal epithelium (ANOVA, $P < 0.0001$). In contrast, absent to minimal nuclear p53 was observed in the ductal epithelium, and in PanINs-1 and -2 (a histologic score of 0–1.86), with a significant upregulation (corresponding to mutational inactivation) seen only at the stage of PanIN-3 and invasive neoplasia (histologic scores of 4.00 and 4.22). Nuclear p53 accumulation in cancers was associated with attenuation of the ATM–Chk2 checkpoint and a restitution to 'baseline' levels. To conclude, activation of the ATM–Chk2 checkpoint pathway is commonly observed in PanINs, likely in response to the accumulating DNA damage from events such as oncogene mutations and telomere dysfunction. Loss of p53 function appears to be a critical determinant for bypassing this checkpoint and the subsequent progression to invasive adenocarcinoma.

Modern Pathology (2009) **22**, 1439–1445; doi:10.1038/modpathol.2009.114; published online 7 August 2009

Keywords: pancreatic cancer; pancreatic intraepithelial neoplasia; DNA damage; ATM; Chk2

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Received 26 May 2009; revised 23 June 2009; accepted 26 June 2009; published online 7 August 2009

Adenocarcinoma of the pancreas affects approximately 38 000 individuals each year in the United States of America, and nearly all patients die within months of diagnosis.¹ A multistep model has recently been proposed for pancreatic adenocarcinomas, in which non-invasive precursor lesions in the pancreatic ducts undergo histological and genetic progression toward invasive cancer.^{2,3} These

morphologically distinct non-invasive lesions have been classified under a uniform nomenclature scheme termed Pancreatic Intraepithelial Neoplasia (PanIN). We and others have shown that PanINs share many of the genetic aberrations associated with invasive adenocarcinomas, underscoring their classification as 'neoplasms' rather than a reactive/hyperplastic process.⁴⁻¹⁰ Some of the genetic alterations are nearly ubiquitous (eg, oncogenic *KRAS2* mutations and telomere dysfunction),^{4,5} suggesting that these are early events in the ductal epithelium, whereas others such as loss-of-function of the tumor suppressor gene, *BRCA2*, or upregulation of the GPI-anchored protein, mesothelin, occur only in the most advanced PanIN lesions that precede invasive cancer.^{7,10}

Autopsy studies have confirmed that PanIN lesions are, surprisingly, common in the general population, with more than 50% of the general population over 60 years harboring one or more low-grade lesions in their pancreata.¹¹ Nevertheless, despite this remarkably high prevalence and the presence of clonal genetic alterations,¹² the overwhelming majority of low-grade PanINs do not progress to invasive adenocarcinoma, as obviously gauged by the annual incidence rates for this disease. One can speculate, therefore, that these low-grade PanINs either undergo apoptosis and are 'shed' from the body, or that intracellular checkpoint mechanisms come into effect, forestalling or entirely preventing their progression to higher-grade PanIN lesions (carcinoma *in situ*) and invasive cancer. Recent seminal studies have identified the DNA damage repair protein ataxia telangiectasia mutated (ATM) and its downstream target, the human homolog of the bacterial checkpoint, Chk2, as a pervasive checkpoint in human epithelial precancerous lesions.¹³⁻¹⁵ A variety of inciting factors, such as telomere dysfunction and oncogene-induced 'replication stress' can cause DNA damage in pre-cancerous lesions, activating the ATM-Chk2 checkpoint, thereby impeding their progression to invasive malignancy.^{16,17}

We hypothesized that activation of the DNA damage response (DDR) checkpoint in the most common non-invasive precursor lesions of pancreatic adenocarcinoma could provide a putative explanation for the lack in connection between PanIN prevalence in the general population and the incidence of invasive adenocarcinoma. Herein, we confirm that activation of the ATM-Chk2 checkpoint is widespread in human PanIN lesions, including in the lowest grade (PanIN-1) lesions. This phenomenon appears to be a consequence of DNA replication stress and the occurrence of double-strand breaks, as measured by the progressive accumulation of phospho-histone γ H2AX, which forms a scaffold at double-strand breaks.¹⁸ We also provide evidence that loss of p53 function is a critical threshold event in the multistep progression of pancreatic cancer, occurring mostly at or

beyond the stage of PanIN-3, allowing the neoplastic epithelium to bypass DDR-induced checkpoints and progress unimpeded into invasive adenocarcinoma.

Materials and methods

Tissue microarrays were prepared from the archival formalin-fixed paraffin-embedded sections of 81 surgically resected primary pancreatic adenocarcinomas, as previously described;^{19,20} this 'cancer tissue microarray' also included 73 cores of the non-neoplastic pancreatic ductal epithelium. An independent set of 58 PanIN lesions (31 PanIN-1, 14 PanIN-2, and 13 PanIN-3) was also arrayed on a 'PanIN tissue microarray', as previously described.^{4,7} For tissue microarray construction, representative areas containing morphologically defined cancers or PanINs were circled on the glass slides and used as a template. The tissue microarrays constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD, USA) and a 1.4 mm core was punched from the donor block to ensure that adequate lesional tissue could be incorporated into the spot.

Immunohistochemistry was carried out as previously described.⁷ Briefly, unstained 5- μ m sections were cut from the paraffin block selected and deparaffinized by routine techniques. Thereafter, the sections were quenched with 3% H₂O₂ for 10 min. The slides were steamed in 10 mM citrate buffer (pH 6.0) to unmask the epitopes for 20 min at 95°C, and then allowed to cool down for 20 min to room temperature. Before incubating with the primary antibody, the slides were blocked for 30 min with a 10% fetal bovine serum solution (Invitrogen, Carlsbad, CA, USA). The following primary antibodies were used for this study: anti-phospho γ H2AX^{Ser139} (Upstate-Millipore, Millipore, MA, USA; dilution 1:200), anti-phosphoATM^{Ser1981} (Rockland Immunochemicals, Boyertown, PA, USA; dilution 1:100), anti-phosphoChk2^{Thr68} (Cell Signaling Technology, Beverly, MA, USA; dilution 1:100), and anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200). The specific phospho antibodies for γ H2AX, ATM, and Chk2 were selected based on the published association of phosphorylation at these sites with functional status of the respective protein.^{13,14,17,21} Labeling was detected with the the PowerVision + Poly-HRP IHC kit (Immunovision Technologies, Norwell, MA, USA) following the standard protocol. Slides were counterstained with Harris's hematoxyline solution. Negative controls (primary antibody replaced by serum from appropriate species) were used for each antibody in each run.

Immunohistochemical labeling was scored using a previously described histologic score (also known as HistoScore) scheme^{6,22,23} that takes into consideration both the area and intensity of labeling in the appropriate (nuclear) compartment. Specifically,

intensity of labeling was designated as 0–3 for absent, weak, moderate, and strong, and area of labeling was designated as 0–3 for <5%, 5–25%, 26–50% and >50%, respectively. The lesional histologic score was calculated by the product of area and intensity, and subsequently, the average histologic scores for the individual histological grades of PanIN lesions, adenocarcinomas, and normal ductal epithelium were determined.

Statistical analyses were carried out using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). Differential expressions of phospho- γ H2AX^{Ser139}, phospho-ATM^{Ser1981}, phospho-Chk2^{Thr68}, and p53 proteins in normal pancreatic ductal epithelium, various grades of PanINs, and pancreatic ductal adenocarcinomas were compared by ANOVA and Duncan's multiple range tests. A $P < 0.05$ was considered statistically significant.

Results

The mean histologic scores of phospho- γ H2AX^{Ser139}, phospho-ATM^{Ser1981}, phospho-Chk2^{Thr68}, and p53 are summarized in Table 1a, whereas a graphical representation is provided in Figure 1. Statistically significant differences between histologic scores for PanINs-1, -2, -3, or adenocarcinoma and that observed in non-neoplastic ductal epithelium for each of the four proteins are indicated in Table 1b (calculated using Duncan's multiple range test, level of significance at $P < 0.05$).

A progressive increase in phospho- γ H2AX^{Ser139} labeling, consistent with escalating DNA damage, was observed in PanIN lesions (histologic scores of

4.34, 6.21, and 7.50, respectively for PanIN-1, -2, and -3), compared with ductal epithelium (histologic score 2.36) (ANOVA, $P < 0.0001$). It is interesting to note that although invasive cancers had a significantly higher phospho- γ H2AX^{Ser139} histologic score (4.53) than non-neoplastic ductal epithelium ($P < 0.05$, Duncan's test), it was significantly lower than that observed in both PanINs-2 and -3, respectively. In conjunction with escalating double-strand breaks, a progressive activation of the ATM-Chk2 checkpoint was observed along the histological continuum of PanIN lesions. Specifically, phospho-ATM^{Ser1981} histologic scores for PanIN-1, PanIN-2, and PanIN-3 were 4.83, 5.14, and 7.17, respectively, versus 2.33 for ductal epithelium (ANOVA, $P < 0.0001$); the corresponding histologic scores for phospho-Chk2^{Thr68} were 5.43, 7.64, and 5.44 in PanINs-1, -2, and -3, respectively, versus 2.75 in ductal epithelium (ANOVA, $P < 0.0001$). As indicated in Table 1b, the histologic score for each histological grade of PanIN was significantly higher than the corresponding histologic score in the ductal epithelium, for both proteins ($P < 0.05$, Duncan's test). In both instances, attenuation of the checkpoint was observed in invasive cancers (histologic scores of 4.84 and 2.43, respectively, for phospho-ATM^{Ser1981} and phospho-Chk2^{Thr68}), such that in the case of phospho-Chk2^{Thr68} no significant difference in histologic scores was observed between cancer and the ductal epithelium. In contrast to the aforementioned proteins, absent to minimal nuclear p53 was observed in the ductal epithelium, as well as in PanINs-1 and 2 (histologic score of 0–1.86), with a significant upregulation (corresponding to mutational inactivation) seen only at the stage of

Table 1a Summary of histologic scores for DDR markers in normal ductal epithelium, PanINs, and pancreatic ductal adenocarcinomas

Antibody	Normal duct (N = 73)	PanIN-1 (N = 31)	PanIN-2 (N = 14)	PanIN-3 (N = 13)	Adenocarcinoma (N = 81)	ANOVA P-value
p γ H2AX ^{Ser139}	2.36	4.34	6.21	7.50	4.53	<0.0001 ^a
pATM ^{Ser1981}	2.33	4.83	5.14	7.17	4.84	<0.0001 ^a
pChk2 ^{Thr68}	2.75	5.43	7.64	5.44	2.43	<0.0001 ^a
p53	0	1.41	1.86	4.00	4.22	<0.0001 ^a

DDR, DNA damage response and PanIN, pancreatic intraepithelial neoplasia.

^aSignificant at the level of P -value < 0.05.

Table 1b Statistically significant differences in histologic scores for DDR markers in PanINs and in adenocarcinomas, compared to histologic score in the non-neoplastic ductal epithelium

Antibody	PanIN-1 (N = 31)	PanIN-2 (N = 14)	PanIN-3 (N = 13)	Adenocarcinoma (N = 81)
p γ H2AX ^{Ser139}	$P < 0.05^a$	$P < 0.05^a$	$P < 0.05^a$	$P < 0.05^a$
pATM ^{Ser1981}	$P < 0.05^a$	$P < 0.05^a$	$P < 0.05^a$	$P < 0.05^a$
pChk2 ^{Thr68}	$P < 0.05^a$	$P < 0.05^a$	$P < 0.05^a$	NS
p53	NS	NS	$P < 0.05^a$	$P < 0.05^a$

DDR, DNA damage response; NS, not significant and PanIN, pancreatic intraepithelial neoplasia.

^aSignificant at the level of P -value < 0.05, *Post-hoc* Duncan's multiple range test.

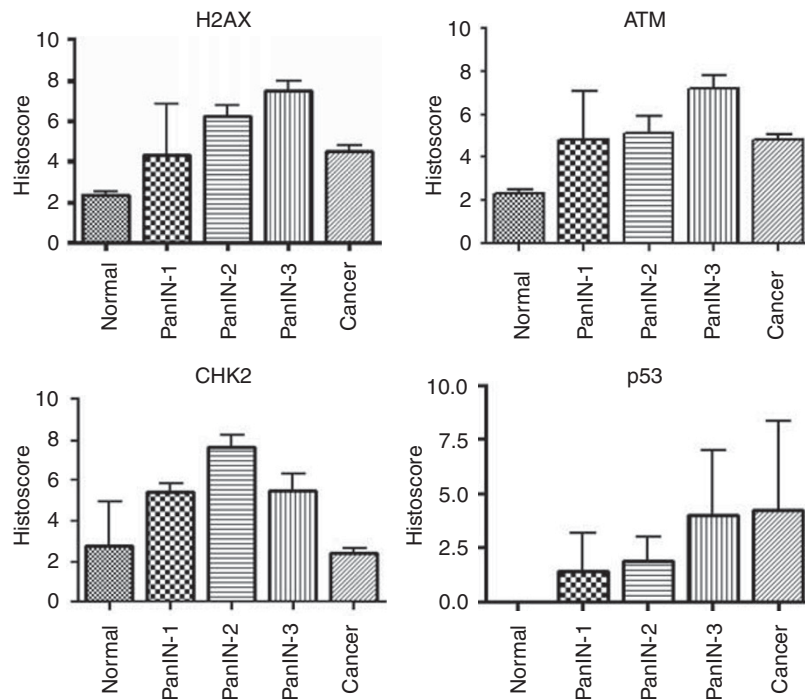


Figure 1 Histograms illustrating the histologic scores for each of the four proteins analyzed in this study, including phospho- γ H2AX^{Ser139}, phospho-ataxia telangiectasia mutated^{Ser1981} (ATM^{Ser1981}), phospho-Chk2^{Thr68}, and p53. The histologic scores are stratified by the normal ductal epithelium, pancreatic intraepithelial neoplasias (PanINs)-1, -2, and -3, and invasive cancer. The mean histologic score and s.d. are represented for each grade of lesion. See text for details and statistical analyses.

PanIN-3 and invasive neoplasia (histologic scores of 4.00 and 4.22). Representative photomicrographs showing expression of these four proteins along the PanIN progression model culminating in invasive cancer are illustrated in Figure 2.

Discussion

A diverse array of intracellular signals may activate the so-called DDR checkpoint in cells, including DNA damage itself, as well as critical telomere shortening and oncogene activation (reviewed in ref. 24–28). Telomere dysfunction and oncogene activation appear to precipitate so-called ‘replicative stress,’ leading to DNA damage, and culminating in activation of the DDR checkpoint.^{16,17,25} The principal DNA damage phenotypes observed in the setting of the DDR are double-strand breaks, and these foci can be recognized by the binding of phosphorylated histone γ H2AX to the damaged chromatin.^{18,29} The phosphorylated γ H2AX forms a scaffold for the DNA repair machinery to engage at the site of double-strand breaks, and therefore serves as a surrogate readout for DNA damage in cells. In mammalian cells, ATM, and its target, the bacterial checkpoint homolog protein Chk2, are the most important ‘sensors’ of double-strand breaks.^{28,30} Activation of ATM was originally described as an intracellular response to ionizing radiation, which, in turn, results in the activation of Chk2 protein through

phosphorylation of a Thr⁶⁸ moiety.^{31,32} As countless examples in experimental animal models and cognate human scenarios have documented, abrogation of the DDR checkpoint itself, or secondary defects in p53, enable cells to escape bypass this checkpoint even in the face of genomic damage (reviewed in refs. 27, 30, 33, 34).

In recent years, evidence has emerged to support aberrant activation of the DDR checkpoint in human epithelial pre-cancerous lesions. For example, Bartek *et al*¹⁴ described widespread abnormalities of the ATM–Chk2 axis in non-invasive precursor lesions of the human bladder, colon, and breast cancers, whereas Gorgoulis *et al*¹⁵ described comparable findings in the context of lung and epidermal tissues. In all of these instances, DDR checkpoint activation was accompanied by evidence of DNA double-strand breaks, as assessed by phosphorylated γ H2AX expression. It was noted that p53 function was generally retained in the non-invasive precursor lesions, whereas progression to invasive cancer was accompanied by p53 inactivation, underscoring a selection pressure for clones with p53 dysfunction.^{14,15} Further, DDR in pre-cancerous lesions was observed before the onset of genomic instability that characterizes invasive cancer, suggesting that widespread allelic imbalances were not the underlying basis for checkpoint activation within the epithelium.

In this study, we report that histological progression along the PanIN continuum is associated with

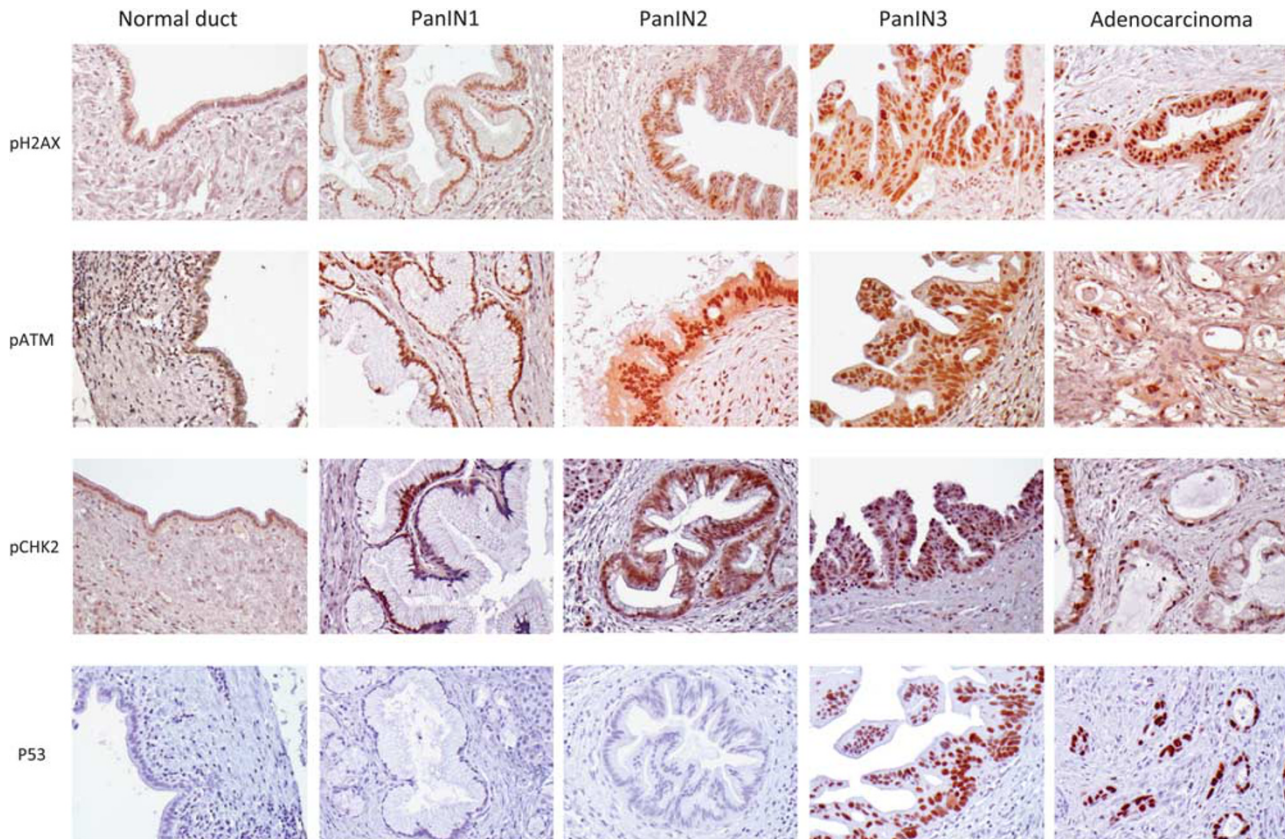


Figure 2 Representative photomicrographs illustrating the expression of phospho- γ H2AX^{Ser139}, phospho-ataxia telangiectasia mutated^{Ser1981} (ATM^{Ser1981}), phospho-Chk2^{Thr68}, and p53 in various histological grades of the pancreatic ductal lesions.

an escalating degree of DNA damage, as assessed by phosphorylated γ H2AX expression, as well as activation of the ATM–Chk2 checkpoint. For three of these proteins (phospho- γ H2AX^{Ser139}, phospho-ATM^{Ser1981}, and phospho-Chk2^{Thr68}), we found significant differences in the histological scores between non-neoplastic ductal epithelium and even PanIN-1, implying that DDR activation is one of the earliest molecular events in the multistep progression of pancreatic cancer. Nuclear accumulation of p53 is a reliable surrogate for mutational inactivation,³⁵ and this was minimally observed up to the stage of PanIN-2, consistent with retained p53 function. In contrast, a significant upregulation of nuclear p53 was seen in PanIN-3 and in invasive cancer, reinforcing the need for loss of p53 function to bypass the DDR checkpoint. In our series, abrogation of p53 and progression to invasive adenocarcinoma was associated with a restitution of activated Chk2 expression to ‘baseline’ levels (ie, no significant differences in histologic scores between adenocarcinoma and non-neoplastic epithelium). Thus, the results described here are comparable to those observed in precursor lesions at other epithelial sites,^{14,15} and provide a unifying model for containing the unimpeded progression of precursor lesions to invasive cancer. A pictorial representation of these interdependent processes

(DNA damage, DDR, and p53 accumulation) along the PanIN histological continuum is presented in Figure 3, and underscores the temporal significance of p53 mutations in bypassing the ATM–Chk2 checkpoint.

One pertinent question that remains unanswered is the inciting event(s) leading to DDR within the pancreatic ducts, as genomic instability alone is unlikely to explain the rather widespread nature of the response. We believe that the reasons are multifactorial, with *KRAS* mutations and telomere dysfunction being the most likely culprits, as both are known to induce DNA damage.^{16,17,21,24,26,36} In fact, our group has previously shown that telomere attrition is present in >90% of PanIN-1,⁴ providing a rational basis for ‘replicative stress’ and induction of DDR in the earliest precursor lesions. Re-activation of telomerase activity in invasive adenocarcinomas, as well as the consequent reduction in replicative stress, might underlie the paradoxical attenuation of double-strand breaks (ie, phospho- γ H2AX^{Ser139} labeling) observed in the cancer samples, when compared with the levels in higher-grade PanIN lesions. The potential role of mutant *KRAS* in DDR has emerged from a recent mouse model of pancreatic cancer mediated by the expression of mutant *Kras* from its endogenous promoter, wherein markers attributable to senescence are observed in

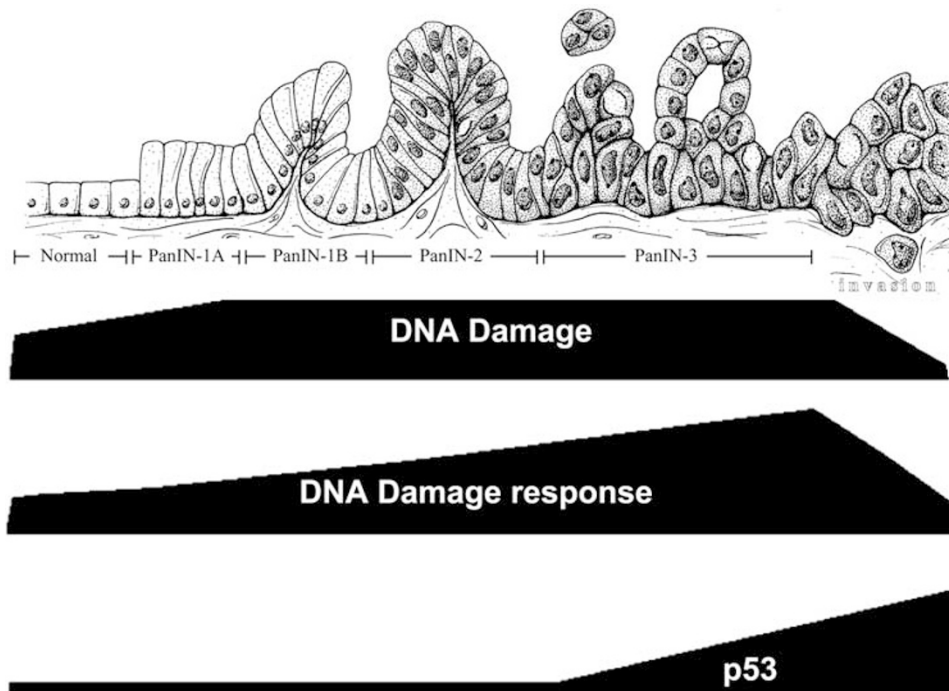


Figure 3 A proposed model of DNA damage response (DDR) mediated by the ataxia telangiectasia mutated (ATM)–Chk2 checkpoint in the pancreatic ductal epithelium. In response to double-strand breaks, DDR can be observed in the earliest pancreatic intraepithelial neoplasia (PanIN) lesions, and the increase in ATM–Chk2 expression parallels the histological progression to high-grade PanIN-3. Inactivation of p53 function at the stage of PanIN-3 and beyond is associated with bypass of the DDR checkpoint and progression to invasive cancer.

the murine PanIN lesions, but are lost on progression to invasive adenocarcinoma.²¹ Furthering this parallel between human and murine disease is the observation that mice expressing mutant *Kras* alone develop invasive cancers in a minority of cases (<10%),³⁷ while cooperating hits that allow cells to bypass checkpoints (eg, loss of *Trp53* or *Ink4a/Arf*) result in complete and accelerated penetrance for the malignant phenotype.^{38–40}

In summary, we report widespread activation of the DDR checkpoint in the most common non-invasive precursor lesions of pancreatic cancer, including in the lowest grade PanINs. We observe that a DDR-induced checkpoint in PanINs is contingent on retained p53 function, and that the inactivation of this ‘gatekeeper’ gene is likely one of the most critical events in opening the floodgates to invasive neoplasia. Finally, our results may provide a functional basis to the discordance between the rather common occurrence of PanIN lesions observed in the elderly population and the relatively uncommon incidence of pancreatic adenocarcinoma.

Acknowledgements

Supported by the Sol Goldman Pancreatic Cancer Research Center, the Michael Rolfe Foundation for Pancreatic Cancer Research and the NIH SPOR in GI Cancers P50CA062924. J-BMK is supported by the Netherlands Cancer Research Foundation (KWF)

and International Exchange Program Grant provided by the University Medical Center Utrecht. The authors are grateful to Professor David A. Tuveson (Cambridge Research Institute, UK) for his insightful discussions on this paper.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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