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Frequency of *K-ras* Mutations in Pancreatic Intraductal Neoplasias Associated with Pancreatic Ductal Adenocarcinoma and Chronic Pancreatitis: A Meta-Analysis[1]

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Abstract

Molecular analyses have demonstrated mutations in the *K-ras* gene at codon 12 in the majority of pancreatic ductal adenocarcinomas (PDACs). In order to determine whether the *K-ras* mutation rate increases parallel to the grade of dysplasia in duct lesions, we performed a meta-analysis of the studies published between 1988 and 2003 that provide information on *K-ras* mutations in hyperplastic and dysplastic duct lesions in the pancreas. The described duct lesions were reclassified according to the nomenclature for pancreatic intraepithelial neoplasia (PanIN), and the molecular methods for detecting K-ras were reviewed. In PanIN lesions from pancreata of patients with PDAC, there was a stepwise increase in *K-ras* mutations that correlated with the grade of dysplasia of the PanIN lesion. *K-ras* mutations were found in 36%, 44%, and 87% of PanIN-1a, 1b, and 2–3 lesions, respectively (trend statistic $P < .001$). Mutation-enriched polymerase chain reaction (PCR) resulted in higher rates of *K-ras* mutations in PanIN than plain PCR did. The incidence of *K-ras* mutations in PanIN lesions associated with chronic pancreatitis (CP) or normal pancreas was low (around 10%). In CP, *K-ras* mutations were only found after a disease duration of 3 years. The correlation of the incidence of *K-ras* mutations with the grade of dysplasia in PanIN and the occurrence of these mutations in CP with a duration of more than 3 years underlines the importance of this genetic change for the development of PDAC.

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Keywords: *K-ras* mutation, pancreatic ductal carcinoma, chronic pancreatitis, pancreatic intraepithelial neoplasia, meta-analysis.

Introduction

Because of its late detection and poor response to therapy, pancreatic ductal adenocarcinoma (PDAC) ranks as one of the most lethal tumors [1]. Numerous genetic studies have been conducted to identify a suitable marker for the early recognition of PDAC [2,3]. This has resulted in the identification of a number of PDAC-associated genes [4,5]. Among these, the first gene studied in detail was the *K-ras* gene. It soon turned out that in the majority of PDACs [6], it shows mutations in a hot spot at codon 12, exon 1. When *K-ras* mutations were also found in *in situ* components of PDAC and PDAC-associated duct lesions [7–9], they were thought to be potential tumor precursors [10], and it was hoped that mutated *K-ras* might serve as a marker of the developing PDAC [11]. However, further studies revealed *K-ras* mutations even in the normal-appearing duct epithelium of the inflamed and noninflammed non-neoplastic pancreas [12,13]. Similar results were obtained in extrapancreatic adenocarcinomas, which were also found to have an elevated *K-ras* mutation rate [14]. These findings reduced the significance of K-ras as a specific tumor marker and raised the question of the role of K-ras in pancreatic carcinogenesis. Another problem was that the frequency of *K-ras* mutations found in duct lesions and PDACs differed from study to study, most likely because of differences in the molecular methodology [15] and the histopathologic terminology used for the analyzed duct lesions.

The aim of this study was to determine if there is a correlation between pancreatic intraepithelial neoplasia (PanIN) severity and the frequency of *K-ras* mutation in patients with and without PDAC by reviewing the published data on *K-ras* mutations in duct lesions [16] using the new PanIN classification. This recently proposed classification is based on the hypothesis that hyperplastic pancreatic duct lesions have a preneoplastic potential [10]. Hence, the duct lesions were given the name PanIN. The designation seems to be correct because three studies demonstrated an increase in loss of heterogeneity (LOH) for p16, p53, and DPC4 according to the grade of

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dysplasia of the PanINs [17–19]. In order to make these LOH data comparable to those of mutated *K-ras* in duct lesions, we applied the PanIN classification to the old histologic terms used to describe these lesions in the various studies. We also included studies on patients with chronic pancreatitis (CP) to find out whether the K-ras positivity rate in the PanINs is similar to that in the PanINs associated with PDACs.

We present a combined histopathologic and molecular meta-analysis that attempts to better define the role that both PanIN and K-ras may play in the pathogenesis of PDAC and the progression of CP to PDAC.

Materials and Methods

Data Acquisition

The study was conceived as a histopathologic meta-analysis [16]. We conducted a computer search of the Medline database for articles on *K-ras* mutations in pancreatic duct lesions between 1988 and 2003. We evaluated only studies published in peer-reviewed journals that presented precise data on the histopathology of the duct lesions in the form of either illustrations or detailed descriptions. Therefore, short reports, abstracts, and conference reports, which are usually included in the data acquisition of a meta-analysis, were excluded [16]. Using search terms such as *dysplasia*, *hyperplasia*, *metaplasia*, and *intraductal tumor* in conjunction with *pancreas* and *pancreatic*, we identified 15 original contributions with all the relevant information (Table 1). The way the tissue was obtained (surgery, postmortem, and microdissection), the associated condition (ductal adenocarcinoma, CP, and normal pancreas), and the polymerase chain reaction (PCR) technique were recorded [15]. Duct lesions reported in association with intrapancreatic mucinous tumors (IPMTs) were excluded. A quality assessment protocol [20] was generated, defining the minimum criteria necessary for a study to be included in the systematic review. These were: 1) detailed histopathologic description of the lesions; 2) histologic evidence of examples of such pancreatic lesions in printed figures; and 3) detection of *K-ras* mutations. The suitability of a given paper for inclusion in this study was assessed by two of the authors.

Evaluation of Histopathologic Data

On the basis of the PanIN classification, all studies with histopathologic reports were reevaluated and the lesions were classified [10,21] by two independent pathologists (G.K. and J.L.) (Appendix 1).

Evaluation of Molecular Data

The PCR technique employed was characterized as either plain PCR for K-ras without modification or mutation-enriched PCR (ME-PCR) technique employing special enzymes and/or PCR protocols [15]. Further, the method used to verify PCR amplificates (restriction fragment length polymorphism, direct DNA sequencing, and allele-specific oligonucleotide hybridization) was recorded.

Statistical Analysis

We performed a meta-analysis of all the available reports. The confidence interval estimates for the proportion of K-ras–positive samples for each individual study and for the overall estimates were calculated using Wilson's method [22]. For comparison of dichotomized stratified data, we used the chi-square analysis. The Mantel-Haenszel test for linear association was used to assess the significance of the trend among strata. The exact Wilcoxon-Mann-Whitney nonparametric test was used to compare the age distribution and the duration of CP with the presence or absence of *K-ras* mutations.

Results

We identified 15 studies dealing with *K-ras* mutations in pancreatic preneoplastic lesions that were histologically defined. A total of 1519 lesions was included in these studies. In all studies, it was possible to reclassify the pancreatic duct lesions according to the PanIN classification (Appendix 1).

The cases described as simple hyperplasia or mucous hypertrophy corresponded well to PanIN-1A, and those described as ductal papillary hyperplasia to PanIN-1B, as shown by the figures in the various studies (Table 1). The review of our own studies [9,17] also revealed a good correlation between the old and new terminologies. Sixty-one papillary lesions in CP with mild atypia were classified as PanIN-1B. In a study by Yanagisawa et al. [23], histologic illustrations of four lesions described as mucous papillary hyperplasia revealed a high degree of nuclear atypia but no structural abnormality. Hence, they were classified as PanIN-2 lesions. In another investigation [7], six lesions were typed as *in situ* carcinoma. They were accordingly classified as PanIN-3 lesions.

A total of 908 lesions was classified as PanIN-1A, 476 as PanIN-1B, 79 as PanIN-2, and 56 as PanIN-3. The overall rate for *K-ras* mutations in PanIN lesions was 10% in CP (95% CI, 7–14%) and 44% in PDAC (95% CI, 41–48%). There was no correlation between K-ras positivity and the source of the tissue.

The frequency of *K-ras* mutations in the PanIN lesions varied considerably according to the underlying disease of the patients (CP *versus* PDAC). Therefore, we investigated the relation between *K-ras* mutations and the grade of the PanIN lesions separately for each group of patients (PDAC, CP, and normal) (Tables 1 and 2).

In patients with PDAC, the frequency of *K-ras* mutations in the PanIN lesions increased with the PanIN grade: 36% in PanIN-1A lesions, 44% in PanIN-1B lesions, and 87% in PanIN-2–3 lesions (Table 2). Due to the relatively small number of lesions, PanIN-2 and PanIN-3 lesions were combined for statistical evaluation (Table 2). When data from studies using plain PCR were compared with data from studies using ME-PCR, it was found that the latter technique was associated with a higher detection rate of *K-ras* mutation in PanIN-IA and PanIN-IB lesions (Table 2).

Table 1. Frequency of *K-ras* Mutations in PanIN Lesions in the Pancreas of Patients with PDAC (Upper Panel) or CP, or in Normal Disease-Free Pancreas (Lower Panel).

Study	Reference	Patients**	Lesions	Method	*K-ras*$^+$/Total Foci	*K-ras*$^+$ (%)
Lemoine et al. (1992)	[7]	PDAC	PanIN-1b	PCR	0/9	0
		PDAC	PanIN-3	PCR	5/6	83
Motojima et al. (1993)	[54]	PDAC	PanIN-3	PCR	2/2	100
Tabata et al. (1993)	[55]	PDAC	PanIN-1a	PCR	0/10	0
		PDAC	PanIN-2	PCR	5/6	83
Moskaluk et al. (1997)	[8]	PDAC	PanIN-3	PCR	16/17	94
		PDAC	PanIN-1a	PCR	2/7	29
Sugio et al. (1997)	[56]	PDAC	PanIN-3	PCR	17/18	94
		PDAC	PanIN-2	PCR	19/23	83
		PDAC	PanIN-1a	PCR	5/21	24
Z'graggen et al. (1997)	[57]	PDAC	PanIN-1a	PCR	3/11	27
Matsubayashi et al. (1999)	[58]	PDAC	PanIN-1a	Me-PCR	115/266	43
Terhune et al. (1998)	[59]	PDAC	PanIN-1b	Me-PCR	27/44	61
		PDAC	PanIN-3	Me-PCR	7/9	78
Lüttges et al. (1999)	[9]	PDAC	PanIN-1a	PCR	33/123	27
		PDAC	PanIN-1b	PCR	46/112	41
Mulligan et al. (1999)	[60]	PDAC	PanIN-3	Me-PCR	3/4	75
Lemoine et al. (1992)	[7]	CP	PanIN-1b	PCR	0/5	0
Tabata et al. (1993)	[55]	CP	PanIN-2	PCR	0/5	0
		CP	PanIN-1a	PCR	0/5	0
Yanagisawa et al. (1993)	[23]	CP	PanIN-2	PCR	1/4	25
		CP	PanIN-1a	PCR	3/3	60
		CP	PanIN-1b	PCR	6/7	86
Song et al. (1996)	[61]	CP	PanIN-1b	PCR	2/12	17
Rivera et al. (1997)	[62]	CP	PanIN-1a	PCR	2/11	18
Mulligan et al. (1999)	[60]	CP	PanIN-2	Me-PCR	2/15	13
		CP	PanIN-1a	Me-PCR	1/17	6
Lüttges et al. (2000)	[12]	CP	PanIN-2	PCR	0/26	0
		CP	PanIN-1a	PCR	15/140	11
		CP	PanIN-1b	PCR	4/97	4
Song et al. (1996)	[61]	Normal	PanIN-1a	PCR	1/3	33
Tada et al. (1996)	[26]	Normal	PanIN-1a	PCR	19/79	24
Matsubayashi et al. (1999)	[58]	Normal	PanIN-1a	Me-PCR	9/51	18
Terhune et al. (1998)	[59]	Normal	PanIN-1b	Me-PCR	8/17	47
Lüttges et al. (1999)	[9]	Normal	PanIN-1a	PCR	10/153	7
		Normal	PanIN-1b	PCR	6/173	3
Agoff et al. (2001)	[63]	Normal	PanIN-1a	PCR	0/8	0

In some studies, PanINs derived from both PDAC or CP were included but separately analyzed. Therefore, these studies appear twice.
*From patients with PDAC, CP, or without pancreas pathology (normal).

Among patients with CP, the proportion of PanIN lesions harboring *K-ras* mutations was relatively low (10%) and independent of the PanIN grade of the lesion (Table 2). Here, the ME-PCR techniques resulted in similar rates of K-ras positivity in the PanIN-1A lesions and a higher percentage in the PanIN-2–3 lesions. The number of lesions investigated was too low in the ME-PCR group to result in any statistically significant difference.

Only low-grade PanIN (1A/1B) lesions were reported in the pancreas of patients free of pancreatic disease and, again, a low proportion (11%) of these lesions harbored *K-ras* mutations. There were no PanIN-3 lesions in patients

Table 2. PCR Data on *K-ras* Positivity in PanIN Lesions Associated with Various Pancreatic Disorders.

	Technique				Total	
	PCR		Me-PCR			
	K-ras$^+$/Total Foci	*K-ras*$^+$ % (95% CI)	*K-ras*$^+$/Total Foci	*K-ras*$^+$ % (95% CI)	*K-ras*$^+$/Total Foci	*K-ras*$^+$ % (95% CI)
PDAC						
PanIN-1A	43/172	25 (19–32)	115/266	43 (37–49)	158/438	36 (32–41)
PanIN-1B	46/121	38 (29–47)	27/44	61 (46–75)	73/165	44 (37–52)
PanIN–2–3	67/76	88 (78–94)	7/9	78 (40–96)	74/85	87 (78–93)
Chronic pancreatitis						
PanIN-1A	20/159	13 (8–19)	1/17	6 (0–31)	21/176	12 (8–18)
PanIN-1B	12/121	10 (5–17)	–	–	12/121	10 (5–17)
PanIN-2–3	1/35	3 (0–17)	2/15	13 (2–42)	3/50	6 (2–18)
Normal pancreas						
PanIN-1A	30/243	12 (9–17)	9/51	18 (9–31)	39/294	13 (10–18)
PanIN-1B	6/173	3 (1–8)	8/17	47 (24–71)	14/190	7 (4–12)

with CP. The numbers of PanIN-1A and PanIN-1B lesions varied between the two PCR methods; in the ME-PCR group, the number of lesions was again low.

Using the original data reported in a previous study [12], we investigated the possible effect of age and duration of CP on the severity of PanIN lesions and on their K-ras status. Among 25 patients with CP, we found no age difference between those with PanIN-1A lesions ($n = 4$, median age = 47.5, range = 39–54) and those with either PanIN-1B ($n = 8$, median age = 54.5, range = 30–69) or PanIN-2 lesions ($n = 13$, median age = 50, range = 17–63; $P = .59$).

We also found no age difference between CP patients with K-ras–negative PanIN lesions ($n = 14$, median age = 46, range = 17–66) and those with K-ras–positive PanIN lesions ($n = 11$, median age = 53, range = 33–69; $P = .42$).

Unlike patient's age, the duration of CP was significantly associated with K-ras positivity. Of the 16 CP patients for whom information was available, the duration of CP for the eight patients with K-ras–negative PanIN lesions was shorter (median = 2 years, range = 0–17) than that of the eight CP patients with K-ras–positive PanIN lesions (median = 7.5 years, range = 3–34; $P = .021$) (Figure 1). No case of CP with a disease duration of less than 3 years showed *K-ras* mutations.

Discussion

Mutations in the *K-ras* oncogene are rare in the normal disease-free pancreas [13]. Their frequency does not seem to exceed 13% [15], whereas it reaches 80% in PDAC [24]. Our meta-analysis demonstrated that the frequency of *K-ras* mutations in PanIN lesions corresponds to their grade of dysplasia, with PanIN-2 and PanIN-3 lesions displaying a significantly higher frequency (87%) than PanIN-1A (36%) (Table 2). The second major finding was the correlation of K-ras positivity in PanINs with the duration of CP.

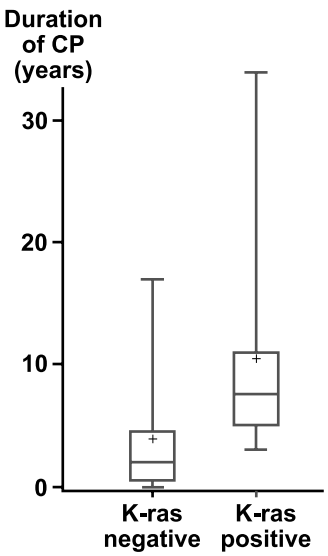

Figure 1. *Boxplot of the duration of CP with and without K-ras mutation. Median duration for K-ras–negative patients = 2 years; K-ras–positive patients = 7.5 years (P = .021).*

We reviewed 15 studies and reclassified 1519 duct lesions. In most studies, the duct lesions could be easily reclassified, particularly when high-quality illustrations were provided. Problems arose in about 15% of the studies. They were due to uncommon phrasing of histopathologic findings and concerned mostly the distinction of PanIN-2 lesions from PanIN-3 lesions. Because of this occasional uncertainty and the small total numbers of PanIN-2 and PanIN-3 lesions reviewed, the statistical analysis was performed on a combined PanIN-2/PanIN-3 group.

In an earlier study on the sensitivity of the molecular method used for the detection of *K-ras* mutations and the rate of positive samples [15], we already found that so-called ME-PCR will result in higher rates of K-ras–positive samples than plain PCR. This study confirmed this result and additionally showed that the high sensitivity of the ME-PCR method was particularly evident in the low-grade PanINs. Whether the results obtained with ME-PCR reflects the *K-ras* mutation status better than plain PCR has been widely debated during recent years [25]. However, in order to avoid false-positive results as far as possible, the use of plain PCR techniques with subsequent DNA sequencing appears to be the method of choice.

Do *K-ras* mutations have a role as a diagnostic marker of PDAC? Considering the methodological ambiguities and the demonstration of K-ras positivity not only in CP but also in the disease-free pancreas [12,13,26,27], the detection of a *K-ras* mutation in a patient does not establish the diagnosis of PDAC. However, the results of our meta-analysis show that a *K-ras* mutation may be indicative of the development of a PDAC, particularly in patients with CP. The latter notion would be supported by our data that *K-ras* mutations were only found in CP of more than 3 years' duration. If, in addition, the patient is a smoker, the risk would further increase. Under these conditions as well as in surveillance programs for patients with hereditary pancreatitis and familial pancreatic carcinoma [28], K-ras analysis should be recommended.

This study clearly demonstrated that in PanINs from patients with PDAC, the gradual increase in K-ras positivity correlated with the grade of dysplasia. Other investigations revealed a rising incidence of LOH for p16, p53, and DPC4/SMAD4 with increasing PanIN grades [17,29]. In addition, shortened telomeres have been demonstrated in PanIN lesions of all grades [30] and were thought to predispose PanINs to accumulate progressive chromosomal abnormalities. Finally, *BRCA2* and *maspin*, two tumor-suppressor genes known to also be involved in breast tumorigenesis, were found to be inactivated in PanIN-3 lesions [31,32]. Based on these findings [24,33–37], a progression model for genetic alteration in PanINs has been proposed. In this model, it is suggested that *K-ras* mutations occur early in the evolution of PanINs [38].

Recently, new experimental evidence for the tumorigenic potential of the *ras* oncogene was provided. Immortalization of bovine pancreatic duct cells from the adult pancreas with SV40 large-T and further transfection of mutated *K-ras* resulted in a malignant phenotype. Upon transplantation into nude mice, PDACs were formed [39]. In a three-step model,

transfection of activated human telomerase (hTERT), SV40 large-T plus, and mutated *H-ras* drove human embryonic kidney cells and fibroblasts into malignancy [40]. More recently, in a study employing a conditionally transgenic mouse model for K-ras, typical PanINs were induced in animals with spontaneous progression to invasive ductal adenocarcinoma [41]. Taken together, these data demonstrate that *ras* does indeed play an important role in tumorigenesis and thus also in the pathogenesis of both PanINs and PDAC. How, precisely, the activated K-ras alters the cell machinery toward malignancy is not yet clear, but recent studies suggest that K-ras stimulates cell proliferation rather than inhibits apoptosis [36,42].

What may induce *K-ras* mutations in the pancreas? As causes of *K-ras* mutations, smoking and also coffee consumption have been discussed [27,43,44]. Whether the same factors may also play a role in the induction of PanINs is not known, but because PanIN-1 lesions may occur early in life [13,45], it is possible that such K-ras–positive PanIN-1 lesions may exist for a long time. Eventually, one of them is suddenly transformed into a higher-degree PanIN and invasive PDAC by accidental accumulation of additional genetic events genes such as *p16*, *p53*, and *SMAD4/DPC4*. Neither the time axis of the progression nor the trigger for these additional mutations is known. Recent findings may provide a clue, at least in alcoholic CP: ethanol has a dramatic synergistic effect with smoking on the formation of acetaldehyde [46]. Acetaldehyde in turn generates reactive oxygen and nitrogen species [47], resulting in reactive aldehydes, which are known to induce DNA adducts and mutations [48]. Some of the reactive aldehydes have been detected in CP already [49]. Alkylating agents may induce specific *K-ras* mutations, namely G–A transitions, as shown in those heavy smokers in the absence of histologic lesions [27]. Besides alkylating agents, methylating agents and organic solvents have been demonstrated to induce *K-ras* mutations. Analyzing patients with PDAC and occupational risks, these G–A transitions could be associated with polycyclic aromatic hydrocarbons and gasoline engine exhausts [50]. For methylating agents, the proposed mechanism lies in the reduction of O^6-alkyl guanine DNA-alkyltransferase (MGMT), as shown in colorectal adenomas [51]. Reduced MGMT has also been described in PDAC [52]. The presumably long phase after the first injury (K-ras) that makes the pancreatic duct epithelium receptive to malignant transformation but unaltered—if not injured for a second time—can be deemed as a ''dance on the volcano'' that is harmless for most people but may be fatal for a few [53].

In summary, using the new PanIN system, we reclassified and then compared a large number of preneoplastic lesions of varying grades in published reports. In PanINs associated with PDAC, we found unambiguous evidence of a stepwise increase in *K-ras* mutations with increasing grade of dysplasia. We also found that the duration of CP was significantly longer in K-ras–positive than in K-ras–negative patients and that the minimum duration of CP associated with K-ras positivity was 3 years. These findings suggest that *K-ras* mutations play an important role in the evolution of PanINs and PDAC. However, detection of *K-ras* mutations as the only test cannot be recommended as a screening tool for PDAC but could be useful in combination with cytology.

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Appendix 1. Histopathologic description and grading of PanIN

Normal: The normal ductal and ductular epithelium is a cuboidal to low-columnar epithelium with amphophilic cytoplasm. Mucinous cytoplasm, nuclear crowding, and atypia are not seen.

Squamous (transitional) metaplasia: A process in which the normal cuboidal ductal epithelium is replaced by mature stratified squamous or pseudostratified transitional epithelium without atypia.

PanIN-1A: These are flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. The nuclei are small and round to oval in shape. When oval, the nuclei are oriented perpendicular to the basement membrane. It is recognized that there may be considerable histologic overlap between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia. Therefore, some may choose to designate these entitles with the modifier term *lesion* (''PanIN/L-1A'') to acknowledge that the neoplastic nature of many cases of PanIN-1A has not been unambiguously established.

PanIN-1B: These epithelial lesions have a papillary, micropapillary, or basally pseudostratified architecture but are otherwise identical to PanIN-1A.

PanIN-2: Architecturally, these mucinous epithelial lesions may be flat but are mostly papillary. Cytologically, by definition, these lesions must have some nuclear abnormalities. These abnormalities may include some loss of polarity, nuclear crowding, enlarged nuclei, pseudostratification, and hyperchromatism. These nuclear abnormalities fall short of those seen in PanIN-3. Mitoses are rare but, when present, are nonluminal (not apical) and are not atypical. True cribiform structures with luminal necrosis and marked cytologic abnormalities are generally not seen and, when present, should suggest the diagnosis of PanIN-3.

PanIN-3. Architecturally, these lesions are usually papillary or micropapillary; however, they may rarely be flat. True cribiforming, the appearance of ''budding off'' of small clusters of epithelial cells into the lumen, and

luminal necrosis should all suggest the diagnosis of PanIN-3. Cytologically, these lesions are characterized by a loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented toward the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses that may occasionally be abnormal, nuclear irregularities, and prominent (macro)nucleoli. The lesions resemble carcinoma at the cytonuclear level, but invasion through the basement membrane is absent.

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