

Temporal sequence of mammary intraductal proliferations, ductal carcinomas *in situ* and adenocarcinomas induced by 1-methyl-1-nitrosourea in rats

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An experimental model for mammary carcinogenesis has been described in which intraductal proliferations, ductal carcinomas *in situ* and adenocarcinomas can be readily detected and the frequency of their occurrence quantified. The objective of the experiment reported in this study was to determine the latency period between carcinogen administration and the occurrence of each of these types of lesion. A total of 150 female Sprague–Dawley rats were injected i.p. with 50 mg 1-methyl-1-nitrosourea (MNU)/kg body wt at 21 days of age. Groups of 30 rats each were killed at 7, 14, 21, 28 and 35 days post-carcinogen. Mammary intraductal proliferations were the first detected lesions and were observed in 20% of the animals at 14 days following carcinogen administration. At 21 days post-carcinogen ductal carcinomas *in situ* and adenocarcinomas were observed. The number of each type of lesion increased with time post-carcinogen, but the temporal pattern of occurrence was different among lesion types. The pattern of lesion occurrence was consistent with intraductal proliferations being a precursor lesion for ductal carcinomas *in situ* and adenocarcinomas. Furthermore, the data imply that ductal carcinomas *in situ* represent one pathway of morphological progression by which intraductal proliferations evolve into invasive carcinomas, but that this lesion type, as currently defined histologically, may not be an obligatory intermediate in morphologic progression. These findings are consistent with emerging evidence of multiple but distinct pathogenetic pathways leading to mammary carcinomas that display different morphological patterns and biological activities.

Introduction

Experimental models in laboratory rodents have provided a great deal of insight about many factors involved in the genesis, prevention and treatment of breast cancer (1–3). There are numerous reports that document that the same morphologically detectable benign, pre-malignant and malignant types of lesions that occur in the human breast also occur in rodent models for mammary carcinogenesis (4–8). Chemically induced models for mammary carcinogenesis in the rat currently are the most widely used systems for investiga-

ting the prevention of this disease process (7); however, there are only a limited number of studies that address the natural history of the disease from its pre-malignant to malignant stages in these models (4–9). The occurrence of mammary intraductal proliferations, ductal carcinomas *in situ* and invasive carcinomas in both 1-methyl-1-nitrosourea (MNU)-induced and 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced models has been reported, and it has been hypothesized that there is stepwise evolution of mammary lesions through these morphologically defined steps (2,5,7,9). However, it has not been possible to investigate this hypothesis vigorously. One reason for the dearth of information on lesion evolution in rat mammary carcinogenesis has been the uncertainty about the appropriate time points following carcinogenic initiation to investigate these events given the long time frame, generally 6 months, over which such experiments are conducted. In addition it has not been feasible to quantify the frequency of pre-malignant lesion occurrence in these model systems. Using a recently published approach to lesion quantification (10), the experiment reported in this study was intended to establish the temporal sequence of events in the occurrence of lesions in the mammary gland following carcinogenic initiation with MNU.

Materials and methods

Female Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. At 21 days of age, animals were injected i.p. with 50 mg MNU/kg body wt as previously described by our laboratory (11). Rats were housed three per cage in an environmentally controlled room maintained at 22°C and 50% relative humidity with a 12 h light–dark cycle. They were fed *ad libitum* a purified diet (AIN-76A) and distilled water (12). Rats were randomized into five groups of 30 animals each following carcinogen administration. One group of animals was killed by gaseous CO₂ inhalation at 7, 14, 21, 28 and 35 days post-carcinogen. At necropsy, rats were skinned and the skin was examined under translucent light. The cervical–thoracic and abdominal–inguinal mammary glands were carefully excised and spread onto clean 50×75 mm pre-labeled microscope slides. These whole mounts were processed for evaluation and photographed as previously described (10). Thereafter all lesions detected by inspection of whole mounts at 2× magnification were dissected using the photograph to provide a permanent identification record of the location and gross morphology of a lesion. Dissected lesions were processed and histologically classified according to the criteria of Russo *et al.* (13) and are illustrated in Figure 1. Accordingly, intraductal proliferations and ductal carcinoma *in situ* were diagnosed based on both histological and cytological criteria. Hyperplastic lesions displaying a spectrum of degrees of proliferation and/or cellular atypia were classified as intraductal proliferations without subcategorization. The requirement that a lesion meet all the criteria for ductal carcinoma *in situ* in two or more cross-sections of the duct, which is used by pathologists in the diagnosis of ductal carcinoma *in situ* in human disease, is not used in the consensus classification scheme of Russo *et al.* (13) and, therefore, was not applied. Statistical analyses were performed for significant differences in the occurrence of intraductal proliferations, ductal carcinomas *in situ* and adenocarcinomas across time. Incidence was evaluated by a Mantel–Haenszel χ^2 test that tested whether the differences in incidence were constant over time (14). Differences in tumor count were assessed by Poisson regression, treating days post-carcinogen as an ordered categorical variable (15).

Results

The injection of MNU at 21 days of age was well tolerated as previously reported and resulted in no mortality (10).

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MNU, 1-methyl-1-nitrosourea.

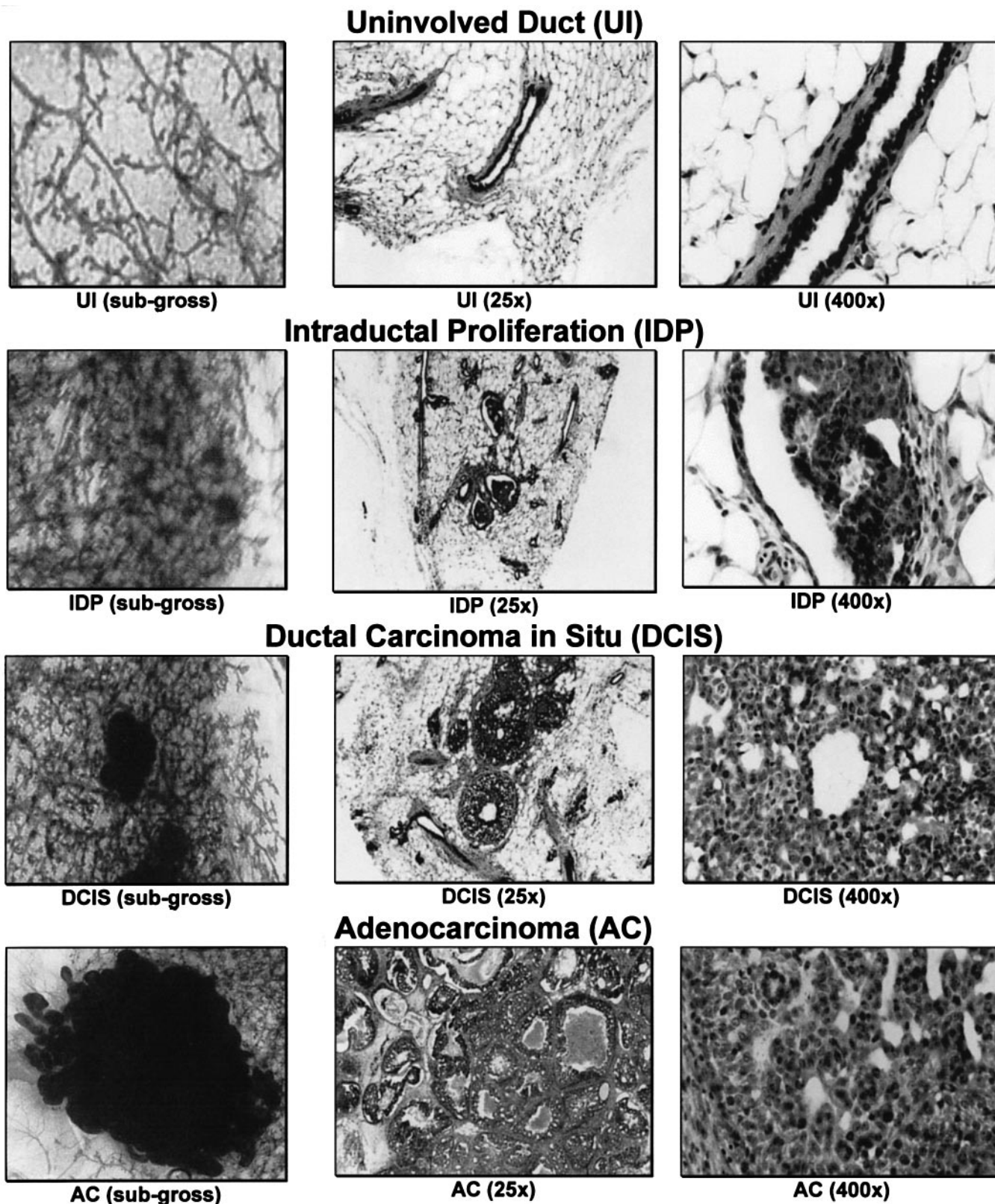


Fig. 1. The sub-gross and histological appearance of uninvolved mammary gland duct (UI), intraductal proliferations (IDP), ductal carcinomas *in situ* (DCIS) and adenocarcinomas (AC). Subgross photomicrographs are from alum carmine stained mammary gland whole mounts. Remaining photomicrographs are from the same tissue shown in the subgross view of each whole mount. The tissue was processed as described in Materials and methods. The 25× and 400× photomicrographs are from hematoxylin and eosin stained sections of the lesions shown in respective subgross panels.

Mammary gland pre-malignant and malignant lesions were observed. Mammary adenoacanthomas also were observed in this study. However, the occurrence of this lesion type was

infrequent and limited to the 28 and 35 day post-carcinogen observation periods; they were not included in the data reported in Tables I and II. Benign mammary lesions were not observed

Table I. Time-related changes in the incidence of pre-malignant and malignant mammary gland lesions following carcinogen administration

Lesion type	Time-post carcinogen ^{a,b}			
	14 days	21 days	28 days	35 days
Intraductal proliferation	20.0 (6)	46.7 (14)	80.0 (24)	80.0 (24)
Ductal carcinoma <i>in situ</i>	0.0 (0)	26.7 (8)	23.3 (7)	40.0 (12)
Adenocarcinoma	0.0 (0)	26.7 (8)	86.7 (26)	93.3 (28)

^aThirty rats were randomized to each group.

^bThe time-related changes in the incidence of each lesion type were evaluated by a Mantel-Haenszel test. The time-related increases in the incidence rates for intraductal proliferations and adenocarcinomas were statistically significant ($P < 0.01$), whereas those for ductal carcinomas *in situ* were not ($P = 0.26$).

Table II. Time-related changes in the number of pre-malignant and malignant mammary gland lesions following carcinogen administration

Lesion type	Time post-carcinogen ^{a,b}			
	14 days	21 days	28 days	35 days
Intraductal proliferation	0.27 (8)	0.73 (22)	1.53 (46)	1.83 (55)
Ductal carcinoma <i>in situ</i>	0 (0)	0.27 (8)	0.30 (9)	0.53 (16)
Adenocarcinoma	0 (0)	0.37 (11)	2.60 (78)	4.10 (123)

^aThirty rats were randomized to each group.

^bThe number of intraductal proliferations increased progressively over time ($P < 0.01$). Following a 2 week latency period, the number of adenocarcinomas increased in a linear manner over time ($P < 0.01$). The pattern for ductal carcinomas *in situ* was similar but not statistically significant ($P = 0.09$).

in this study, and grossly detectable lesions were not observed in other organs at necropsy.

The incidence and multiplicity of mammary intraductal proliferations, ductal carcinomas *in situ* and adenocarcinomas in rats injected with 50 mg MNU/kg body wt at 21 days of age as a function of time post-carcinogen are shown in Tables I and II, respectively. Some mammary adenocarcinomas had a DCIS component; however, these lesions were not reported as a separate category since the natural history of the development of adenocarcinomas with a prominent DCIS component is unclear. No mammary lesions were detected at 7 days post-carcinogen. Twenty percent of the animals were found to have one or more mammary intraductal proliferations 14 days post-carcinogen; however, no other lesion type was observed. The number of animals with intraductal proliferations increased to 14 (46.7%) at 21 days post-carcinogen and to 24 (80%) at both 28 and 35 days post-carcinogen. Ductal carcinomas *in situ* were first observed 21 days post-carcinogen; the incidence rate was 26.7%. A similar percent (23.3%) of animals with ductal carcinomas *in situ* was observed at 28 days post-carcinogen whereas the number of animals with ductal carcinomas *in situ* essentially doubled by 35 days post-carcinogen to 12 (40%). Adenocarcinomas also were first observed at 21 days post-carcinogen; the incidence rate was 26.7%. The incidence of adenocarcinomas had doubled by 28 days post-carcinogen (86.7%), and further increased to 93.3% by 35 days post-carcinogen. The time-related increases in the incidence rates for intraductal proliferations and adenocarcinomas were statistically significant ($P < 0.01$), whereas those for ductal

carcinomas *in situ* were not ($P = 0.26$). The pattern of lesion occurrence observed in the analysis of the incidence data was also observed in the time-related changes in number of lesions (Table II). The number of intraductal proliferations increased progressively over time ($P < 0.01$). Following a 2 week latency period, the number of adenocarcinomas increased in a linear manner over time ($P < 0.01$). The pattern for ductal carcinomas *in situ* was similar (Figure 1) but not statistically significant ($P = 0.09$).

Discussion

The ability to detect and to quantify the types of changes reported in this study was significantly enhanced by the developmental state of the mammary gland during the time-frame investigated. Carcinogenic initiation by chemical insult occurred when rats were 21 days of age. At this age each of the rats 12 mammary glands can be distinguished from one another and the degree of overlap of mammary gland branches is limited; thus the complexity of the mammary gland is low. Although complexity of the gland increased over the 5 week period of observation reported in Tables I and II, the whole mount preparations were still readily evaluated for lesion detection (Figure 2). This situation, coupled with the relatively short time-frame over which essentially all carcinogen-initiated animals in a group are observed to have one or more detectable mammary gland lesions, permitted the evaluation of large numbers of animals for lesion occurrence at regularly spaced time intervals. To our knowledge, a similar experimental approach has not been previously used to investigate the natural history of disease progression in the rat.

The data shown in Tables I and II provide a number of insights about the sequence of occurrence of morphologically detectable changes during chemically induced mammary carcinogenesis. No morphologically detectable lesions occurred in the mammary gland over the first 7 days following carcinogenic insult. At some point between 7 and 14 days post-carcinogen, intraductal proliferations emerged as inferred from finding that 20% of the animals had one or more intraductal proliferations at 14 days post-carcinogen. However, at this time point no ductal carcinomas *in situ* or adenocarcinomas were noted. The emergence of both ductal carcinomas *in situ* and adenocarcinomas at 21 days post-carcinogen with a higher frequency of adenocarcinomas than ductal carcinomas *in situ* was not anticipated, based on the hypothesis that intraductal proliferations evolve through a ductal carcinoma *in situ* stage to become invasive carcinomas. We offer two possible explanations for these findings.

The hypothesis that intraductal proliferations evolve through a ductal carcinoma *in situ* stage to become invasive carcinomas is based largely on various types of observational data from cross-sectional studies and studies of changes in cellular atypia associated with disease progression (4-7,16,17). The statistical analyses of the data presented in Tables I and II can be interpreted to be consistent with this accepted model of morphological progression, although such an interpretation necessitates very rapid progression through ductal carcinoma *in situ* to adenocarcinoma between 14 and 21 days post-carcinogen, to account for the low frequency of morphologically distinct ductal carcinomas *in situ* at 21 days post-carcinogen. While this is clearly a possibility, it is of interest that the latency to emergence of intraductal proliferations, 14 days (Table I), is the same as that reported when

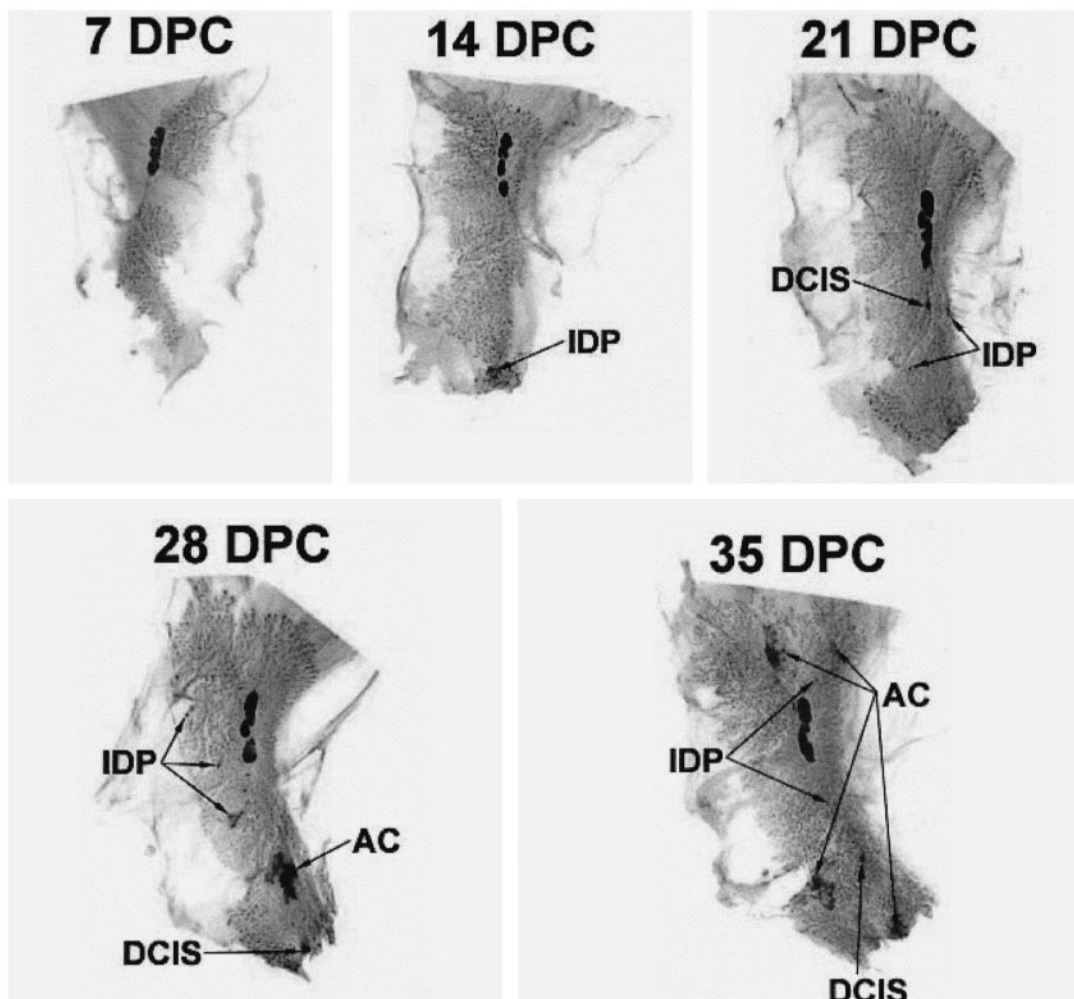


Fig. 2. Photographs of whole mount preparations of the abdominal-inguinal mammary chain at 7, 14, 21, 28 and 35 days post-carcinogen. Whole mounts were selected to illustrate the overall complexity of the mammary gland at the days post-carcinogen that were investigated and to show the gross appearance of various mammary lesions observed following the administration of MNU to 21-day-old female Sprague-Dawley rats. IDP, intraductal proliferations; DCIS, ductal carcinomas *in situ*; AC, adenocarcinomas.

DMBA was administered to 50-day-old rats (7), i.e. the Huggins model, in which a latency period of several months is required to reach the incidence and multiplicity of carcinomas reported at 35 days post-carcinogen in Tables I and II (1). Moreover, the latency to occurrence of ductal carcinomas *in situ* in the Huggins model was reported to be 20 days following carcinogen administration (7), a value that agrees remarkably well with the latency of 21 days reported in Tables I and II. Thus, while emergence of lesions in a group of animals is rapid when animals are injected with MNU at 21 days of age, latency to detection of pre-malignant lesions appears to be similar to that observed when animals are injected at a later age. Consequently, there needs to be caution in accepting the argument that rapid progression accounts for the differences noted above, and that ductal carcinoma *in situ* represents an obligatory intermediate step in morphological progression to invasive carcinomas, at least in this model system.

An alternative explanation of the data presented in Tables I and II and Figure 1 is that some intraductal proliferations may progress to adenocarcinoma without going through a carcinoma *in situ* stage. Consistent with this idea is our finding that intraductal proliferations occurring in this model system display a spectrum of mild to florid hyperplasia with varying degrees

of cellular atypia. This may reflect the existence of different subpopulations of intraductal proliferations some of which progress to adenocarcinoma without manifesting all characteristics of carcinoma *in situ*, whereas other subpopulations proceed via an intermediate step such as ductal carcinoma *in situ*. Given the emerging evidence of multiple pathways to cancer that is defined genetically, it would be surprising if multiple pathways in the morphological progression of mammary neoplasias were not identified. Consistent with this line of reasoning is the existence of morphologically distinct types of carcinomas in this and other model systems (7), and the emerging evidence from transgenic models of mammary carcinogenesis, of which particular morphological patterns are characteristic in carcinomas induced in response to specific genetic defects (18).

In summary, the data presented in the above tables show the latency period from carcinogenic initiation at which various pre-malignant and malignant lesions are present in the mammary gland of the rat. This information should be of considerable value for investigators studying the genesis, prevention and treatment of carcinogenesis and who wish to design experiments with parallels to clinically relevant situations. These data also support the need for further investigation of

the question of whether ductal carcinoma *in situ* is a requisite intermediate step in the morphological progression of intraductal proliferations to adenocarcinomas.

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