Original article

Title

Immunohistochemical analysis of steroidogenic enzymes in ovarian-type stroma of pancreatic mucinous cystic neoplasms: Comparative study of subepithelial stromal cells in intraductal papillary mucinous neoplasms of the pancreas

Short running title

Steroidogenesis of ovarian-type stroma

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Abstract

Mucinous cystic neoplasms (MCNs) are generally defined as cyst-forming epithelial neoplasms that arise in the pancreas and harbor characteristic ovarian-type stroma beneath the epithelium. In this study, we compared the immunoreactivity of steroid-related factors in these subepithelial stromal cells in MCNs to those in intraductal papillary mucinous neoplasms (IPMNs) to further characterize this unique MCN ovarian-type stroma through evaluation of sex steroid biosynthesis. Twenty MCNs and twenty IPMNs were examined. Immunoreactivity of steroid hormone receptors, including estrogen receptor (ERα and ERβ), progesterone receptor (PR, PR-A, and PR-B), and androgen receptor (AR), was more frequently detected in MCN ovarian-type stromal cells than in IPMN stromal cells ($P < 0.01$). The H-scores (mean±SD) of steroidogenic factor (SF)-1 were also significantly higher in MCNs (112.3±33.1) than in IPMNs (0.9±1.2) ($P < 0.01$). The steroidogenic enzymes cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450 17 alpha-hydroxylase (P450c17) and 3β-hydroxysteroid dehydrogenase (3β-HSD) showed immunoreactivity in 9/20 (45.0%), 15/20 (75.0%) and 13/20 (65.0%), respectively, of ovarian-type stroma from MCN cases. These results demonstrate that the ovarian-type stroma of MCNs can express steroidogenic enzymes. Thus, the ovarian-type stroma of MCNs can produce sex steroids that may also act on these cells.

Key words

pancreas, cystic lesion, mucinous cystic neoplasm, ovarian-type stroma, steroidogenesis, immunohistochemistry, steroidogenic factor-1, steroidogenic enzyme

Introduction

Mucinous cystic neoplasms (MCNs) are defined as cyst-forming epithelial neoplasms that arise in the pancreas and are relatively rare, accounting for merely 7.6% of all surgically resected cystic pancreatic neoplasms and tumor-like lesions.[1] Intraductal papillary neoplasms (IPMNs) are another type of pancreatic cystic neoplasm lined by tall, columnar, mucin-producing cells. MCNs are associated with characteristic clinicopathological features, including female predominance, an origin in the body and tail of the pancreas with no communication to the pancreatic ductal system, and, in particular, characteristic subepithelial ovarian-type stroma.[2-4] In contrast, IPMNs occur in both genders equally, predominantly occur in the pancreatic head, and arise from the primary ductal system of pancreas.[4-6] The most characteristic histopathological finding of MCNs is ovarian-type stroma, located just beneath the columnar, mucin-producing epithelium and composed of highly cellular, spindle-shaped cells intermingled with cells harboring round or elongated nuclei and sparse cytoplasm, all histologically resembling ovarian stroma. Therefore, these histological findings, i.e., cysts lined by mucin-producing epithelial cells harboring ovarian-type stroma beneath the epithelium, are generally considered the hallmarks of MCNs in the histological differentiation from IPMNs.[7]

Ovarian-type stromal cells of MCNs have been reported to be immunohistochemically positive for smooth muscle actin (SMA), desmin, calponin,

h-caldesmon, and alpha-inhibin.[8, 9] The presence of progesterone receptor (PR) and estrogen receptor (ER) in these stromal cells has also been reported.[3, 9, 10] In addition, results of recent oligonucleotide microarray analysis in MCN revealed that both the steroidogenic acute regulatory protein (STAR) and estrogen receptor 1 (ESR1) genes are expressed in these ovarian-type stroma,[11] which is consistent with the capacity of these cells to produce sex steroids.

In the human ovarian steroidogenesis pathway, specific steroidogenic enzymes, such as cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), cytochrome P450 17 alpha-hydroxylase (P450c17), and 3β-hydroxysteroid dehydrogenase (3β-HSD), all play pivotal roles in overall steroid production. In addition, the spatial and temporal distribution of these steroidogenic enzymes could account for continual human ovarian remodeling in premenopausal women.[12, 13] A steroidogenic cell-specific transcription factor (SF-1) activates the transcription of the steroidogenic P450 genes and may also regulate steroidogenic P450 expression in the human ovary.[14, 15]

Therefore, in this study, we attempted to evaluate the details of sex steroid biosynthesis of ovarian-type stroma of MCNs to examine its possible correlation with development and histogenesis of the lesion. We also compared these findings with those of IPMN subepithelial stromal cells to further characterize the unique ovarian-type stroma of MCNs.

Materials and methods

Cases

We studied twenty MCNs and twenty IPMNs that had been retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan). All MCNs were from female patients; their mean age at the time of diagnosis was 43 years (range, 24-68 years) and they histologically demonstrated subepithelial ovarian-type stroma (Figure 1A, 2A). IPMNs were from 15 male and 5 female patients with a mean age at diagnosis of 67 years (range, 53-77 years). The research protocol was approved by the ethics committee of Tohoku University School of Medicine (No. 2004-227).

Primary antibodies

Steroid hormone receptors examined included ER-alpha (ERα) using the monoclonal antibody EP1 (DAKO Cytomation, Glostrup, Denmark), ER-beta (ERβ) using the monoclonal antibody 14C8 (Gene Tex, San Antonio, TX), PR with the monoclonal antibody PgR636 (DAKO), PR-A with the monoclonal antibody hPRa7 (NeoMarkers, Fremont, CA), PR-B with the monoclonal antibody hPRa2 (NeoMarkers), and AR with the monoclonal antibody AR441 (DAKO). SF-1 was detected using the monoclonal antibody N1665 from Perseus Proteomics, Inc. (Tokyo, Japan), and was used as a general regulator of all

steroidogenic P450. P450scc and P450c17 were detected by polyclonal antibodies from BEX Co., LTD. (Tokyo, Japan), and 3β-HSD was detected using a polyclonal antibody generously provided by Dr. J. I. Mason (University of Edinburgh, Edinburgh, Scotland).

Immunostaining

All specimens had been fixed in 10% formalin and embedded in paraffin wax. Sections (3 μm) were cut and mounted on poly-L-lysine–coated glass slides (Matsunami, Tokyo, Japan). Immunohistochemical analysis of ERα and P450scc was achieved using the EnVision + system (DAKO Cytomation, Glostrup, Denmark), while immunostaining of other markers was carried out as previously described using the Histofine Kit (Nichirei, Tokyo, Japan) for the streptavidin-biotin amplification method.[13, 15] The following dilutions of primary antibodies against these proteins were used: ERα, no dilution; ERβ, 1/1,000; PR, 1/2,000; PR-A, 1/200; PR-B, 1/200; AR, 1/100; SF-1, 1/1,00; P450scc, 1/2,00; P450c17, 1/5,00; 3β-HSD, 1/2,500. The antigen-antibody complex was visualized with 3,3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2) and counterstained with hematoxylin. Immunostaining for ERα, ERβ, PR, PR-A, PR-B, AR, and SF-1 was performed by placing the sections in an autoclave for 5 min for antigen retrieval. Positive controls included breast cancer tissue for ERα, healthy endometrium for ERβ, PR, PR-A, and PR-B, and healthy adrenal gland for AR. As a negative

control, normal rabbit or mouse IgG was used instead of the designated primary antibodies; no specific immunoreactivity was detected in the negative control sections. Details about the immunohistochemical detection of the steroidogenic enzymes P450scc, 3β-HSD, and P450c17, including immunohistochemical procedures, have been described elsewhere.[13]

Immunoreactivity scoring

Immunoreactivity scoring was performed using the H-score for ERα, ERβ, PR, PR-A, PR-B, AR, and SF-1 as reported by McCarty et al. with some modifications by Suzuki et al.[16, 17] Briefly, > 500 tumor cells were counted in each case, and H-scores were subsequently generated by summing 2 x the percentage of strongly stained nuclei, and 1 x the percentage of weakly stained nuclei, giving a possible range of 0-200. The analysis was performed using Microsoft Excel 2013 version (Microsoft, Redmond, WA, USA). Statistical significance was evaluated using a Mann-Whitney U-test and $P < 0.01$ was considered significant.

Immunoreactivity of the steroidogenic enzymes P450scc, 3β-HSD, and P450-c17 was considered to be positive with cytoplasmic expression regardless of staining area. Each slide was reviewed by two authors (K.I. and H.S.) through a multi-headed microscope, and a consensus was reached in all cases.

Results

Steroid hormone receptors

Table 1 summarizes the comparison of steroid hormone receptor immunoreactivity in stromal cells between MCNs and IPMNs. Immunoreactivity of all steroid receptors was detected in the nuclei of ovarian-type stromal cells of MCNs (Figure 1B-G). In MCN ovarian-type stromal cells the H-score values (mean±SD) of ERα, ERβ, PR, PR-A, PR-B, and AR were 99.5±39.2, 67.8±32.0, 119.5±46.2, 45.5±30.5, 93.7±39.7, and 78.5±37.3, respectively, and 2.8±3.3, 2.8±2.3, 2.1±3.1, 2.1±2.9, 7.1±3.9, and 2.0±2.9, respectively, in IPMN subepithelial stromal cells. H-scores of all steroid hormone receptors examined were significantly higher in the ovarian-type stromal cells of MCNs than in the subepithelial stromal cells of IPMNs ($P < 0.01$).

SF-1 in MCN and IPMN stromal cells

The SF-1 immunoreactivity results in stromal cells of MCNs and IPMNs are summarized in Table 2. SF-1 was detected in the nuclei of ovarian-type stromal cells of MCNs (Figure 1H, 2B). The H-score of SF-1 was significantly higher in the ovarian-type stromal cells of MCNs (112.3±33.1) than in the subepithelial stromal cells of IPMNs (0.9±1.2) ($P < 0.01$) (Supplemental Figure).

Steroidogenic enzymes in MCN ovarian-type stromal cells

The immunoreactivity of steroidogenic enzymes in the ovarian-type stromal cells of MCNs is summarized in Table 3 (Figure 2C-E). The ovarian-type stromal cells in 9 MCN cases (45.0%) were immunohistochemically positive for P450scc, 15 cases (75.0%) were positive for P450c17, and 13 cases (65.0%) were positive for 3β-HSD. No IPMN subepithelial stromal cells were positive for P450scc, P450-c17 or 3β-HSD (data not shown) (Supplemental Figure).

Discussion

The diagnostic pathologic hallmarks of MCNs include an epithelium lined by tall, columnar, and mucin-producing cells, and a subepithelial ovarian-type stroma. However, the possible roles of ovarian-type stroma in the development and/or histogenesis of pancreatic MCN have remained unknown. Our present immunohistochemical analysis of ovarian-type stroma in MCNs revealed that sex steroid hormone receptors such as ER, PR, and AR are expressed in ovarian-type stromal cells. These receptors were rarely detected in the stromal cells of IPMNs, another type of mucin-producing cystic neoplasm that originates in the pancreas. Therefore, various sex steroids could exert their effects on ovarian-type stroma of MCN. In MCN, PR and ER have been reported to be expressed in 60-90% and 30% of stromal cells, respectively;[9, 10, 18] however, this is the first study to demonstrate the presence of AR in these ovarian-type stromal cells. In the normal human ovary, AR has been detected in

stromal cells regardless of the distance from the follicles or corpora lutea; however, PR is sporadically detected and ER expression is negative in these stromal cells.[19] Therefore, the results of the present study demonstrate that the expression of AR in ovarian-type stromal cells in pancreatic MCNs is similar to that in stromal cells of the ovary, while expression of PR and ER clearly differs between these tissues. AR belongs to the steroid hormone receptor superfamily,[20] and androgens are considered to play important roles in the stromal cells of pancreatic MCNs. Of particular interest, a relatively high accumulation of androgens in the pancreas of castrated male rats has been reported.[21] Therefore, these androgens could play a role in the pathogenesis and development of pancreatic MCNs through the manipulation of stromal cell functions; clarification of this role awaits further investigation.

SF-1 plays an important role in the embryonic differentiation and biological function of steroidogenic tissues such as the gonads or adrenal cortex, and has been reported to activate the transcription of all steroidogenic P450 genes, including the cytochrome P450s P450scc, P450c17, and 3β-HSD.[14, 22] The presence of SF-1 mRNA has also been reported in the testis, ovary, placenta, brain, and adipose tissue in addition to the adrenal gland.[23] In the ovarian steroidogenesis pathway, a cascade of steroidogenic enzymes is involved in the production of biologically active sex steroids. Results of the present study demonstrate for the first time that SF-1 is expressed in the ovarian-type stromal cells in all MCN cases. This finding indicates that the ovarian-type stroma of MCN can express steroidogenic enzymes. In normal human

ovary, SF-1 immunoreactivity has been detected sporadically in the granulosa cells and adjacent stromal cells in the preantral follicles. However, SF-1 immunoreactivity has not previously been identified in normal stromal cells.[15, 24] It was, however, reported that SF-1 immunoreactivity was detected in the nuclei of intratumoral stromal cells in primary epithelial and metastatic ovarian tumors, particularly those adjacent to the invasive carcinomatous glands.[25] Therefore, the expression of SF-1 in the stroma of ovarian neoplasms has been considered in the context of a carcinoma-stromal tissue interaction or an ovarian stromal reaction toward invasion. SF-1 was immunohistochemically negative in the stromal cells of ovarian mucinous cystadenoma in this previous report; in contrast, in the present study, SF-1 immunoreactivity was detected in the ovarian-type stromal cells of MCNs, which were classified as low-grade dysplasia and thus conceptually the same as adenoma. Therefore, ovarian-type stroma of MCNs could occur as part of the tumor, whereas the stroma of primary epithelial and metastatic ovarian tumors could develop in response to tumor invasion; further studies are required for clarification.

Whether MCN ovarian-type stromal cells with SF-1 expression could synthesize or metabolize steroid hormones has become a topic of interest. A previous gene expression analysis revealed that MCN ovarian-type stroma overexpresses the ESR1 and STAR genes, in contrast to the reactive stromal cells of chronic pancreatitis.[11] In our present study, immunoreactivity of the steroidogenic enzymes P450scc, P450c17 and 3β-HSD was detected

in 9/20, 15/20 and 13/20, respectively, of ovarian-type stroma in MCN cases. IPMNs occasionally exhibited foci characteristic of desmoplasia, which simulate ovarian-type stroma, but SF-1 and steroidogenic enzymes were immunohistochemically negative in the stromal cells of all IPMNs examined. Therefore, ovarian-type stroma was specific to MCN and by no means represents reactive fibroblasts in general. The expression of 3β-HSD and P450c17 has previously been reported in the ovarian-type stroma of MCN.[26] P450scc catalyzes the conversion of cholesterol to pregnenolone, while 3β-HSD further converts pregnenolone and P450c17 to 17-hydroxyprogesterone. In conjunction with SF-1 immunoreactivity in stromal cells, the immunoreactivity of P450scc, 3β-HSD, and P450c17 in ovarian-type stroma of MCNs suggests that these cells could produce sex steroids in situ. Thus, evaluation of whether the results for intraturmoal steroidogenic enzymes could be correlated with clinical manifestations of individual patients would be of interest. In particular, the status of these steroidogenic enzymes in MCNs could serve as surrogate markers for selecting endocrine therapies, although additional investigation would be needed to confirm this possibility.

The origin of these ovarian-type stromal cells remains unknown. Zamboni et al. reported two theories regarding the developmental pathways that underlie the histogenesis of ovarian-type stroma of MCNs.[18] First, the ovarian-type stroma may have been endodermally derived from periductal epithelium and primitive mesenchyme in the pancreas and proliferated in response to female sex steroids. The expression of hormone receptors and SF-1

in ovarian-type stroma of MCNs in the present study is consistent with this developmental pathway. In addition, clinical features of pancreatic MCNs, such as the predominance in middle-aged woman, are also similar to those of mucinous cystic neoplasms in the retroperitoneum and liver.[27-29] Sasano et al. reported that mucinous carcinoma exhibited a significantly higher number of SF-1–positive stromal cells than other histological types of ovarian carcinoma.[25] However, the expression of SF-1 was predominantly correlated with invasion. Furthermore, none of the epithelial cells demonstrated steroid hormone receptors or SF-1 in MCNs in the present study. Therefore, the first theory still remains a hypothesis. The second theory suggests that ectopic ovarian stroma in the pancreas could possibly release hormones and growth factors that stimulate endodermally-derived epithelium in its vicinity to proliferate and form cystic neoplasms.[18] However, it is also true that the pancreatobiliary tree is within the embryologic pathway and participates in germ cell descent.[28] Therefore, despite the morphological similarities between stromal cells of MCNs and the ovary, the results of our present study indicate that the ovarian-type stroma of MCNs may express steroidogenic enzymes as do the ovaries.[15, 24]

In summary, the ovarian-type stroma of MCNs was immunoreactive for sex steroid hormone receptors such as AR, and the presence of SF-1 and steroidogenic enzymes indicates that these lesions have the ability to produce and secrete biologically active sex steroids. These findings indicate that the ovarian-type stroma of MCNs may play a role in tumor

development through tumor-stromal interaction.

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Disclosure statement

The authors have no conflicts of interest to declare.

Figure legends

Figure 1. MCNs harboring ovarian-type stroma (A, 200x; hematoxylin and eosin-stained section). Immunoreactivity of steroid hormone receptors and SF-1 (B-H, 200x). B, ERα; C, ERβ; D, PR; E, PR-A; F, PR-B; G, AR; H, SF-1. Immunoreactivity of all steroid receptors and SF-1 was detected in the nuclei of ovarian-type stromal cells of MCNs (B-H).

Figure 2. Representative immunoreactivity of SF-1 and steroidogenic enzymes in ovarian-type stroma of MCN (A-E, 200x). A, hematoxylin and eosin-stained section; B, SF-1; C, P450scc; D, P450c17; E, 3β-HSD. Immunoreactivity of SF-1 with nuclear expression and steroidogenic enzymes with cytoplasmic expression was observed in the same location as in ovarian-type stroma of MCNs (B-E).

Supplemental Figure. Representative immunoreactivity of SF-1 and steroidogenic enzymes in the stromal cells of IPMN (A, 40x; B-F, 200x). A, B, hematoxylin and eosin-stained section; C, SF-1; D, P450scc; E, P450c17; F, 3β-HSD. SF-1 and steroidogenic enzymes were immunohistochemically negative in stromal cells of all IPMNs (C-F).

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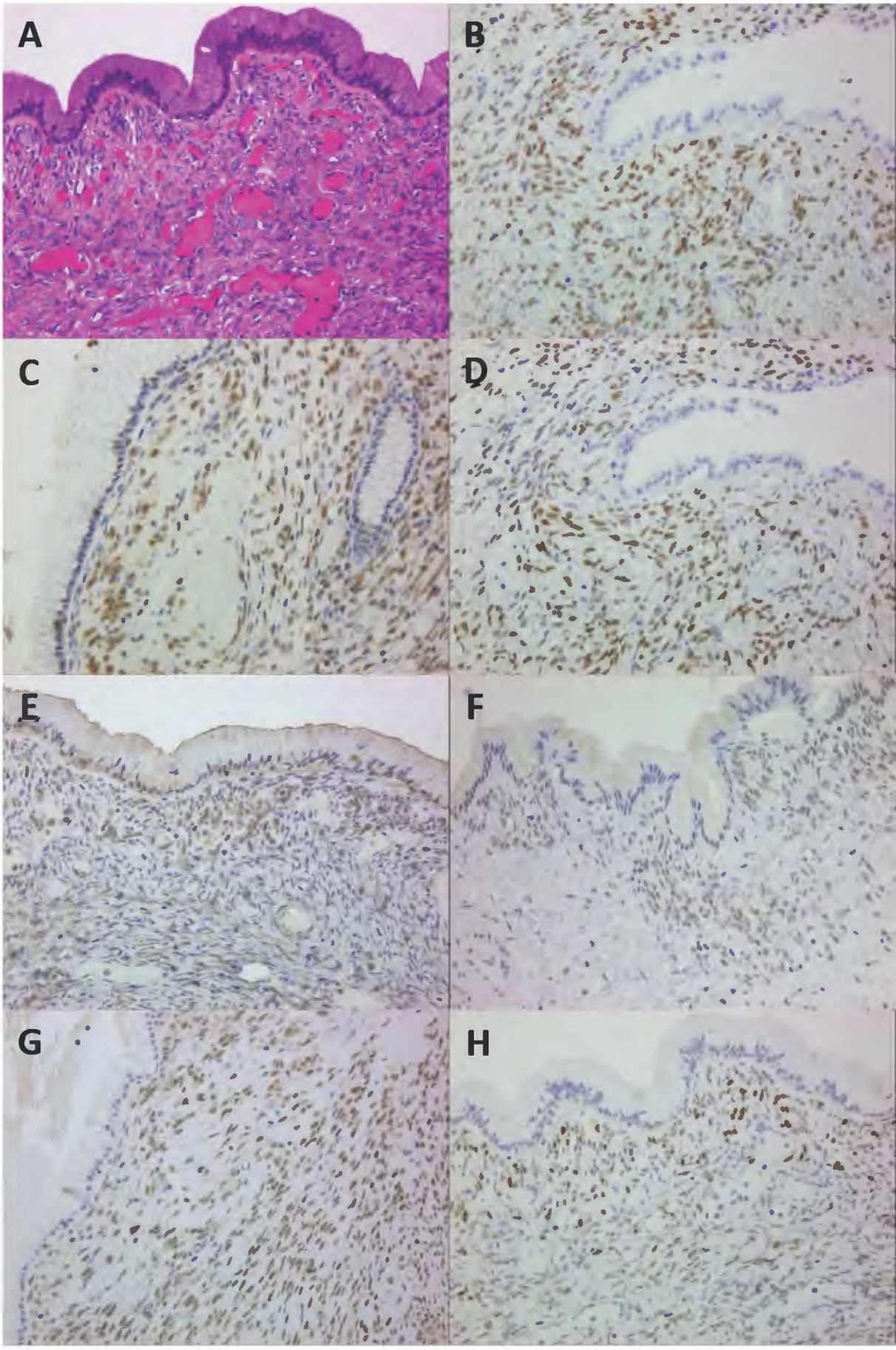


Fig.1

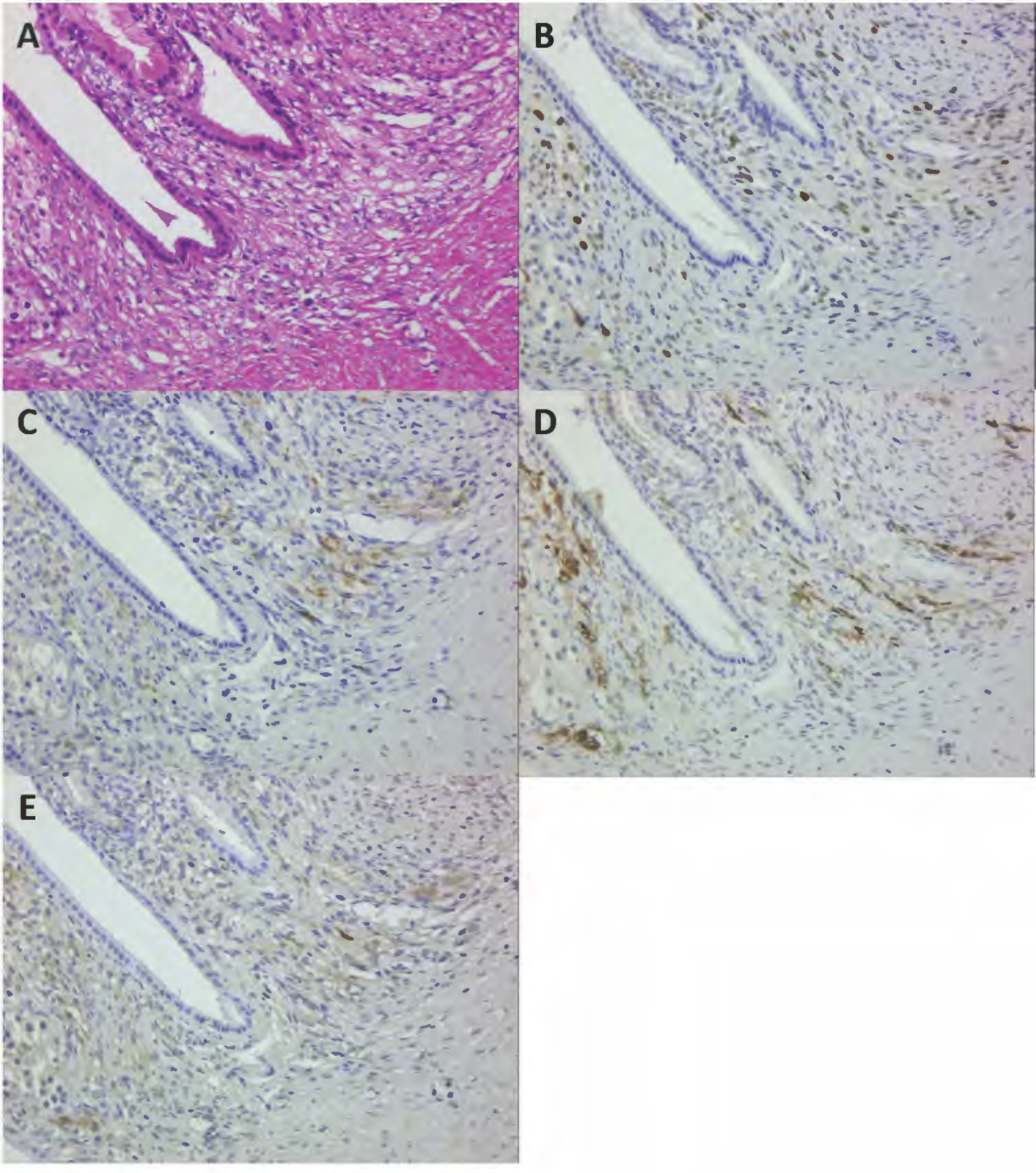


Fig. 2

Table 1. Comparison of steroid hormone receptor immunoreactivity in MCN and IPMN stromal cells.

	MCNs (n=20)	IPMNs (n=20)	*P* value
ERα	99.5 ± 39.2	2.8 ± 3.3	< 0.001
ERβ	67.8 ± 32.0	2.8 ± 2.3	< 0.001
PR	119.5 ± 46.2	2.1 ± 3.1	< 0.001
PR-A	45.5 ± 30.5	2.1 ± 2.9	< 0.001
PR-B	93.7 ± 39.7	7.1 ± 3.9	< 0.001
AR	78.5 ± 37.3	2.0 ± 2.9	< 0.001

Numbers indicate H-scores (mean ± SD).

Table 2. Comparison of SF-1 immunoreactivity in MCN and IPMN stromal cells.

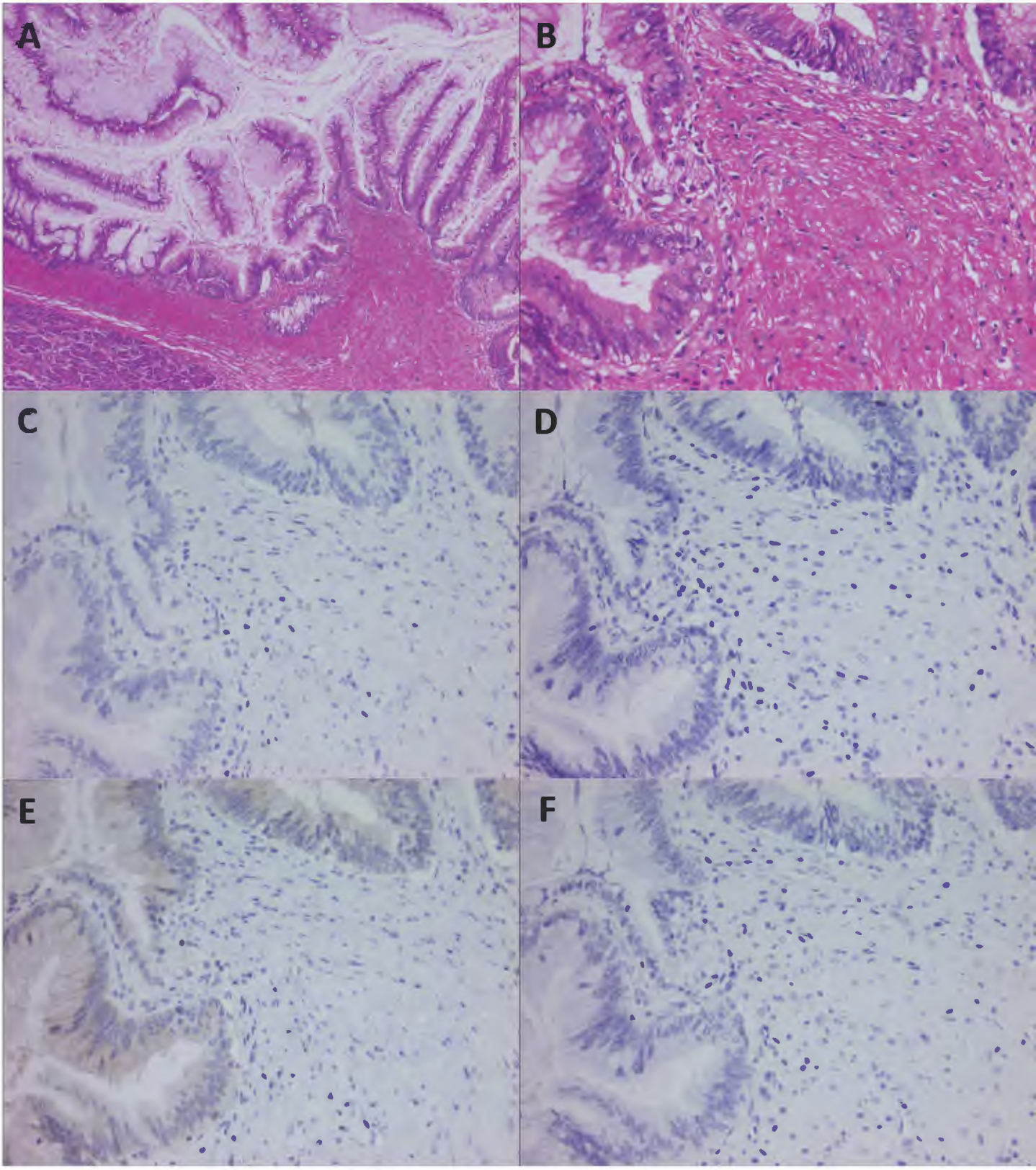
	MCNs (n=20)	IPMNs (n=20)	*P* value
SF-1	112.3 ± 33.1	0.9 ± 1.2	< 0.001

Numbers indicate H-scores (mean ± SD).

Table 3. Steroidogenic enzyme immunoreactivity in MCN stromal cells.

Steroidogenic enzymes	MCNs
P450scc	9/20 (45.0%)
P450c17	15/20 (75.0%)
3β-HSD	13/20 (65.0%)

Numbers indicate positive cases.



Supplemental Fig.