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Caveolin-3 Null Mutation in Family with Barrett's Esophagus and Esophageal Adenocarcinoma

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ABSTRACT

INTRODUCTION: Aggregation of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) in families has been termed familial Barrett's esophagus (FBE). Analysis of single, large FBE families can enable the identification of genetic susceptibility to complex diseases such as BE and EAC.

METHODS: Phenotypes of BE and EAC were ascertained in a large FBE family with 7 affected members: 4 men with EAC, 1 man with BE and high grade dysplasia, and 2 women with non-dysplastic BE by review of endoscopy and surgical pathology reports. Whole exome sequencing was performed on germline DNA from 3 affected members to identify variants in coding genes that segregated with disease. Formalin fixed paraffin embedded tissue from an affected family member as well as non-familial subjects with BE and EAC was examined with regular histology and immunohistochemistry. The CAV3 gene with a variant segregating in the family was further characterized in a porcine model of esophageal injury using immunofluorescence.

RESULTS: Using a whole exome sequencing approach on an exceptional FBE family we identified a segregating nonsense mutation in the gene Caveolin-3 (*CAV3*). Histologic examination of a formalin fixed paraffin embedded (FFPE) esophagectomy specimen from an individual carrying the *CAV3* null mutation revealed esophageal submucosal glands (ESMG) that showed acinar metaplasia with marked atypia and absence of myoepithelial cells, distinctly different from acinar metaplasia seen in ESMG of non-familial subjects with BE and high grade dysplasia. Immunofluorescence studies of ESMG in porcine esophagus revealed the presence of CAV3 in selected cells in a distribution that was consistent with myoepithelial cells. Experimental injury of the porcine esophagus using radiofrequency ablation revealed that CAV3 expression increased markedly within ESMGs, ESMG ductal epithelium, and overlying healing neosquamous epithelium 10 days after injury.

CONCLUSIONS: We theorize that *CAV3* expression, perhaps through myoepithelial cells within ESMGs, controls the differentiation and proliferation of squamous epithelial precursor cells in response to injury. Furthermore, the truncating nonsense *CAV3* mutation discovered in a family disrupts normal squamous healing and the organization of ESMGs, making affected family members susceptible to the proliferation and development of metaplastic columnar BE and EAC.

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The majority of esophageal adenocarcinomas (EACs) originate in Barrett's esophagus (BE), a pre-malignant metaplastic columnar epithelium, which replaces the normal stratified squamous epithelium of the injured esophagus [1-9]. BE is believed to develop as a reparative response to injury of the distal esophagus from gastroesophageal reflux disease (GERD) [8, 10-12]. BE progresses from metaplasia to dysplasia to cancer. BE and EAC are likely complex diseases caused by a combination of genetic and environmental factors.

BE and EAC aggregate in a proportion of families [13-19]. A family history of BE and/or EAC, termed Familial Barrett's Esophagus (FBE), is present in 7% of probands with BE or EAC [20]. Segregation analysis suggests that FBE is consistent with dominant transmission of one or more incompletely penetrant major Mendelian alleles [21]. The discovery of susceptibility genes for BE and EAC will lead to molecular insights into the esophagitis-metaplasia-dysplasia-cancer progression, providing targets for chemo-preventive and therapeutic drug development.

The Aim of this study was to identify a germline genetic mutation segregating with disease in an exceptionally large FBE family and determine the potential function of the segregating gene.

METHODS

Family Ascertainment

The family in this study was identified and recruited in institutional review board (IRB) approved studies at University Hospitals of Cleveland Medical Center (UHCMC) and Hospital of University of Pennsylvania (HUP) using previously described approaches [7]. Definitions of phenotype were - BE = intestinal metaplasia on biopsy plus $\geq$ 1 cm segment on EGD and EAC was defined as adenocarcinoma on biopsy report involving tubular esophagus. Blood lymphocytes were collected, immortalized, and banked at the Rutgers University DNA Repository (RUCDR) as a source of germ-line DNA from family members who were alive.

Archived FFPE Tissues

Formalin fixed paraffin embedded (FFPE) specimens were obtained with consent from deceased family members with EAC. These FFPE specimens were used for IHC. Furthermore, DNA was extracted from normal tissues contained within the resected FFPE specimens from deceased NN-0001 family members and used for Sanger sequencing to confirm the presence of mutations identified through whole exome sequencing.

Whole-exome capture and deep sequencing

Whole exome capture, library preparation, and deep sequencing were performed as previously described.[22] Target sequence enrichments were performed using the Illumina TruSeq Exome Enrichment Kit as per the manufacturer's protocols (Illumina Inc). Briefly, sample DNAs were quantified using a picogreen fluorometric assay and 3 µg of genomic DNA were randomly sheared to an average size of 300 bp using a Covaris S2 sonicator (Covaris Inc). Sonicated DNA was then end-repaired, A-tailed, and ligated

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with indexed paired-end Illumina adapters. Target capture was performed on DNA pooled from six indexed samples, following which the captured library was PCR amplified for 10 cycles to enrich for target genomic regions. The captured libraries were precisely quantified using a qPCR-based Kapa Biosystems Library Quantification Kit (Kapa Biosystems) on a Roche Lightcycler 480 (Roche Applied Science). Deep sequencing of the capture enriched pools was performed on an Illumina HiSeq 2000 instrument with 100 bp, paired-end reads to an average read-depth of 70X per sample.

Immunohistochemistry and In situ hybridization

Immunohistochemistry (IHC): Archival formalin-fixed paraffin-embedded tissue 5 um serial sections from esophageal resections were deparaffinized, rehydrated and immunostained with anti-human antibodies to caveolin 3 (CAV3), cytokeratin 7 (CK7, a marker for BE and columnar epithelium) and p63 (a nuclear marker for squamous differentiation and myoepithelial cells). Vendor sources, antibody dilutions and antigen retrieval methods used are designated in Table 1. Sections were incubated for 60 minutes at room temperature followed by an HRP-polymer detection system (Biocare Medical ®) appropriate for each host species, was applied for 30 minutes, followed by visualization with DAB chromagen and counterstained briefly with Gill's Hematoxylin. Positive tissue controls (per manufacturer recommendation) and negative antibody control (antibody omission) were included in each staining run.

Porcine ESMG

Briefly, Yorkshire pigs (Sus scrofa) were cared for under North Carolina State University and Duke University IACUC146 approved protocols (NCSU 13-116-B, Duke A120-14-05). Esophagus was injured using endoscopic radiofrequency ablation (RFA) as previously described.[23] Tissue blocks were created from injured and uninjured esophagus. Slides from tissue blocks were put into a solution containing 50% methanol and 50% acetone for 10 minutes at -20^{0}C , then into 1X PBS (pH 7.4) solution for 10 minutes at room temperature, blocked with 1% BSA in PBS for 30 minutes room temperature and incubated overnight at 4^{0}C with primary antibodies for CAV3 immunofluorescence prepared in 0.1% BSA in PBS with the dilutions (Table 1). The following morning, after washing 1X PBS for 20 minutes, respective secondary antibodies prepared in 0.1% BSA in PBS were added to the slides for 45 minutes at room temperature followed by a wash and DAPI stain (1:5000) for 10 minutes. After a PBS wash, 2 drops of ProLong Gold antifade reagent and a coverslip was added. Slides were imaged and processed using the EVOS microscope and ImageJ software.

RESULTS

Family FBE-NN-0001 Pedigree (FIGURE 1)

The proband (Figure 1, arrowhead) was diagnosed with BE and high grade dysplasia when he had upper endoscopy at age 52. The family reported Eastern European ancestry. The proband's brother had died of EAC at age 50 and mother had BE diagnosed at age 60 and breast cancer at age 68. The mother provided the family

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history and diagnoses. Two maternal uncles were diagnosed with EAC at age 67 and 72, a maternal male cousin was diagnosed with BE at age 40 and early EAC at age 57, and a maternal aunt had BE diagnosed at age 46 and breast cancer at age 57. The diagnoses were confirmed by review of medical records and histological review of archived formalin fixed paraffin embedded (FFPE) biopsies from the deceased members with EAC. Both affected women with BE also had a history of breast cancer. There was no known obesity or smoking history in the family. Family members also denied any history of muscular dystrophy or cardiomyopathy, autosomally inherited diseases associated with missense mutations in *CAV3*.[24-26]

Identification of Segregating *CAV3* Variant

Whole exome sequencing of 3 affected individuals – proband, maternal cousin with EAC, and and maternal aunt with BE – from family NN-0001 revealed rare, i.e., reported allele frequencies < 1% in Thousand Genome, missense/splice site/indel/null variants shared by all three affecteds in 3 coding genes – CAV3, MYO1E, and RCL1. Out of these 3, the C19X null variant in *CAV3* was the only completely private deleterious variant, i.e. not reported in dbSNP, Thousand Genome, or Exome Aggregation Consortium (ExAC), that segregated in all three affected family members. Sanger sequencing of the shared rare and private variants found in these 3 coding genes was performed in the seven affected and four unaffected members of the family. The private variant in *CAV3* was the only variant that segregated in all but one of the 11 phenotyped family members. The maternal uncle who did not carry the *CAV3* null variant was the oldest affected individual in the family. Missense variants in the other two genes, MYO1E and RCL1, did not segregate in at least three of the 11 members whose affection status was known. For this single family, assuming a dominant one-locus two allele model at zero recombination fraction, the lod score for the putative causative *CAV3* null variant is 0.56 (p=0.023).

Histology of ESMG in Family Member with *CAV3* Variant

The archived FFPE specimen from esophagectomy performed for an early esophageal cancer on the proband's maternal cousin who carries the C19X *CAV3* variant was available for analysis. Histological examination (FIGURE 2) showed that focal ESMGs (deep to luminal esophageal cancer) with metaplastic acini contain strikingly distorted acini with proliferating atypical cells focusing our attention on the possible role of CAV3 in ESMGs. IHC confirmed the presence of CK7, a marker for metaplastic ESMGs in these metaplastic atypical acini, although the CK7 was absent in the more atypical looking acini. IHC and ISH did not find *CAV3* protein or mRNA expression in these atypical ESMGs. Characterization of these atypical ESMGs using p63 IHC found a marked decrease in myoepithelial cells. Furthermore, the acini themselves showed a paucity of nuclear p63 staining myoepithelial cells. The ESMG with acinar metaplasia in this subject with null CAV3 mutation (Figure 2) was distinctly different from ESMG with acinar metaplasia from non-familial subjects with BE and high grade dysplasia (Figure 3). As reported previously, ESMGs with acinar metaplasia are associated with BE and EAC and show distinctive CK7 immunostaining.[27] Furthermore, these ESMGs showed a small number of scattered mesenchymal cells that were CAV3 positive and likely

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represent myoepithelial cells plus p63 IHC showed distinct nuclear staining clearly outlining the presence of myoepithelial cells (Figure 3).

Localization of *CAV3* in Esophagus

Interrogation of our RNA sequencing data[28] from 18 non-dysplastic BE, 56 pre-treatment EACs, 20 normal esophageal squamous, and 11 normal gastric biopsies revealed that *CAV3* was not expressed in mucosal tissues from normal esophagus, normal stomach, BE or EAC.

Expression of *CAV3* in porcine ESMGs following injury

IF staining of ESMGs from porcine esophagus showed that in the quiescent state *CAV3* expression is present in selected cells in a distribution that is consistent with myoepithelial cells (Figure 4). There are no CAV3 expressing cells in interlobular ducts or in squamous epithelium. Ten days following injury during the reparative phase CAV3 expressing epithelial cells are noted in dilated acini of ESMGs near the healing wound (Figure 4); CAV3 expressing cells also appear in the epithelium of the interlobular ducts; and CAV3 expressing cells are present throughout the healing neosquamous epithelium.

DISCUSSION:

There are several theories for the origin of BE: trans-differentiation of squamous cells, a special population of cells in the gastric cardia, and emergence from ESMGs.[29] Whole exome sequencing of an exceptional FBE family with multiple members affected with EAC and BE identified an inactivating null mutation in *CAV3* segregating with disease. Histologic examination of an esophagectomy specimen from an affected member demonstrated a disordered ESMG - focusing our attention on the role of *CAV3* within ESMG. Studies in the porcine injury model suggest that *CAV3* expression perhaps in ESMG myoepithelial cells is involved in normal squamous healing. Loss of CAV3 function may disrupt normal esophageal homeostasis and permit the development of alternative metaplastic repair after reflux injury of the distal esophagus.

Caveolins are small proteins that are the main component of caveolae, small raft like invaginations of the plasma membrane, implicated in a variety of cell functions including membrane organization, sensing, and cell signaling.[26] Three different mammalian caveolins act as caveolar scaffolds by forming hairpin structures within caveolar membranes. *CAV1* is the predominant caveolar protein in most cells, *CAV2* is associated with *CAV1,* and *CAV3,* which is highly homologous to *CAV1,* is the predominant caveolar protein in striated and cardiac muscle. Caveolae play a role in a variety of cellular processes including cell migration, differentiation, proliferation, and signal transduction that are implicated in carcinogenesis.[30-32]. Furthermore, dominant negative *CAV1* mutations are associated with breast cancer, possibly through increased migration and proliferation of breast stem cells.[33, 34] Thus, caveolin biology makes *CAV3* a particularly interesting gene to consider in terms of a role in BE origin and EAC carcinogenesis.

CAV3, which is predominantly expressed in muscle and missense mutations in *CAV3,* has been associated with muscular dystrophies and cardiomyopathies.[26] These

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missense mutations may cause disease by affecting oligomerization and scaffolding of *CAV3*. Family NN-0001 was found to have a protein truncating null variant that is clearly deleterious. Yet this family has no known muscular dystrophy or cardiomyopathy suggesting that *CAV3* is pleiotropic. An archived esophagectomy specimen was available from one affected member of the NN-0001 family. Histological examination of this specimen revealed a markedly atypical ESMG (Figure 2) focusing our attention to the role of *CAV3* in ESMGs.

The theory that ESMGs offer a protected source of esophageal progenitors has gained increased attention.[35] In humans, shared clonality has been identified, linking underlying ESMGs and ducts with both squamous epithelium and BE.[36] The shared clonality strongly suggests a progenitor cell source within ESMGs that activates in the setting of profound overlying esophageal injury. A canine model of esophageal injury from the 1980s included such profound injury via stripping of the squamous mucosa; notably, there were areas of abnormal repair to columnar metaplasia in the esophagus directly above ESMGs.[37] It is important to recognize that while rodent models have been useful for studying some potential cellular origins of BE, because rodents lack ESMGs, alternative models are needed for relevance to human disease. Indeed acinar ductal metaplasia within ESMGs has been identified in humans in association with esophageal injury (ulcer), BE and EAC.[27]

BE develops as a reparative response to chronic reflux injury. The distal esophagus appears to have two competing reparative pathways, one that leads to squamous healing and an alternative pathway that promotes metaplastic columnar healing. *In vivo* studies revealed that as part of the repair response following injury, ESMGs that undergo acinar ductal metaplasia have increased expression of columnar BE and progenitor markers. [38] Injury in the porcine model induces expression of CAV3 in ESMG acinar cells in a cellular compartment that is distinct from CAV1. Furthermore, CAV3 expressing cells appear in the glandular ducts and in neo-squamous epithelium in the healing wound suggesting that CAV3 expression in ESMG epithelial cells is vital to neo-squamous healing. These results suggest a hypothesis that the deleterious CAV3 allele in the NN-0001 family impairs squamous healing following reflux making members of this family more susceptible to BE and EAC. Furthermore, we hypothesize that the CAV3 expressing cells may represent induced myoepithelial cells that could undergo mesenchymal epithelial transition and migrate up the duct to heal the injured epithelium, similar to submucosal glands of the trachea where myoepithelial cells demonstrate the plasticity to migrate, differentiate and repair damaged epithelium [39, 40].

In summary, exome sequencing identified a deleterious null CAV3 variant segregating in an exceptional family with multiple affected members with BE and EAC. Histologic examination of an esophagectomy specimen of a family member with BE and HGD revealed atypical ESMGs with marked proliferation of dilated metaplastic acini and loss of myoepithelial cells. Experiments on the porcine model suggest that CAV3 expressing cells from ESMGs participate in neosquamous healing. CAV3 expression may be a marker for the precursor cells in ESMGs that heal injured esophageal epithelium. Loss of CAV3 function by an inactivating germline mutation led to an increased susceptibility to BE and EAC by impairing normal healing after chronic reflux injury in this unique family with a high penetrance. These findings further suggest that research into the

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caveolin/myoepithelial complex may identify molecular factors related to the development of BE in both high-risk families and BE in the general population.

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Table 1 – Antibodies used for Immunohistochemistry (human) and Immunofluorescence (IF)

Target antigen	Antibody source/ cat#	Antibody type	Dilution	AR
CAV3 (human)	Abcam/ ab182759	Rabbit monoclonal	1:100	TRIS-HCL
CAV3 (pig)	Abcam/ ab2912	Rabbit polyclonal	1:40	N/A
CAV1 (pig)	Novus/NB100-615	Mouse monoclonal	1:200	N/A
CK7 (human)	Dako/ OV-TL	Mouse monoclonal	1:1600	BC* Diva
P63 (human)	Biocare/ CM163	Mouse monoclonal	1:400	BC* Reveal

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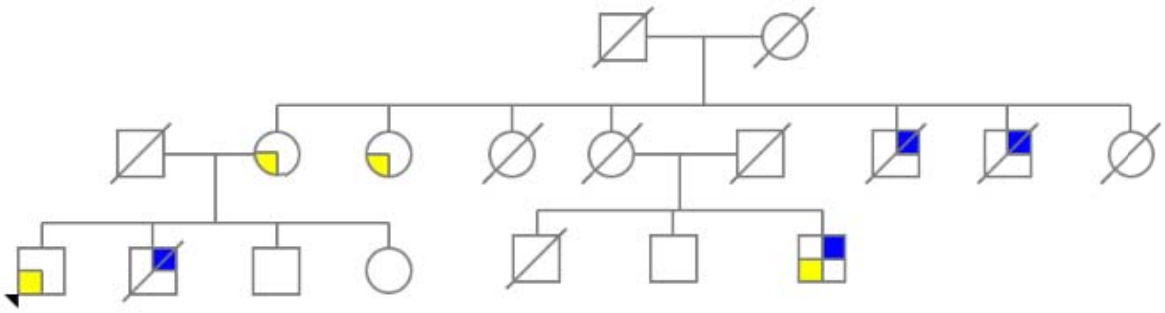

Fig. 1: Pedigree NN-0001 Yellow square in left lower quadrant = BE; Blue right upper quadrant = EAC. Proband is indicated with arrowhead

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Figure 2 – Esophageal submucosal gland (ESMG) histology (20X magnification) from subject with a CAV3 null mutation who had esophagectomy. H&E upper left shows distorted metaplastic acini with atypical proliferating cells; CAV3 immunostaining (upper right) shows no signifcant CAV3; ESMG shows CK7 immunopositivity (lower left); p63 immunostaining (lower right) shows relative paucity of p63 positive myoepithelial cells, but with presence of variable intracytoplasmic p63 staining in acinar cells.

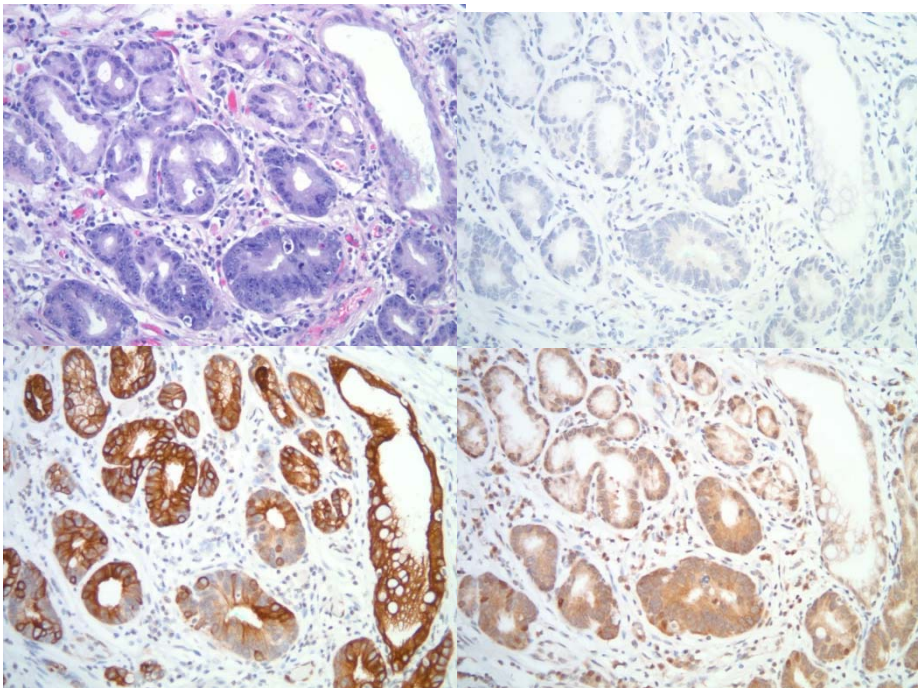

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Figure 3 – Esophageal submucosal gland (ESMG) histology (20X magnification) from non-familial subject with BE and high grade dysplasia who had esophagectomy showing ESMG with acinar metaplasia. H&E upper left shows acinar metaplasia; CAV3 immunostaining (upper right) shows a few scattered cells in mesenchyma with CAV3; ESMG shows strong CK7 immunopositivity (lower left); p63 immunostaining (lower right) shows p63 positive myoepithelial cells.

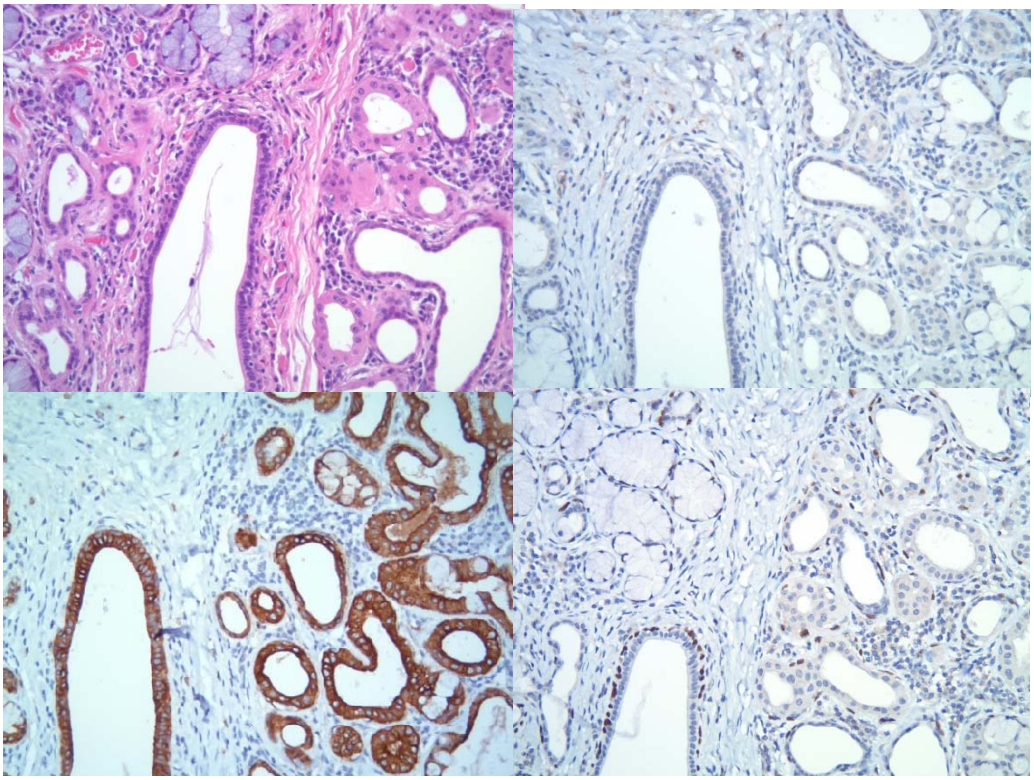

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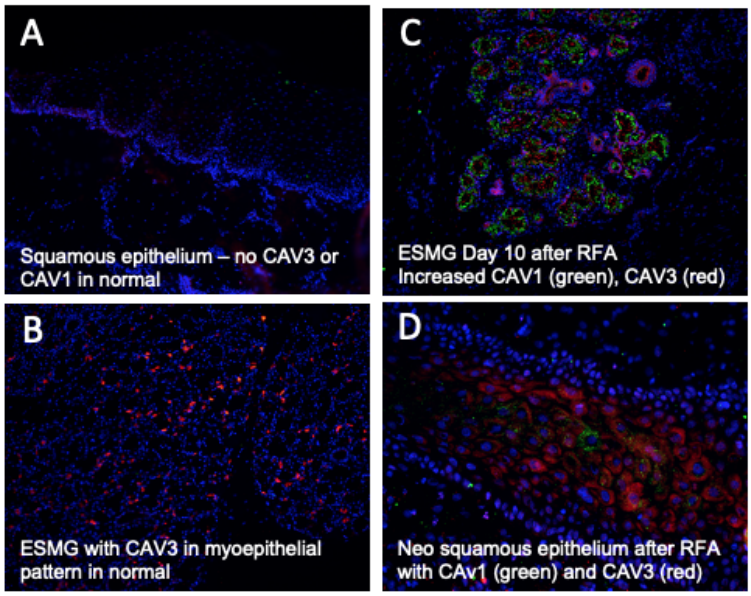
Figure 4 – Immunofluorescent imaging of normal procine squamous epithelium in esophagus (A) shows no CAV1 or CAV3, blue DAPI nuclear staining. Normal esophageal submucosal gland (B) shows CAV3 (red) expressing cells consistent with distribution in myoepithelial cells. Ten days after radiofrequency ablation injury ESMG (C) show CAV3 (red) expression within acini distinct ly different compartment than CAV1 (green). Interlobular duct and healing neosquamous epithelium (D) show CAV3 epxression.



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