Functional Consequences of CAPN14-Mediated Proteolysis of Catenins in the Esophageal Epithelium in Eosinophilic Esophagitis

Hemchandra Patel[1], Dr. Marc Rothenberg[2], Dr. Julie Caldwell[2], Dr. Mark Rochman[2]
University of Cincinnati College of Medicine[1], Cincinnati Children's Medical Center - Department of Allergy and Immunology[2]

Background

Eosinophilic esophagitis (EoE) is a food-related, chronic, allergic disorder that affects 1 in 2000 individuals and costs the United States about 1 billion dollars annually to treat and manage. EoE is characterized by chronic esophageal inflammation that can make it difficult and painful to swallow. In children, this inflammatory process leads to decreased appetite, resulting in inadequate nutrition and ultimately failure to thrive. Patients must be placed on a restricted diet and undergo numerous endoscopies throughout their lifetime, resulting in a low quality of life. At a cellular and molecular level, esophageal tissue exhibits epithelial barrier dysfunction, increased epithelial cell proliferation, and a block in epithelial differentiation. Through genome-wide association studies, the calpain 14 (*CAPN14*) locus was identified to be associated with EoE risk. Additionally, CAPN14 is upregulated in esophageal epithelial cells in response to IL-13 stimulation. However, the substrate of CAPN14 and its effects on epithelial barrier dysfunction remain unknown. In this study, we aimed to test the hypothesis that catenin proteolysis is mediated by CAPN14 in esophageal epithelial cells in EoE, resulting in impaired barrier function, increased proliferation, and altered differentiation in the esophageal epithelium.

Hypothesis: Alpha-catenin proteolysis is mediated by CAPN14 in esophageal epithelial cells in EoE, resulting in impaired barrier function

Allergen Exposure → ↑IL-13 → CAPN14 expression → ??? → Epithelial Barrier Dysfunction

Methods

First, EPC2 cells were cultured with and without IL-13, a cytokine shown to upregulate expression of CAPN14. We generated protein lysates from these cells and conducted SDS-PAGE followed by western blot analysis to identify any change in catenin levels or size in the presence of CAPN14. Next, we co-expressed CAPN14 and alpha catenin in HEK293T cells by transfecting them with mammalian expression constructs. We then generated protein lysates and conducted SDS-PAGE and western blot analysis to determine if cells co-expressing CAPN14 and alpha catenin had lower levels of alpha catenin compared to controls. Lastly, we obtained esophageal biopsies from patients with and without active EoE, from which we generated lysates and conducted SDS-PAGE and western blot analysis. Additionally, we used immunofluorescence staining to observe the pattern of alpha catenin in patients with active EoE.

Cell culture and transfection:
- EPC2 cells were grown in keratinocyte serum-free medium (KSFM) for 24 hours. Experimental cells were then incubated in KSFM media with 1.8mM Ca^{2+} with or without IL-13 [100ng/ml] for the given time course.
- HEK293T cells were grown in DMEM. Transfection was conducted using the TransIT-LT1 transfection reagent (Mirus) per the manufacturer's instructions.
- Mammalian expression constructs contained the open reading frame of CTNNA1 or CTNNA1 that was HA-tagged under the control of a CMV promoter. A construct containing no open reading frame was used as a negative control.

Ionomycin treatment:
- When indicated, prior to cell harvest, cells were incubated with 20uM ionomycin for 45 minutes.

Western Blot Analysis:
- Cell lysates were collected, and an equal volume of lysate for each sample was solubilized in loading buffer, loaded onto a 4-12% Tris-Bis polyacrylamide gel, and subjected to electrophoresis. Proteins were transferred to a nitrocellulose membrane, blocked for 30-60 minutes, and then incubated with primary antibodies overnight. Primary antibodies used were anti-GAPDH (Origene), anti-CTNNA1 (Cell Signaling, N-terminus), anti-CTNNB1 (Cell Signaling), and anti-CAPN14 (Sigma). Membranes were washed, incubated with secondary antibodies conjugated to infrared fluorophores (Jackson ImmunoResearch Laboratories, Inc.), and then washed prior to visualization of the infrared signals using the Odyssey Clx scanner (LiCOR).

Immunofluorescence:
- Tissue was deparaffinized, placed in pressure cooker for 15 minutes, blocked in 10% donkey serum, incubated in primary antibodies at 4 degrees Celsius for 5 hours, washed with PBS, placed in DAPI and secondary antibody for 2 hours, washed with PBS, mounted with 15uL Prolong Gold Antifade and dried overnight. Fluorescent signal visualized with confocal microscope.

Results

IL-13 induction of CAPN14 in EPC2 cells has no effect on endogenous CTNNA1

Fig 1: EPC2 cells were grown in normal KSFM media or KSFM with 1.8 mM Ca^{2+}, with or without IL-13 100 ng/mL. Cells were harvested, and protein lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and western blot was performed to detect CTNNA1 (100 kDa), CAPN14 (75 kDa), and GAPDH (37 kDa) as a loading control. **A)** IL-13 incubation for 24 hours. **B)** IL-13 incubation for 24 or 48 hours.

Alpha catenin expression was unaffected when co-transfected with CAPN14 in HEK293T cells

Fig 2: A) HEK293T cells were co-transfected with CTNNA1 and CAPN14 constructs. Cells were then incubated with 0uM or 20uM ionomycin as a potential CAPN14 activator. Cell lysates were collected followed by SDS-PAGE and western blot analysis for the HA epitope (to detect CTNNA1-HA, 100 kDa) and GAPDH (37 kDa) as a loading control. **B)** Quantification of Western blot signal for lanes 4-9. All conditions graphed include expression of CTNNA1.

Patient biopsy lysates exhibit no change in CTNNA1 quantity in patients with active EoE

Fig 3: A) Patient esophageal biopsy lysates were collected and subjected to SDS-PAGE and western blot analysis with anti-CTNNA1 and anti-CTNNB1. Control (CTL) biopsies demonstrated no histological evidence of EoE. EoE biopsies had histological evidence of active disease process. **B)** Quantification of western blot signal. The bar graphs represent the mean of each group, and error bars show the standard deviation. Dots represent CTNNA1/GAPDH values for each individual biopsy.

Immunofluorescence staining of patient biopsies demonstrate no change in CTNNA1 expression pattern in patients with active EoE

Control Biopsies: DAPI Stain, CTNNA1 Antibody

EoE Biopsies: DAPI Stain, CTNNA1 Antibody

Fig 4: Immunofluorescence staining of patient esophageal biopsies with anti-CTNNA1. Sections of formalin-fixed, paraffin embedded esophageal tissue were mounted on slides. Antigen retrieval was performed, and tissue was incubated in blocking solution followed by incubation with primary antibody (anti-CTNNA1). Tissue was washed and then incubated with secondary antibody and DAPI. Mounted with Prolong Gold Antifade. Fluorescent signal was visualized using a confocal microscope.

Conclusions

- In EPC2 cells incubated with Ca^{2+} and IL-13, there was no significant decrease in CTNNA1 quantity or molecular weight regardless of time control.
- In HEK293T cells that were co-transfected with CAPN14 and CTNNA1, there was no decrease in CTNNA1 quantity or molecular weight, regardless of the presence of ionomycin.
- In patient biopsy lysates there was no decrease in CTNNA1 quantity or expression pattern in patients with active EoE.
- Immunofluorescence staining of patient biopsies demonstrated no change in CTNNA1 expression pattern or localization in patients with active EoE when compared to controls.

Overall, we did not find evidence that alpha-catenin was a target of calpain 14 proteolysis.

Future Directions

- Determine if proteolytic products of CTNNA1 identified in preliminary screening study are sufficient to promote barrier dysfunction.
- Explore alternative targets of CAPN14
- Preliminary lab data suggest several alternative targets.
- One target of interest is Annexin A1, a protein involved in anti-inflammatory responses and an inhibitor of the NF-kB signaling pathway.
- Annexin A1 is significantly decreased on the RNA level in patients with active EoE, and preliminary lab data demonstrates there may be lower protein levels in patients with active EoE.

Acknowledgements

I would like to thank Dr. Marc Rothenberg, Dr. Mark Rochman, and Dr. Julie Caldwell for their mentorship throughout the duration of this project.

Supported by the University of Cincinnati MSSRP – NIH grant T35 DK06066 and Insert any additional grants here