Metallothionein-3 is a clinical biomarker for tissue zinc levels in nasal mucosa.

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Running title: MT3 is a biomarker for zinc in nasal mucosa.

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Metallothionein-3 is a clinical biomarker for tissue zinc levels in nasal mucosa.

ABSTRUCT

Objective: Recently, depleted tissue zinc levels were found in nasal mucosa from patients with chronic rhinosinusitis (CRS) in correlation with tissue eosinophilia, however, no clinical biomarkers for tissue zinc levels have been identified. Metallothionein-3 (MT3) is an intracellular zinc chelator and previous data showed MT3 mRNA levels to be reduced in CRS patients with nasal polyps (CRSwNP). In this study, we examined the correlation between MT3 expression and zinc levels in nasal mucosa and primary human nasal epithelial cells (HNECs) to investigate whether MT3 could be a clinical biomarker for tissue zinc levels.

Method: Tissue was harvested from 36 patients and mounted on tissue micro-array (TMA) slides. MT3 expression and tissue zinc fluorescence intensity were measured at different areas within the mucosa (surface epithelium and lamina propria) and compared between controls, CRSwNP and CRS without nasal polyps (CRSsNP) patients. MT3 mRNA and protein expression were examined in zinc-depleted HNECs by qPCR and immunofluorescence microscopy.

Results: MT3 expression in CRSwNP was significantly decreased in both surface epithelium ($p<0.001$ to controls) and lamina propria ($p=0.0491$ to controls). There was a significant positive correlation between tissue zinc levels and MT3 expression in nasal mucosa ($r=0.45$, $p=0.007$). In zinc-deplete HNECs, MT3 expression was significantly decreased at mRNA ($p=0.02$) and protein level ($p<0.01$). There was a significant positive correlation between tissue zinc levels and MT3 expression within individual HNECs ($r= 0.59$, $p<0.001$).

Conclusions: MT3 expression reflects intramucosal zinc levels in both nasal mucosa

and HNECs indicating MT3 could be used as a clinical biomarker for monitoring intracellular zinc levels in the nasal mucosa.

Key words: chronic rhinosinusitis, human nasal epithelial cells, zinquin, and nasal polyps

INTRODUCTION

Chronic rhinosinusitis (CRS) is defined as a sinonasal inflammation persisting for more than 12 weeks, characterized by infiltration of inflammatory cells into the nasal mucosa, tissue remodeling, and often accompanied by asthma(1, 2). CRS is clinically classified into two subtypes depending on the presence or absence of nasal polyps; CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP)(3).

Zinc is an essential trace element required for the normal functioning of many organ systems including the immune system. Zinc deficiency decreases the function of Th1 cells, but not that of Th2 cells and is associated with Th2 polarisation and eosinophilic inflammation (4-6). Zinc depleted diet has been shown to promote eosinophil infiltration into the lung in mice (7). Zinc depletion has been postulated to be involved in the pathogenesis of bronchial asthma and atopic dermatitis(8-10).

Recently, depleted tissue zinc levels were reported in the nasal mucosa of patients with CRS(11). Interestingly, whilst tissue zinc levels were decreased, there was an increase in mucus zinc levels at sites of inflammation and no evidence of systemic zinc depletion (11, 12). Mucosal zinc depletion is thought to be involved in multiple aspects of the pathophysiology of CRS and is more severe in CRSwNP than CRSsNP (11, 12). Reduced zinc levels impair epithelial barrier function at the level of the nasal mucosa and promote pro-inflammatory cytokines expression(11). Mucosal zinc levels are inversely correlated with eosinophil infiltration and affect tissue remodeling, which concerns a cycle of deposition and removal of extracellular matrix (ECM) proteins(12). Tissue eosinophil infiltration is seen in up to 70% of CRSwNP patients and those patients often have extensive disease and poor prognosis compared to non-eosinophilic CRS patients(13, 14).

Despite evidence of altered homeostasis of zinc in eosinophilic CRSwNP patients and the role of zinc in the pathophysiology of CRS, there are no methods to evaluate mucosal zinc levels in clinical practice. Serum zinc levels and zinc transporter genes in nasal mucosa are not useful biomarkers, because there were no significant differences in serum zinc levels and zinc transporter genes between healthy controls and CRS patients(11, 12).

Metallothionein-3 (MT3) is a zinc chelator gene, the expression of which is regulated by intracellular zinc levels. Our previous data showed MT3 mRNA was significantly reduced in CRSwNP compared to controls (11). These findings indicate that MT3 expression could be a new biomarker for mucosal zinc levels. However, there are no reports on MT3 expression in nasal mucosa from CRS patients in relation to mucosal zinc levels. It is also unknown whether MT3 expression reflects intracellular zinc levels in primary human nasal epithelial cells (HNECs).

In this study, we examined the relationship between MT3 expression and tissue zinc levels using a tissue micro array (TMA). We also investigated the effect of zinc depletion on MT3 expression in HNECs using zinc-depleted medium.

MATERIALS AND METHODS

Patients and tissue collection

The study was approved by The Queen Elizabeth Hospital Human Research Ethics Committee (reference HREC/15/TQEH/132). Written informed consent was obtained from study participants prior to tissue or cell collection. Nasal mucosa was taken from those patients undergoing endoscopic sinus surgery (ESS) for CRS or skull base tumor resections. Donors were divided into control patients (patients with skull base tumor), and CRSsNP or CRSwNP, depending on the absence or presence of nasal polyps respectively, as defined by the European Position Paper(3). Ethmoidal mucosa was used in control and CRSsNP patients and nasal polyps from middle meatus were used for CRSwNP patients. Control patients were only included in the absence of radiographic or endoscopic evidence of CRS. Patients with immunosuppression and treatments with oral antibiotics or corticosteroids in the week prior to study initiation were excluded. The sample pathologically diagnosed as respiratory epithelial adenomatoid hamartoma (REAH) or nasal tumor were excluded.

Primary human nasal epithelial cell cultures

Primary human nasal epithelial cells (HNECs) were taken from the nasal mucosa of donors in a method as previously described(15-17). The cells were suspended in Bronchial Epithelial Growth Medium (BEGM, CC-3170, Lonza, Walkersville, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port Washington, NY, USA). Monocytes were depleted from the cell suspension using anti-CD68 antibody (Dako, Glostrup, Denmark) coated cell culture dishes. HNECs were incubated in 37°C, humidified, 5% CO_2 in collagen-coated flasks (Thermo Scientific, Walthman, MA, USA), until passage 2. The cells were confirmed of being

epithelial lineage by reactivity to PAN-cytokeratin and CD45 antibodies, and by morphological examination with Diff-Quik analysis by qualified cytologists (IMVS Cytology Department, The Queen Elizabeth Hospital, Adelaide, Australia).

Zinc depleted media

5 grams of analytical grade Chelex 100 chelating ion-exchange resin (C7901, Sigma, Saint Louis, MO, USA) in two separate 50 ml falcon tubes was washed twice with Milli-Q water for 10 minutes. 50 ml of BEGM was added into the first tube and incubated for 2 hours on a rotator at room temperature (RT), following transfer into the other tube. After incubation overnight on a rotator at 4°C, the Chelex beads were discarded and the media was collected. The depleted molecules except zinc were added to the media ($CaCl_2$=22.19mg, $Fe(NO_3)_3$=0.00014mg, $FeSO_4$=0.022mg, $MgSO_4$=2.4mg, $MgCl_2$=4.28mg). The pH of the media was adjusted to original BEGM.

RNA extraction, Reverse transcription and qPCR.

HNECs were incubated in 6-well plates in normal and zinc deplete BEGM for 48 hours. Cells were collected and washed in phosphate buffered saline (PBS), followed by RNA extraction using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN Pty Ltd., Australia). An on-column DNAse treatment was performed with an RNase-Free DNase Set (QIAGEN Pty Ltd., Australia). Extracted RNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Franklin, MA, USA). RNA was reverse transcribed into cDNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany) with a MyCycler Thermal Cycler (BioRad Laboratories Inc., Gladesville,

Australia). The resulting cDNA was subjected to qPCR with TAQman primer/probe sets for each target gene, Taqman Universal Master Mix II (Thermo Fisher Scientific, Scoresby, Australia) and nuclease-free water. The average threshold cycle (Ct) was determined from three independent experiments and the level of gene expression relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined with the comparative CT method. Taqman Gene Assays used for gene expression analysis were: Hs00359394_g1 (MT3) and Hs02758991 (GAPDH).

Tissue Microarray

The analyses were conducted with the approval of the local Human Research Ethics Committee. Archival formalin-fixed paraffin-embedded sinus tissue blocks collected from the patients who underwent sinonasal surgery were used to construct the tissue microarray (TMA) for immunofluorescence. To guide the location of tissue cores, a 4 μm section was sliced from each tissue block and stained using Haematoxylin and Eosin (H&E). The tissue cores were punched using a 2 mm disposable biopsy punch (Kai medical, Kai Europe GmbH, Solingen, Germany) and manually put into Quick-RayTM paraffin recipient block 2 mm × 60 wells (IHC World, Woodstock, MD, USA).

Immunofluorescence

Tissue samples were cut in 4 μm sections from the TMA block. The slice was rehydrated, followed by antigen retrieval by submerging the slides in sodium citrate buffer (10 mM sodium citrate buffer, pH 6.8) and incubating in a 1100W microwave for 10 minutes. After cooling, sections were incubated with Serum Free Blocker (SFB, Dako, Glostrup, Denmark) for 60 minutes at RT, followed by permeabilization with

1% SDS in PBS. The sections were blocked with SFB for 60 minutes at RT. Rabbit polyclonal metallothionein 3 (MT3) antibody (A27974, Sigma-Aldrich, St Louis, MO, USA), diluted to 4 μg/mL in Tris-Buffered Saline-0.5% Tween (TBST), was added and incubated overnight at 4 °C followed by washing with TBST to remove excess primary antibody. 2 μg/mL Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Labs Inc, West Grove, PA, USA) was then added and cells were incubated for 1 hour at RT, followed by washing in TBST three times. 25 μmol/L of zinquin ethylester (Sigma-Aldrich, St. Louis, MO, USA) was added for 30 minutes at 37°C. The slides were washed, mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and visualized by using a LSM700 Confocal Laser Scanning Microscope (Zeiss Microscopy, Oberkochen, Germany) using the 20x objective. Excitation and emission values for MT-3 and zinquin were 555/570 nm and 405/460 nm, respectively. All images were acquired with identical microscopy parameters and taken sequentially to minimise cross-talk between fluorophores. Processing was performed using ZEN Imaging Software (Carl Zeiss AG, Oberkochen, Germany). The threshold of images was set for each channel to subtract autofluorescence and background fluorescence due to unspecific binding of antibody. For each specimen, at least 3 areas of the epithelium and the lamina propria were randomly selected as a region of interest (ROI) as described(18-20), then quantification of zinquin intensity was performed at the area. Quantification of MT-3 fluorescence intensity was acquired within the same ROI where zinquin intensity was measured (Supplemental Figure 1). Results are expressed as mean arbitrary fluorescence units, provided by the ZEN imaging software.

For immunofluorescence of HNECs, the cells were fixed with 2.5% formalin in PBS for 10 minutes followed by washing with TBST four times. The cells were

permeabilized with 1% SDS in PBS and blocked with SFB. The following process was identical as described above.

Statistical Analysis

All data was expressed as mean ± Standard Deviation (SD). The intensity of MT3 fluorescence was compared with Kruskal-Wallis test. When 3 or more groups were compared, one-way Analysis Of Variance (ANOVA) followed by Tukey's test was used to analyze differences among the groups. The Pearson correlation test was used to determine the correlation among MT3 and zinquin intensity. P-values of <0.05 were considered statistically significant. All the analyses were performed by using the JMP® 11 (SAS Institute Inc., Cary, NC, USA).

RESULTS

MT3 expression is decreased in the mucosa of CRSwNP patients.

First, we examined MT3 expression and zinquin fluorescence within the mucosa using immunofluorescence assays on a TMA slide that included tissue cores from 8 controls, 15 CRSsNP, and 13 CRSwNP. MT3 and zinquin fluorescence intensity were measured at different areas within the mucosa (surface epithelium and lamina propria) and compared amongst patient groups (Figure 1A). MT3 intensity in CRSwNP tissue was significantly decreased in both surface epithelium ($p=0.0006$ to controls, Figure 1B) and lamina propria ($p=0.0491$ to controls, Figure 1C). MT3 intensity in CRSsNP tissue was also significantly decreased in the surface epithelium compared to controls ($p=0.003$, Figure 1B).

MT3 expression positively correlated with zinc levels in nasal mucosa.

Next, we determined the relation of labile zinc levels with the expression of MT3 using immunofluorescence analysis of the TMA. There was a significant positive correlation between MT3 and zinquin fluorescence intensity in nasal mucosa ($r=0.45$, $p=0.007$, Figure 2).

MT3 expression correlates with zinc levels in nasal epithelial cells.

Next, we investigated the effect of zinc depletion of HNECs on MT3 expression. We established zinc deplete cells by culturing HNECs in zinc deplete medium for 48 hours as previously reported(11, 12). Compared to control cells, in zinc-deplete cells, MT3 fluorescence intensity was significantly decreased (control 57.83±10.83 vs zinc-deplete cells 36.68±4.51, $p<0.01$, Figure 3A and 3B). MT3 mRNA in zinc-depleted cells was also significantly decreased (0.45±0.26 fold change,

p=0.02, Figure 3C). There was a significant positive correlation between zinquin and MT3 fluorescence intensity within individual cells ($r= 0.59$, $p<0.001$) (Figure 3D).

DISCUSSION

This study showed that MT3 fluorescence intensity and mRNA expression were significantly decreased in zinc-depleted HNECs in relation to zinc levels. In addition, MT3 expression and labile zinc levels were significantly decreased in the mucosa of CRSwNP patients, corresponding to previous reports showing zinc depletion with decreased MT3 mRNA expression in CRSwNP(11). These results suggest that MT3 mRNA and protein expression could be a new biomarker for tissue zinc levels in nasal mucosa.

Zinc is an essential micronutrient and has a critical role in physiologic, enzymatic and structural functions(21). Consequently, altered zinc homeostasis affects multiple organs and systems and causes impaired immune function. In fact, zinc depletion has been reported in many diseases, including rheumatoid arthritis(22), hypertension(23), neurodegenerative diseases(24), Inflammatory Bowel Disease (IBD)(25), type 2 diabetes mellitus(26, 27), atopic dermatitis(8), bronchial asthma(28, 29), and chronic rhinosinusitis (CRS)(10-12, 30).

Recently, altered zinc homeostasis has been thought to be involved in multiple aspects of the pathophysiology of CRS, especially in CRSwNP patients(10). This includes impaired epithelial barrier function, eosinophil infiltration and tissue remodeling(11, 12). CRSwNP patients have been shown to present with lower mucosal zinc levels in association with a reduced expression of the tight junction protein Zonula Occludens-1 (ZO-1) compared to controls(11). This finding is supported by *in-vitro* results showing that zinc-depleted HNECs formed leaky mucosal barriers (11).

Depleted zinc levels also affect immune responses in the nasal mucosa and have been shown to promote pro-inflammatory cytokine production in HNECs(12).

Also, significant negative correlations between tissue zinc levels and eosinophil infiltration into the nasal mucosa of CRS patients have been demonstrated, in line with previous findings of Th2 polarisation and eosinophilia in the context of zinc deficiency(4-6). Tissue zinc levels are also correlated with collagen abundance in nasal mucosa and zinc depleted-fibroblasts synthesized less collagen compared to control cells. Considering that eosinophil infiltration and decreased collagen expression in nasal mucosa are hallmarks of CRSwNP, it has been postulated that depleted zinc levels might play a role in the formation of nasal polyps(12). Moreover, since tissue eosinophilia is often seen in CRS patients in relation to severity of disease, mucosal zinc levels and MT3 expression might also relate to disease severity and outcomes after surgery in those patients(13, 14). More research into the potential relationship between disease severity and mucosal zinc levels is needed to evaluate this hypothesis.

So far, there have been no standard methods to examine mucosal zinc levels in daily practice. Zinquin, the ultraviolet-excitable zinc-specific fluorophore, has been used to visualize tissue zinc in airways, but only for research purposes. It requires special equipment such as confocal laser microscopy and advanced imaging techniques and is a semi-quantitative technique to evaluate labile zinc levels.

It has been well documented that MT3 expression is strictly regulated by intracellular zinc levels through metal response elements (MRE) present in the promoter region of metallothionein genes. Intracellular zinc activates MRE-binding transcription factor-1 (MTF-1). The activated MTF-1 binds to MRE and promotes MT3 expression (Figure 4)(31-33). In contrast, depletion of intracellular zinc levels suppresses the activity of MTF-1, resulting in decreased MT3 expression. These

known mechanisms of transcriptional regulation of MT3 expression by intracellular zinc levels support the potential for MT3 mRNA expression to be used as a biomarker for tissue zinc levels in nasal mucosa. Interestingly, this theoretical framework of MT3 gene regulation matches our findings of reduced MT3 mRNA and protein levels in relation to reduced zinc levels in HNECs as well as results of our previous study showing a reduced MT3 mRNA expression in CRSwNP patients in relation to depleted tissue zinc levels(11).

From a clinical viewpoint, it could be more applicable to examine MT3 mRNA than MT3 or zinquin fluorescence intensity, as mRNA is more readily examined with qPCR and does not require special equipment such as confocal laser microscopy and advanced imaging techniques. Also, analysis of MT3 mRNA allows for more precise quantitation as compared to semi-quantitative methods relying on fluorescence. In general, immunofluorescence provides MT3 or zinquin intensity as relative values to other samples put on the same slide. In this research, we constructed the TMA slide containing 36 samples so that fluorescence intensity of the different samples could be compared under the same condition. Whereas immunofluorescence analysis using TMA slides therefore more accurately define relative protein/cellular content, the use of such techniques is not applicable in clinical practice. In contrast, intramucosal zinc levels could be more readily predicted by MT3 mRNA levels allowing more accurate comparison among different samples measured at different times and places.

It is unknown whether reduced MT3 expression plays a role in the pathophysiology of CRSwNP. MT3 has been shown to protect against airway inflammation induced by Ovalbumin(34), indicating a potential beneficial role for

MT3 in allergic airway disease. MT3 might have a protective role for airway inflammation in the nasal cavity as well. Further study will be necessary to unravel the exact role of MT3 in the pathogenesis of CRS.

CONCLUSION

MT3 expression reflects intramucosal zinc levels in both nasal mucosa and HNECs indicating MT3 could be used as a clinical biomarker for monitoring intracellular zinc levels in the nasal mucosa.

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AUTHORSHIP CONTRIBUTION

MS, YN, and SV designed the experiments. AJP, AH, and PJW supervised the project. MS and MR performed most of the experiments and compiled the data. CC and KO performed sample preparation. MS, MR, KO, and SV wrote the manuscript. All authors provided feedback on the manuscript.

DISCLOSURE STATEMENT

None of the authors have any conflicts of interest or financial disclosures that are relevant to this study.

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FIGURE LEGENDS

Figure 1. MT3 expression is decreased in the surface epithelium and lamina propria in CRSwNP.

A. Immunofluorescence of representative control, CRSsNP and CRSwNP patients using Zinquin (blue staining) and MT3 antibody (red staining). B and C. MT3 fluorescence intensity between the different patient groups was compared in the surface epithelium (B) and the lamina propria (C) in nasal mucosa. *$p<0.05$, Wilcoxon test.

Figure 2. MT3 expression positively correlated with zinc levels in nasal mucosa.

Pearson's correlation coefficient between MT3 and Zinquin fluorescence in the lamina propria of nasal mucosa is shown in a scatter diagram with 99% (red), 95% (green), 90% (blue), and 50% (purple) probability eclipses, respectively. Skyblue, orange, and gray points stand for CRSwNP, CRSsNP and control, respectively.

Figure 3. MT3 expression correlates with zinc levels in nasal epithelial cells.

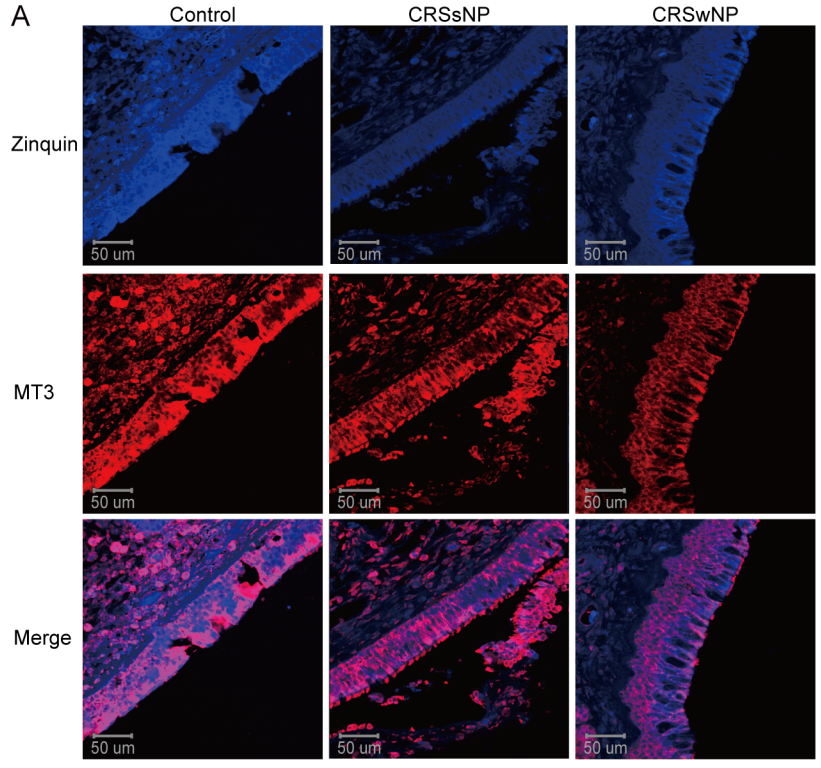
A-D. HNECs were incubated for 48 hours in zinc depleted (ZD) or control media followed by immunofluorescence using MT3 antibodies (A, B, and D) and by qPCR using MT3 primers (C). Data are means ± standard deviation (s.d.) of values from at least three independent experiments. P values for indicated comparisons were determined by t-test. *$p<0.05$. D. Zinquin and MT3 fluorescence intensity per HNEC was shown in a scatter diagram. The correlations between the zinquin and MT3 fluorescence intensity were tested using Pearson's correlation coefficient.

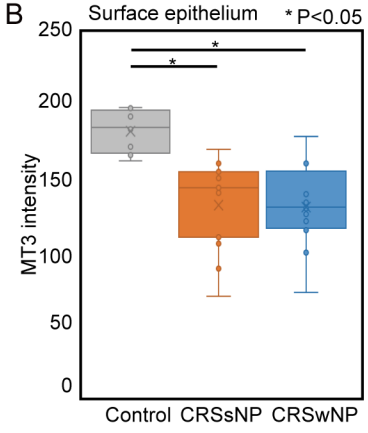
Figure 4. MT3 expression correlates with intracellular zinc levels.

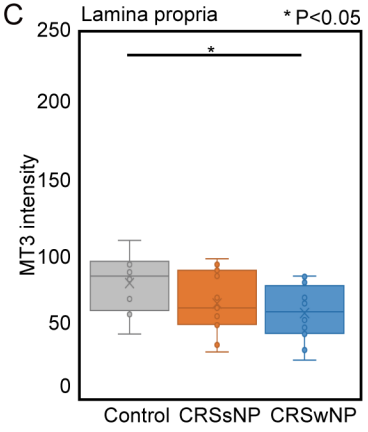
Free intracellular zinc activates metal response elements (MRE)-binding transcription factor-1 (MTF-1). MT3 expression is regulated by binding of activated MTF-1 to MRE located in the promotor region of MT-3 promoting MT3 mRNA expression (31-33). Depleted intracellular zinc fails to activate MTF-1 resulting in decreased MT3 expression.

Supplementary Figure 1. MT3 expression correlates with intracellular zinc levels. For each specimen, at least 3 areas of the epithelium and the lamina propria were randomly selected as a region of interest (ROI), then quantification of zinquin and MT-3 fluorescence intensity was performed at the area.

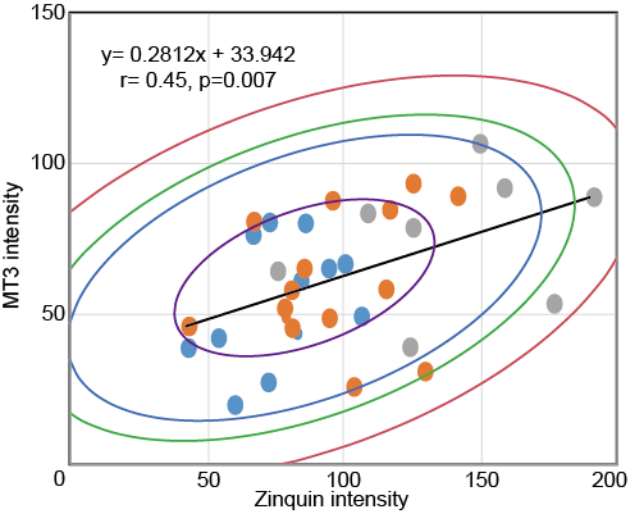
M Suzuki, MT3 figure 1



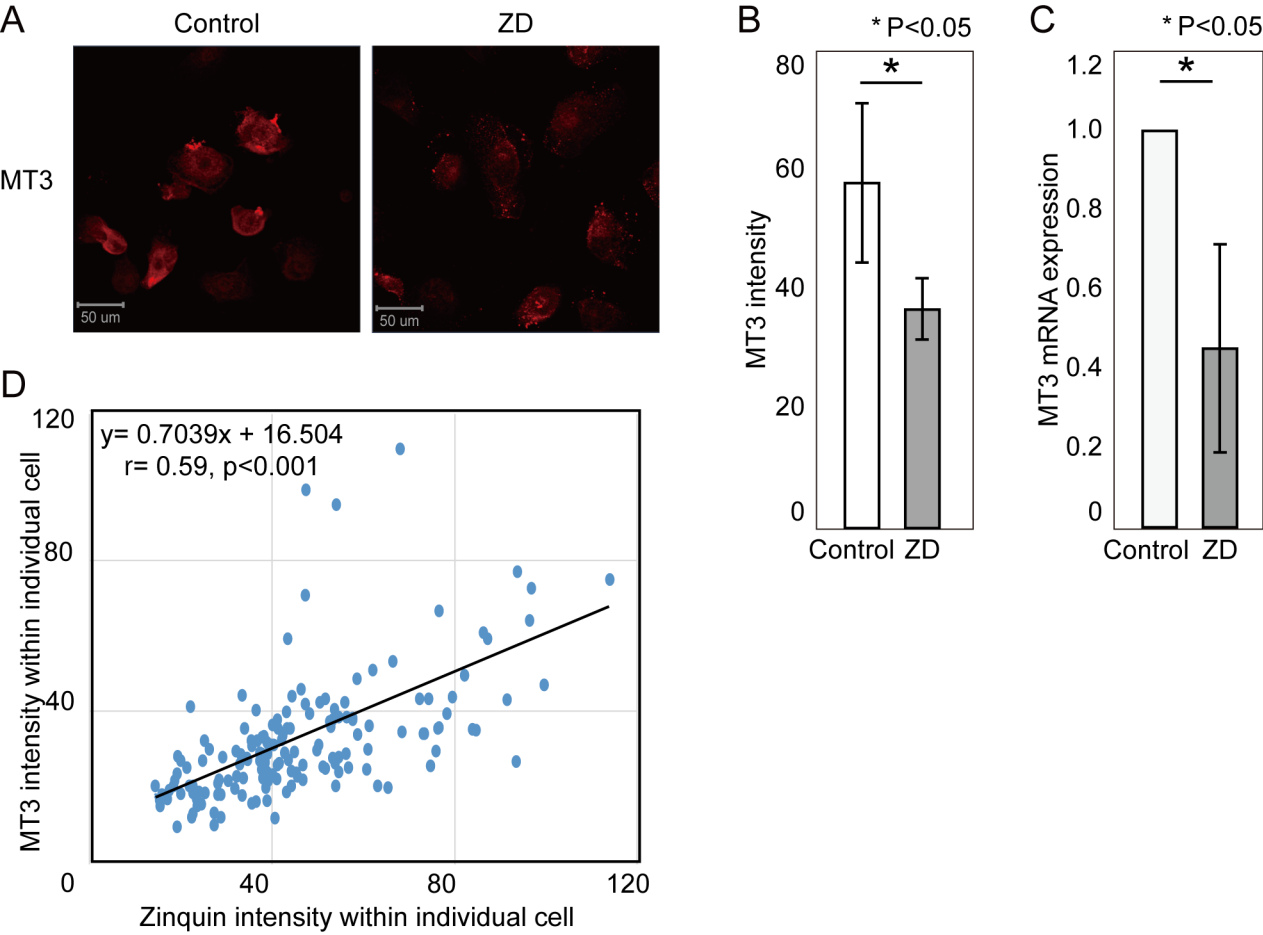




M Suzuki, MT3 figure 2



M Suzuki, MT3 figure 3



M Suzuki, MT3 figure 4