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# The discovery and validation of biomarkers for the diagnosis of esophageal squamous dysplasia and squamous cell carcinoma

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## Abstract

**Background—**The 5-year survival rate of esophageal cancer is less than 10% in developing countries, where more than 90% of these cancers are squamous cell carcinomas (ESCC). Endoscopic screening is not feasible on the scale required however non-endoscopic cell-sampling techniques may be feasible. Coupling endoscopic biopsies or non-endoscopic samples with biomarker analysis could reduce the subjectivity associated with both methods and thus improve diagnostic accuracy. The aims of this study were therefore to identify biomarkers for esophageal squamous dysplasia and carcinoma.

**Methods—**A publically available dataset was used to identify genes with differential expression in ESCC compared with normal esophagus (NE). Each gene was ranked by a support vector machine separation score. Expression profiles were examined, before validation by qPCR and immunohistochemistry.

**Results—**800 genes were overexpressed in ESCC compared to NE ($p<10^{-5}$). Of the top 50 genes, 33 were expressed in ESCC epithelium and not in NE epithelium or stroma using the Protein Atlas website. These were taken to qPCR validation and 20 genes were significantly overexpressed in ESCC compared to NE ($p<0.05$). TNFAIP3 and CHN1 showed differential expression with immunohistochemistry. TNFAIP3 expression increased gradually through NE, mild, moderate and severe dysplasia, and SCC ($p<0.0001$). CHN1 staining was rarely present in the top third of NE epithelium and extended progressively towards the surface in mild, moderate, and severe dysplasia, and SCC ($p<0.0001$).

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**Conflict of Interest:**

Rebecca C Fitzferald and Pierre Lao-Sirieix are named inventors on patents pertaining to a non-endoscopic screening device, the Cytosponge, and related assays. The technology was licensed to Covidien GI Solutions (now Medtronic). RCF and PLS have not received any financial benefits to date. Since December 2013, PLS is now employed partly by Covidien GI Solutions. All other authors have no conflicts of interest to declare.

**Conclusions—**Two novel promising biomarkers for ESCC were identified, TNFAIP3 and CHN1.

**Impact—**CHN1 and TNFAIP3 may improve diagnostic accuracy of screening methods for ESCC.

### Keywords

screening; esophageal squamous cell; cancer; carcinoma; biomarker

## Introduction

Cancer of the esophagus is the 6th most common cause of cancer death in the world (1). The squamous-cell carcinoma (ESCC) subtype has been declining in the West but remains the most common subtype in the rest of the world. ESCC accounts for 82% of esophageal cancer cases worldwide, including 90% of cases in the highest risk areas of Iran and China, but only around 30% of cases in the UK and USA (1). Patients with ESCC usually present late, with locally advanced disease or metastases, resulting in very poor survival. The 5-year relative survival rate in the USA is 18% (2), but in most high risk populations, where the medical infrastructure is less well developed, it is <10% (3). Early detection and treatment is associated with improved survival (4, 5). Although lead-time bias must be accounted for, a recent 10-year prospective community assignment study in China has reported a significant reduction in ESCC mortality following a single endoscopic screening followed by appropriate therapy (6). Rapid advances in imaging (7), minimally invasive endoscopic therapies (8–10), and novel chemoradiotherapy regimes (11) provide the opportunity to improve patient outcomes when disease is discovered at an early stage.

In high incidence areas for ESCC screening is an important consideration (12). Endoscopy is the gold standard for diagnosis, and identification of dysplasia can be assisted using Lugol's iodine staining (12, 13). However, on a population wide scale, endoscopy based methods are not logistically or economically feasible as a method of screening due to their high cost and requirement for expertise (12, 14–16). Non-endoscopic cell-sampling techniques are less invasive and costly, though the sensitivity and specificity of cytological assessment have been disappointing (12, 17–19). Coupling a pan-esophageal non-endoscopic cell-collection device with analysis of biomarkers could improve diagnostic accuracy. Equally, biomarker analysis of endoscopic specimens could reduce both the requirement for histopathological expertise and the risk of sampling bias because of the molecular field defect, thus potentially reducing both the procedure length and the number of samples required. Hence the biomarker assisted analysis could reduce the cost of endoscopic diagnosis to a level where it could be considered for screening high risk populations (15, 20).

There is currently a lack of suitable diagnostic biomarkers for ESCC (15, 21). Recent work has begun to identify candidate genes for differentiating ESCC pre-malignant changes (22–28), however the studies have different designs and rarely examine the same genes, making cross comparisons difficult, and the studies are very limited in quantitative analysis (15).

We hypothesised that protein biomarkers for squamous cell dysplasia and ESCC could be identified that would be suitable for application to clinical human tissue samples. The aims

of this study were therefore to identify candidate genes that are upregulated in ESCC and squamous dysplasia compared to normal esophageal epithelium and then to validate the putative targets at both the RNA and protein levels on samples from a cohort of patients with ESCC and healthy controls.

## Methods

### Microarray analysis

The publically available cDNA microarray data set described in Greenawalt et al. (29) was used to identify gene expression profiles from 65 samples (26 ESCC and 39 normal esophageal epithelium controls, Figure 1). A total of ~9,400 unique cDNA clones were available. Data was available in the form of normalised test:reference hybridisation signal intensity ratios. These were converted to $\log_2$ values and three clear outliers were excluded (1 from normal control, 2 from ESCC). A one-dimensional support vector machine (SVM) separation score for each gene was calculated for high expression in ESCC compared to low expression in normal controls with a soft 1-norm margin with weight C=1000. For each gene these scores were divided by the fold change between the geometric average of low and high expression and the geometric average fold change of high expression against control.

In this way, genes were ranked using a support vector machine (SVM) separation score, with a low score reflecting: i) consistent expression in each group of samples, ii) a good separation between normal and ESCC expression levels, and iii) a satisfactory level of expression in ESCC samples.

### Protein Atlas Evaluation

The 50 genes with the lowest SVM separation scores were assessed using the expression profiles on the Protein Atlas website (http://www.proteinatlas.org/) to ensure their suitability for paraffin embedded tissues. Genes were excluded based on reported protein expression in the epithelium of normal oesophagus or if they were known to be solely expressed in the stroma.

### Human specimens

The putative genes were validated using real-time polymerase chain reaction (RT-PCR) in 30 samples each of: normal esophagus from patients who were endoscopically normal (NN), ESCC from the tumour site of patients (T), and normal esophagus taken from the same patients with ESCC, from as far from the tumour site as possible (NT). The NT and T groups were a ‘matched cohort’, as the corresponding NT and T samples were paired from each patient.

The protein expression of putative biomarkers validated by RT-PCR was confirmed by immunohistochemistry on paraffin-embedded sections from the NT and T samples from the matched cohort, and on 34 paraffin-embedded biopsies of normal esophagus (NE), 31 mild dysplasia (Mild), 31 moderate dysplasia (Mod), and 31 severe dysplasia (Sev) samples from a ‘dysplastic cohort’.

In this study, the NN samples came from patients attending endoscopy at Addenbrooke's Hospital, Cambridge UK for routine diagnostic procedures with endoscopically-normal esophagus. The NT and T samples came from esophagectomy speciments used in a previous study in Linxian, China (30), and the biopsies of the "dysplastic cohort" came from another previous study in Linxian, China (19). All of the original studies, and the use of collected specimens for future evaluations, were approved by the appropriate IRBs.

### RNA extraction and real-time PCR

Total RNA was extracted from frozen samples using an AllPrep DNA/RNA Mini kit (QIAGEN Ltd, Manchester, UK) was then was reverse transcribed using the QuantiTect Reverse Transcription kit (QIAGEN Ltd, Manchester, UK). Quantitative PCR was performed using the LightCycler 480 SYBR Green I Master mix according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). PCR consisted of 45 cycles of 95°C denaturation (10s), 60°C annealing (10s) and extension (10s). Positive controls were identified for each primer pair. The cycle threshold (Ct) was determined for each sample and the average Ct of the triplicate samples was calculated. The expression of each gene relative to the geometric mean of the triplicate average Ct values for β-actin and 40S ribosomal protein S18 (RPS18) was determined as ΔCt. A melt curve was constructed for each primer.

### Immunohistochemistry

Sections of 3.5μm each were stained using a Bond system with the Bond Polymer Refine™ detection kit according to manufacturer's instructions (Leica Microsystems, Milton Keynes, UK). Origin of the primary antibodies as well as staining conditions are detailed in Table S1. A negative control was performed by omission of the primary antibody.

Each slide was double scored on both extent and intensity. For all genes except CHN1, extent was scored based on percentage of stained epithelium: 0 if absent, 1 for one cell to 33%, 2 for 34–66%, and 3 for ≥67%. For CHN1, extent was scored based on staining from the basal membrane to the epithelial surface: 0 if absent, 1 for any staining in the basal third of the epithelium, 2 for staining in the basal two thirds of the epithelium, and 3 for staining in the superficial third of the epithelium. Intensity was scored as 0 if absent, 1 for weak, 2 for medium, and 3 for strong staining.

### Statistical analysis

A one-way ANOVA analysis with a Dunn's multiple comparisons test was performed to analyse difference in mRNA expression. A Kruskal-Wallis one-way analysis of variance by ranks was performed to analyse difference in IHC scoring between all sample groups. A Wilcoxon matched-pairs signed-rank test was performed to analyse difference in IHC scoring between NT and T samples from the matched cohort. A Mann-Whitney test was used to compare IHC scoring between NN and NT samples. All statistics were performed using Prism (GraphPad Software).

## Results

### Identification of putative targets

The SVM separation score analysis (examples shown in figure S1) yielded 800 genes which were overexpressed in ESCC compared to normal esophagus ($p < 10^{-5}$, adjusted for multiple comparison). Expected expression profiles were evaluated using the Protein Atlas website for the 50 most significant genes: 9 genes were found to be expressed in normal esophagus (BAP1, SERPINH1, GUCY1A3, LAMC2, MMP2, SQSTM1, MMP10, NT5E and SOCSE) and 8 were expressed only in the stroma and would therefore not be suitable for a biopsy or cytology screening test (POSTN, CSPG2, MFAP2, CDH11, COL4A2, PODXL, SPARC and D2S448), (figure 1). These 17 genes were excluded from the study. A total of 33 genes were taken to mRNA validation.

### mRNA validation

Altered expression in ESCC compared to normal esophagus was confirmed at the mRNA level by real-time qPCR in 30 histopathologically verified tissues each from normal esophagus (NN), normal esophagus from ESCC patients (NT), and ESCC (T). The matched cohort of NT and T samples allowed analysis for the specificity of biomarkers compared to histologically confirmed ESCC within and between cancer patients.

A suitable primer pair could not be designed for E2Ig4. Validation of this target gene was therefore not taken any further. Eight genes (LUM, THY1, PLAU, TIA-2, PTGS2_2, UCHL1, PTSG2_1, LAP1B) were not detected in either normal (NN, NT) or ESCC (T) samples. Four genes (ALCAM, LAMA3, LTBR, ERBB2) had no statistical difference in expression between groups (figure 1 and 2A). These genes were excluded from the study.

The expression of 20 genes was significantly higher in ESCC compared to normal samples (range $p = 0.0002$ to $p < 0.0001$) but despite the significant difference in expression, 10 of these genes (FST, ITGA6, F2R, NELL2, DUSP6, SULF1, IL1B, FRP1, PLAT, IGFBP7) displayed marked overlap in expression between sample groups. It was therefore unlikely that the difference in mRNA expression would translate to a clear difference in protein expression and were therefore not taken forward to validation at the protein level (figure 1 and 2B). It is very interesting to note that 11 out of these 20 genes also displayed a significant over expression in NT compared to NN (Figure 1, Figure 2 and Table S2) suggesting a strong field defect around ESCC which could be advantageous for a non-endoscopic cytology sample method.

The remaining 10 genes contained groups of analogous genes: tumour necrosis factor alpha-induced protein 3 and 6 (TNFAIP3, TNFAIP6), collagens type III alpha 1 and type I alpha 2 (COL3A1, COL1A2), and C-C motif chemokine ligands 18 and 3 and 3-like 1 (CCL18, CCL3, CCL3L1). Only TNFAIP3, COL3A1 and CCL18 were taken to protein validation, based on better separation in expression between sample groups compared to TNFAIP6, COL1A2, and CCL3 and CCL3L1 respectively; the remainder were excluded from the study (figure 1 and 2C).

Therefore, 6 genes (CCL18, COL3A1, TNFAIP3, CHN1, CTSL, TNC) which were all overexpressed in ESCC compared to normal samples ($p < 0.0001$) were selected to be taken to protein validation (figure 1 and 3). It is interesting to note that for 5 of these genes (CCL18 $p = 0.0233$, COL3A1 $p = 0.0008$, CHN1 $p = 0.0004$, CTSL $p < 0.0001$, TNC $p < 0.0001$), their expression in normal esophagus from normal patients was lower than in normal esophagus from cancer patients. Again, this suggests some degree of field defect in pathologically normal epithelium adjacent to the cancer.

### Protein validation

Increased expression in ESCC compared to normal esophagus was validated at the protein level by immunohistochemistry in histopathologically verified sections from the 'matched' and 'dysplastic' cohorts of samples, containing normal esophagus from ESCC patients (NT), ESCC (T), normal esophagus (NE), and mild, moderate and severe dysplasia. CCL18 was not statistically overexpressed in ESCC compared to matched normal (figure 1 and figure S2). While TNC was statistical upregulated in ESCC compared to normal esophagus ($p<0.0002$), in nearly 38% of cases TNC was not expressed and its expression was limited to small foci of tumour cells in 45% of samples (figure 1 and figure S2). Therefore both CCL18 and TNC were excluded from further IHC (figure 1 and figure S2).

COL3A1 (figure 4A) showed a significant difference ($p = 0.0001$) in staining across the normal, dysplastic and cancer groups, however a Dunn's multiple comparisons test showed this was only true for the normal samples copared to any of the other groups, with no change in staining with progression through severity of dysplasia. COL3A1 was expressed mainly in the stromal compartment with very limited epithelial staining (figure S3). COL3A1 was therefore decided to be unsuitable for use as a diagnostic biomarker. CTSL (figure 4B) showed no significant difference ($p = 0.3586$) in staining across the normal, dysplastic and cancer groups. CTSL was expressed through out the tissue in both stroma and epithelium in normal and cancer samples (figure S4). In contrast, staining of CHN1 (figures 4C and 5) showed a progressive extent of staining towards the superficial layers of the esophageal epithelium, with staining in the superficial third of the epithelium (i.e. a score of 3) in 25% of normal esophagus, 34% of mild dysplasia, 65% of moderate dysplasia, 84% of severe dysplasia, and 97% of ESCC samples ($p < 0.0001$). TNFAIP3 (figure 4D and 5) also demonstrated stronger staining with increasing dysplasia ($p < 0.0001$), with staining scores of ≥1 (i.e. at least 1 cell staining positively) in 16% of normal esophagus, 18% of mild dysplasia, 30% of moderate dysplasia, 55% of severe dysplasia, and 63% of ESCC samples.

## Discussion

We have demonstrated that microarray analysis can be applied to biomarker discovery in the context of squamous-cell carcinoma of the esophagus. We identified CHN1 andTNFAIP3 as candidate biomarkers for ESCC using commercially available cDNA microarray data. We demonstrated that these were overexpressed in ESCC compared to normal esophageal epithelium at the mRNA and protein levels.

Initial validation was chosen to be at the mRNA level as reverse transcription quantitive polymerase chain reaction (RT-qPCR) is notably cheaper and less labour intensive than

immunohistochemistry (IHC), allowing parallel throughput of multiple prospective biomarkers. RNA species are relatively unstable, however, and rarely survive the paraffin embedding process, so samples must be snap frozen. Proteins are much more stable and are readily tested in paraffin embedded samples, so they make for more clinically suitable biomarkers, particularly in remote rural high incidence areas which is why the secondary validation was performed by IHC.

Out of the 50 markers selected for validation, only two were validated at the protein level. This may appear like a low validation rate especially given that 20 out of 33 genes validated at the mRNA level. This is a example of the difficulties faced when trying to identify biomarkers for a particular cancer. While significant differences can be seen in mRNA expression, they do not necessarily translate to levels of protein expression. While the expression level or staining extent of TNFAIP3 or CHN1 respectively increased along the progression from normal esophagus to SCC, neither marker is perfect at defining the dysplastic or cancer states. Combining both markers might however offer a specific and sensitive test for esophageal dysplasia and early squamous cell cancer of the esophagus.

There were some limitations to the microarray experiments conducted, however these did not detract from the results obtained. The microarray experiments were not designed specifically to identify markers distinguishing between ESCC and normal esophagus, but rigorous statistical measures were employed to reduce the effect of this shortfall which also reduced the number of putative genes. It is interesting to note that only 20 of the 33 targets were validated by qPCR. Eight of the excluded genes were due to undetectable expression levels in biopsy samples. This is most likely due to the amplification of the RNA extracted from samples in the microarray protocol (29) which could account for the observed difference in base expression values. In the microarray data available to us there was no data on dysplasic tissue. This could have allowed us to identify more appropriate biomarkers for identifying high-grade squamous dysplasia, which is the primary screening target for ESCC.

The expression level of 13 out of the 20 increased gradualy between normal oesophagus from normal patients, normal oesophagus from cancer patients and cancer samples (figures 2 and 3). The pathology of all samples was confirmed by an expert pathologist, it is therefore unlikely that dysplasia or cancer was present in the normal samples from cancer patients. This intermediate level of SCC biomarkers suggests that a field defect exists in SCC patients. This field defect could be utilised diagnostically. Even in the event of biopsy or cytological collection that misses the area of cancer and/or dysplasia, an abnormal biomarker could still be detected and patients could be recalled for further investivation.

The biological reason for alterations in the expression of CHN1 and TNFAIP3 remain unclear. Chimerin 1 (CHN1) is expressed in neurones and is predominantly found in the cerebral cortex. It codes for a Rho GTPase-activating protein which is important for dendritic morphology (31) and axon guidance (32). Missense mutations in CHN1 have been associated with variants of Duane’s retraction syndrome (33, 34) and cranial nerve abnormalities (35). The possible role of CHN1 in aiding cellular remodelling in dysplastic and cancer cells would need to be investigated to fully comprehend the full impact of the striking increase in expression of the gene along the progression to cancer. Tumour necrosis

factor, alpha-induced protein 3 (TNFAIP3) codes for a ubiquitin editing enzyme which inhibits NFκB and TNF-mediated apoptosis. It is associated with many autoimmune conditions (36–39) and has been noted to have tumour suppressor functions in lymphomas and colorectal cancer (40–43). However, TNFAIP3 also has oncogenic properties, with implication in tamoxifen resistance in breast cancer and developing resistance to apoptosis to promote cancer cell survival (44). It would interesting to understand the role of TNFAIP3 in squamous cell cancer and its link with possible resistance to chemotherapy.

In summary, the biomarker discovery/validation pipeline successfully identified markers for esophageal squamous dysplasia and squamous cell carcinoma of the esophagus. A clinical study applied to endoscopic biopsies or non-endoscopic cell collaction device of the biomarkers CHN1 and TNFAIP3 is now warranted. If used in high risk regions through out the world, such an approach has the potential to identify most patients with moderate or severe dysplasia who would benefit most from endoscopic treatments to prevent the development of invasive squamous cell cancers.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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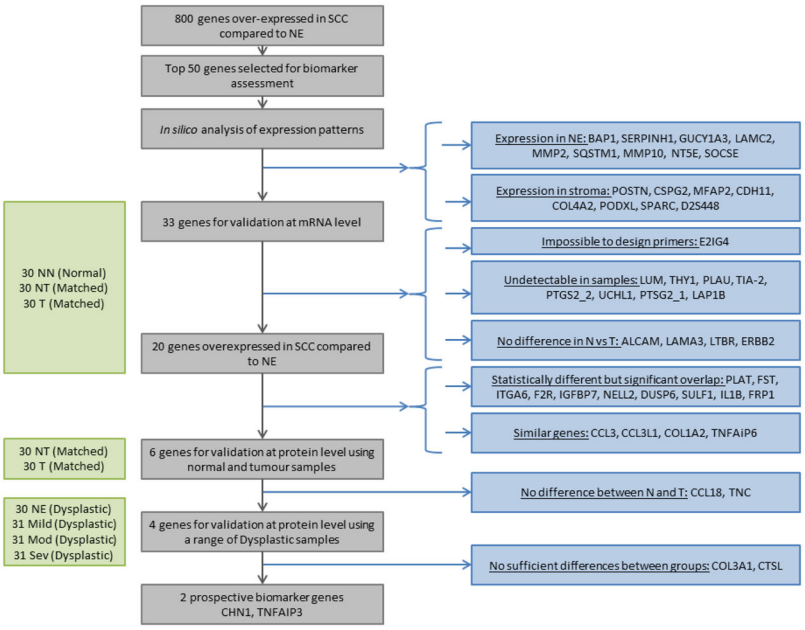


**Figure 1.**

Overview of discovery-validation pipeline and results at each stage. Samples used at each stage are displayed on the left, with the sample cohort shown in brackets. NN = Normal esophagus from endoscopically normal patients (normal cohort); T = Tumour, NT = Normal Esophagus from ESCC patients (matched cohort); NE = Normal Esophagus, Mild = mild dysplasia, Mod = moderate dysplasia, Sev = severe dysplasia (dysplastic cohort).

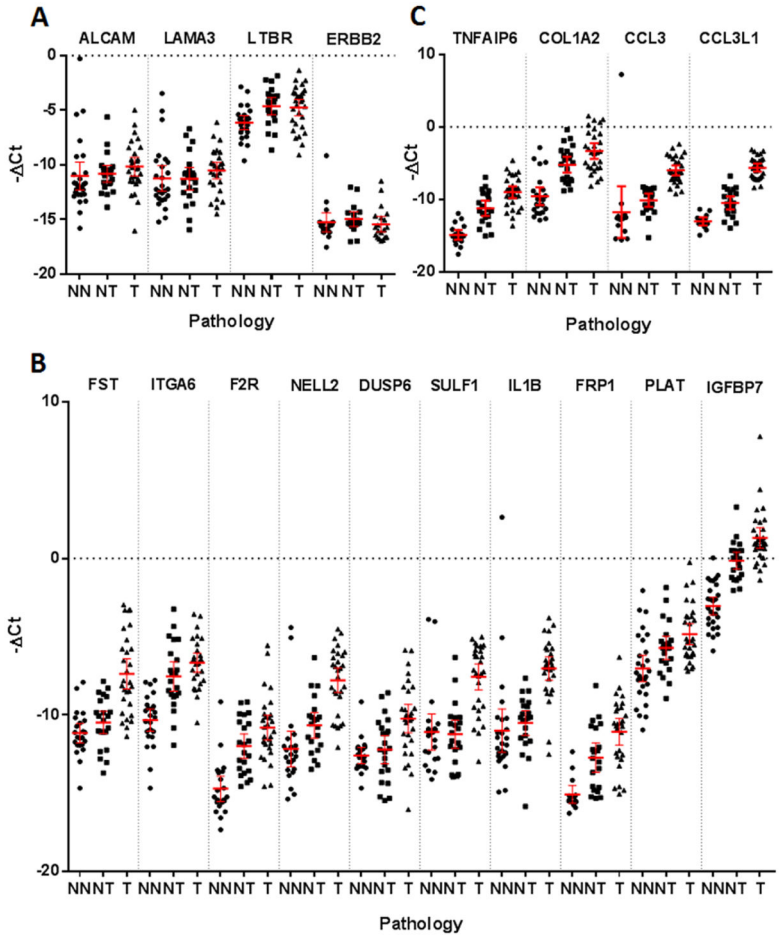


**Figure 2.**

−ΔCt qPCR data relative to housekeepers β-actin and RPS18 from normal esophagus (NN), normal esophagus from ESCC patients (NT) and ESCC (T) samples. The mean and 95% confidence intervals are displayed. **A:** Genes with no statistical difference in expression between sample groups. **B:** Genes which showed significant overlap in expression between sample groups. **C:** Genes with less clear separation compared to their analogous gene counterparts.

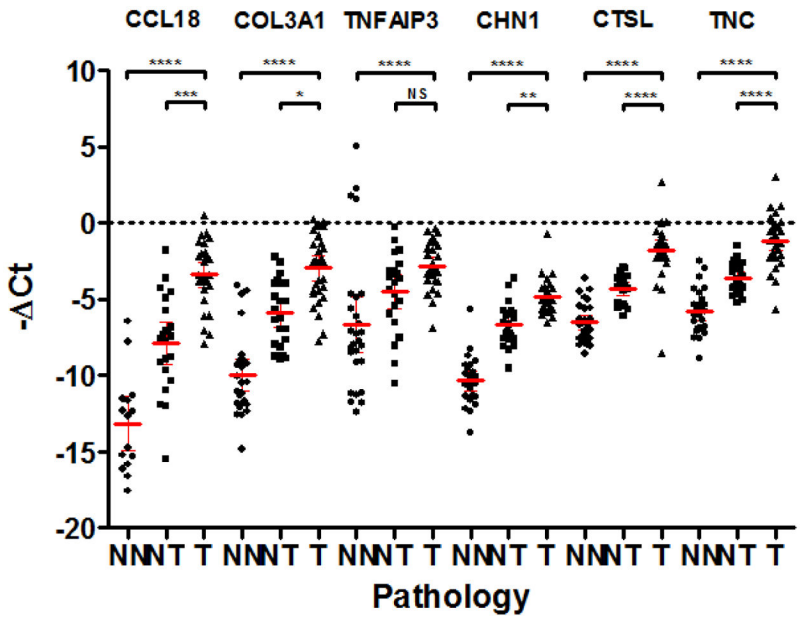


**Figure 3.**

−ΔCt qPCR data from normal esophagus (NN), normal esophagus from ESCC patients (NT) and ESCC (T) samples relative to housekeepers β-actin and RPS18. The mean and 95% confidence intervals are displayed. Adjusted p values are as follows: **** $p<0.0001$, *** $p=0.0003$, ** $p=0.0025$, * $p=0.01$, NS $p>0.05$.

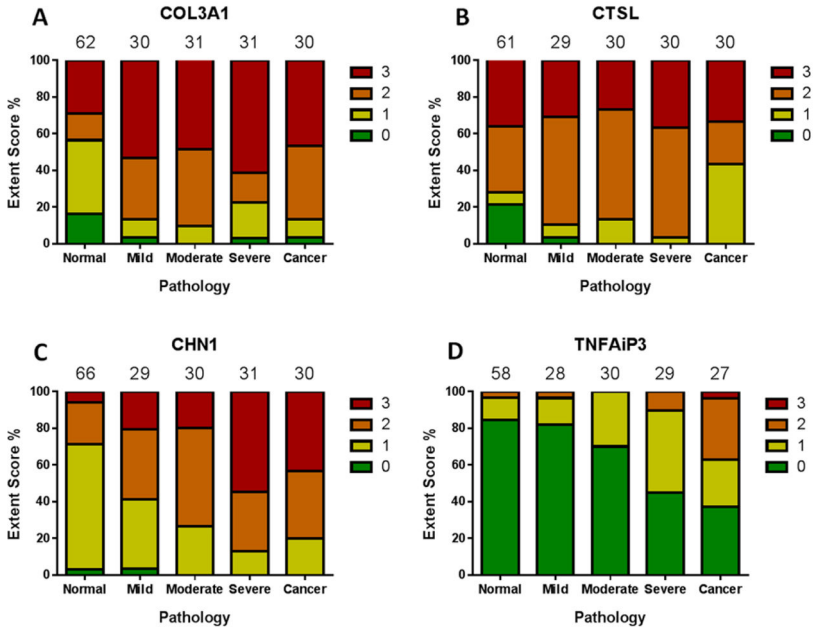


**Figure 4.**

IHC staining extent scoring for putative genes across matched and dysplastic cohorts of samples. The number of samples in each pathology group is noted above each column. The ‘normal’ group is comprised of NT samples from the matched cohort and NE samples from the dysplastic cohort.

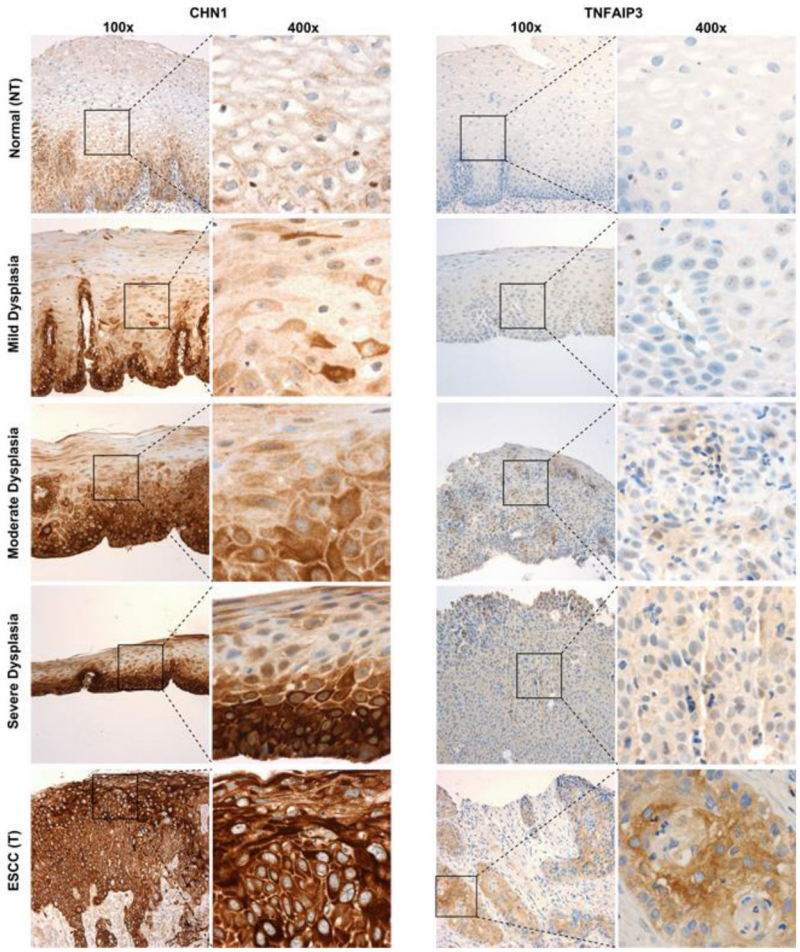


**Figure 5.**

Representative images of immunohistochemistry staining for normal esophagus from ESCC patients (NT), mild, moderate, and severe dysplastic, and ESCC samples at 100x and 400x magnification.