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**EDITORIAL**

- 5451** Wilson's disease: Prospective developments towards new therapies

Ranucci G, Polishchuck R, Iorio R

- 5457** Are our endoscopy patients at risk for pyogenic liver abscess?

Cerwenka H, Schemmer P

REVIEW

- 5460** Pancreatitis: Preventing catastrophic haemorrhage

Evans RPT, Mourad MM, Pall G, Fisher SG, Bramhall SR

MINIREVIEWS

- 5469** Treating children with inflammatory bowel disease: Current and new perspectives

Guariso G, Gasparetto M

- 5486** Influence of gut microbiota on neuropsychiatric disorders

Cenit MC, Sanz Y, Codoñer-Franch P

ORIGINAL ARTICLE**Basic Study**

- 5499** Everolimus halts hepatic cystogenesis in a rodent model of polycystic-liver-disease

Temmerman F, Chen F, Libbrecht L, Vander Elst I, Windmolders P, Feng Y, Ni Y, De Smedt H, Nevens F, van Pelt J

- 5508** MicroRNA profile in neosquamous esophageal mucosa following ablation of Barrett's esophagus

Sreedharan L, Mayne GC, Watson DI, Bright T, Lord RV, Ansar A, Wang T, Kist J, Astill DS, Hussey DJ

- 5519** Expression of Interleukin-26 is upregulated in inflammatory bowel disease

Fujii M, Nishida A, Imaeda H, Ohno M, Nishino K, Sakai S, Inatomi O, Bamba S, Kawahara M, Shimizu T, Andoh A

- 5530** Autophagic cell death induced by reactive oxygen species is involved in hyperthermic sensitization to ionizing radiation in human hepatocellular carcinoma cells

Yuan GJ, Deng JJ, Cao DD, Shi L, Chen X, Lei JJ, Xu XM

- 5538** Yangzheng Sanjie decoction regulates proliferation and apoptosis of gastric cancer cells by enhancing let-7a expression

Deng HX, Yu YY, Zhou AQ, Zhu JL, Luo LN, Chen WQ, Hu L, Chen GX

Case Control Study

- 5549 Crohn's disease environmental factors in the developing world: A case-control study in a statewide catchment area in Brazil

Salgado VCL, Luiz RR, Boechat N, Schorr BC, Leão IS, Nunes T, Zaltman C

Retrospective Cohort Study

- 5557 Postoperative bleeding in patients on antithrombotic therapy after gastric endoscopic submucosal dissection

Sato C, Hirasawa K, Koh R, Ikeda R, Fukuchi T, Kobayashi R, Kaneko H, Makazu M, Maeda S

Retrospective Study

- 5567 Serous pancreatic neoplasia, data and review

Dietrich CF, Dong Y, Jenssen C, Ciaravino V, Hocke M, Wang WP, Burmester E, Möller K, Atkinson NSS, Capelli P, D'Onofrio M

- 5579 Pancreaticoduodenectomy for duodenal papilla carcinoma: A single-centre 9-year retrospective study of 112 patients with long-term follow-up

Lian PL, Chang Y, Xu XC, Zhao Z, Wang XQ, Xu KS

Clinical Trials Study

- 5589 Efficacy and safety of Xiangsha Liujunzi granules for functional dyspepsia: A multi-center randomized double-blind placebo-controlled clinical study

Lv L, Wang FY, Ma XX, Li ZH, Huang SP, Shi ZH, Ji HJ, Bian LQ, Zhang BH, Chen T, Yin XL, Tang XD

Observational Study

- 5602 Combination of acoustic radiation force impulse imaging, serological indexes and contrast-enhanced ultrasound for diagnosis of liver lesions

Sun XL, Yao H, Men Q, Hou KZ, Chen Z, Xu CQ, Liang LW

Prospective Study

- 5610 Incidents and adverse events of endoscopic ultrasound-guided fine-needle aspiration for pancreatic cystic lesions

Du C, Chai NL, Linghu EQ, Li HK, Sun YF, Xu W, Wang XD, Tang P, Yang J

SYSTEMATIC REVIEWS

- 5619 Systematic review of giant gastric lipomas reported since 1980 and report of two new cases in a review of 117110 esophagogastroduodenoscopies

Cappell MS, Stevens CE, Amin M

- 5634 Acute colonic pseudo-obstruction: A systematic review of aetiology and mechanisms

Wells CI, O'Grady G, Bissett IP

Contents

World Journal of Gastroenterology
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Basic Study

MicroRNA profile in neosquamous esophageal mucosa following ablation of Barrett's esophagus

Loveena Sreedharan, George C Mayne, David I Watson, Timothy Bright, Reginald V Lord, Alfiya Ansar, Tingting Wang, Jakob Kist, David StJ Astill, Damian J Hussey

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Abstract

AIM

To investigate the microRNA expression profile in esophageal neosquamous epithelium from patients who had undergone ablation of Barrett's esophagus.

METHODS

High throughput screening using TaqMan® Array Human MicroRNA quantitative PCR was used to determine expression levels of 754 microRNAs in distal esophageal mucosa (1 cm above the gastro-esophageal junction) from 16 patients who had undergone ablation of non-dysplastic Barrett's esophagus using argon plasma coagulation *vs* pretreatment mucosa, post-treatment proximal normal non-treated esophageal mucosa, and esophageal mucosal biopsies from 10

controls without Barrett's esophagus. Biopsies of squamous mucosa were also taken from 5 cm above the pre-ablation squamo-columnar junction. Predicted mRNA target pathway analysis was used to investigate the functional involvement of differentially expressed microRNAs.

RESULTS

Forty-four microRNAs were differentially expressed between control squamous mucosa *vs* post-ablation neosquamous mucosa. Nineteen microRNAs were differentially expressed between post-ablation neosquamous and post-ablation squamous mucosa obtained from the more proximal non-treated esophageal segment. Twelve microRNAs were differentially expressed in both neosquamous *vs* matched proximal squamous mucosa and neosquamous *vs* squamous mucosa from healthy patients. Nine microRNAs (miR-424-5p, miR-127-3p, miR-98-5p, miR-187-3p, miR-495-3p, miR-34c-5p, miR-223-5p, miR-539-5p, miR-376a-3p, miR-409-3p) were expressed at higher levels in post-ablation neosquamous mucosa than in matched proximal squamous and healthy squamous mucosa. These microRNAs were also more highly expressed in Barrett's esophagus mucosa than matched proximal squamous and squamous mucosa from controls. Target prediction and pathway analysis suggests that these microRNAs may be involved in the regulation of cell survival signalling pathways. Three microRNAs (miR-187-3p, miR-135b-5p and miR-31-5p) were expressed at higher levels in post-ablation neosquamous mucosa than in matched proximal squamous and healthy squamous mucosa. These miRNAs were expressed at similar levels in pre-ablation Barrett's esophagus mucosa, matched proximal squamous and squamous mucosa from controls. Target prediction and pathway analysis suggests that these microRNAs may be involved in regulating the expression of proteins that contribute to barrier function.

CONCLUSION

Neosquamous mucosa arising after ablation of Barrett's esophagus expresses microRNAs that may contribute to decreased barrier function and microRNAs that may be involved in the regulation of survival signaling pathways.

Key words: Neosquamous; Barrett's esophagus; Ablation

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Core tip: We report that the microRNA profile of esophageal neosquamous mucosa developing after ablation of Barrett's esophagus is different to normal squamous epithelium, and that the differentially expressed microRNAs in neosquamous mucosa may regulate survival signalling pathways and contribute to decreased barrier function in the esophagus.

Sreedharan L, Mayne GC, Watson DI, Bright T, Lord RV, Ansar A, Wang T, Kist J, Astill DS, Hussey DJ. MicroRNA profile in neosquamous esophageal mucosa following ablation of Barrett's esophagus. *World J Gastroenterol* 2017; 23(30): 5508-5518 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i30/5508.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i30.5508>

INTRODUCTION

The incidence of esophageal adenocarcinoma has increased rapidly in the western world over recent decades, with overall 5-year survival rates of approximately 15%^[1]. A strategy to improve survival outcome is early detection of cancer, or detection at the pre-malignant stage - high grade dysplasia. Barrett's esophagus is the precursor to adenocarcinoma^[2], and results from a metaplastic change of normal esophageal squamous epithelium to columnar epithelium with intestinal differentiation^[3,4]. This is a consequence of chronic gastro-esophageal reflux, and it can be identified in 1%-2% of individuals aged over 60^[4]. Barrett's esophagus progresses to cancer in a sequential manner through low and then high grade dysplasia^[2]. The risk of progression of non-dysplastic Barrett's esophagus to adenocarcinoma has been reported to be 0.2%-0.5% per patient year for patients enrolled in surveillance programs^[1].

Endoscopic surveillance remains the mainstay of cancer prevention in individuals with Barrett's esophagus, and definitive management by surgery or endoscopy is reserved for individuals who develop high grade dysplasia or cancer^[5]. Several endoscopic treatments are widely used for the treatment of high grade dysplasia or early cancer in Barrett's esophagus, including radiofrequency ablation, argon plasma coagulation, and endoscopic mucosal resection.

Generally, endoscopic therapy for Barrett's esophagus aims to completely eradicate any columnar mucosa, although persistent genomic alterations at tumour suppressor loci have been found after ablation^[6]. Even though endoscopic removal of Barrett's esophagus by ablative therapies is possible in the majority of patients^[7], there is still a risk of recurrence and progression to adenocarcinoma following complete eradication of Barrett's esophagus. As a result endoscopically treated patients are maintained under surveillance^[4].

There have also been concerns about the risk of recurrence associated with residual sub-squamous glandular tissue which is not visible with a white light endoscope. However, Basu *et al*^[8] reported that sub-squamous glandular tissue was not associated with recurrence of Barrett's esophagus in patients with effective acid suppression after argon plasma

coagulation ablation, and newer ablation modalities such as radio frequency ablation appear to have largely eliminated this problem^[9], due to increased control of depth and uniformity of tissue ablation^[10]. There have been several reports of patients progressing to cancer in whom Barrett's esophagus has been completely eradicated^[11-13], and this has brought into question the "normality" of the regenerated neosquamous epithelium. In a previous study we observed that the expression levels of cytokeratins CK-8 and CK-14, and microRNA-205, are similar in post-ablation neosquamous epithelium and more proximal normal squamous epithelium from patients with Barrett's esophagus. However, microRNA-143 expression, which is elevated in Barrett's esophagus^[14], was elevated in post-ablation neosquamous mucosa and in squamous mucosa above the metaplastic segment compared to squamous epithelium from healthy patients, suggesting that the regenerated neosquamous mucosa might not be "normal"^[15].

MicroRNAs are short (about 22 nucleotides) non-coding RNA molecules that regulate gene expression. Because a single microRNA can target several mRNAs, dysregulated microRNA expression can impact on key biological pathways and contribute to cancer development^[16]. Dysregulated microRNA expression along the squamous - Barrett's - dysplasia - adenocarcinoma pathway has been reported by several groups^[3,14,17-19]. In the current study we profiled global microRNA expression in esophageal mucosa before and after ablation of Barrett's esophagus using Argon plasma coagulation. Our aim was to further investigate differences in microRNA expression between neosquamous and normal squamous mucosa, and to investigate how these differences might contribute to altered biology using predicted targets and pathway analysis.

MATERIALS AND METHODS

The methods used for the tissue collection and processing are described in our previous study^[15], and reproduced here for completion.

Tissue collection

Esophageal mucosal tissue was collected before and after Argon plasma coagulation ablation of Barrett's esophagus from 16 individuals of median age 54.2 years (range 28.9-68.1) who were enrolled in the treatment arms of previously reported randomised controlled trials of Barrett's esophagus ablation vs endoscopic surveillance^[20]. Barrett's esophagus was defined as columnar epithelium in the distal esophagus with histological confirmation of the presence of intestinal metaplasia. All patients were free of reflux symptoms following treatment of gastro-esophageal reflux, by either high dose proton pump inhibitors ($n = 8$) or a laparoscopic fundoplication ($n = 8$); before

enrolment in the trial, at pre and post-treatment sample collection, and at Barrett's esophagus ablation.

All patients underwent baseline endoscopy and biopsies from the distal esophageal mucosa were collected as described below. Biopsies were assessed by standard histopathological techniques. The presence of intestinal metaplasia and the absence of dysplasia within the Barrett's esophagus segment were confirmed in all patients. The pre-ablation length of Barrett's esophagus ranged from 1-10 cm in length (median 3 cm). Patients underwent endoscopic Argon plasma coagulation ablation following baseline endoscopy. The details of the ablation protocol have been described in detail previously^[20]. Of the patients contributing tissues to the current study, complete ablation of the Barrett's esophagus was achieved in 13 of 16 (82%). In the other 3, 95%, 99% and 95% ablation was achieved. Patient-matched post ablation neosquamous and proximal squamous samples were included in the study.

Biopsy collection

Four quadrant esophageal biopsies were taken commencing from 1 cm above the gastro-esophageal junction and then every 2 cm proximally for the length of the Barrett's segment and sent for histopathology. An additional three biopsies were collected for research purposes from each sampled level of the Barrett's esophagus, and stored in RNeasy[®] (Ambion, Austin, Texas, United States) as per the manufacturer's protocol.

Repeat endoscopy was performed at a median of 6 wk (inter quartile range 4.96-6.5 wk) after the last ablation treatment and biopsies were collected from the post-ablation neosquamous esophageal mucosa using the same biopsy collection protocol. Additional biopsies were collected 5 cm above the proximal margin of the pre-ablation Barrett's mucosa, and the corresponding site post-ablation for use as patient-matched non-regenerated squamous esophageal mucosa.

Biopsies collected from patients who had undergone Barrett's esophagus ablation were selected for analysis from the following sites: (1) pre-ablation Barrett's esophagus mucosa (columnar mucosa with intestinal metaplasia), 1 cm above the gastro-esophageal junction; (2) post-ablation neosquamous mucosa, 1 cm above the gastro-esophageal junction; and (3) post-ablation squamous mucosa, 5 cm above the level of the pre-ablation squamo-columnar junction.

Endoscopic esophageal mucosal biopsies were also collected at endoscopy from fourteen control individuals of median age 51.9 (range 24.1-71.0) with no known esophageal disease. These biopsies were taken from the distal esophagus, at an equivalent distance from the gastro-esophageal junction to the neosquamous mucosal biopsies, to allow direct comparison of the biopsies from normal squamous mucosa from the

control patients to the biopsies of neosquamous mucosa from the patients who underwent ablation. The inclusion criteria for the control patients were: (1) no reflux symptoms; (2) endoscopy was not undertaken for the investigation of reflux; (3) no macroscopic esophagitis seen at endoscopy; (4) gastro-esophageal junction closed when viewed from within the esophagus; and (5) gastro-esophageal junction snug around the endoscope when viewed with the retroflexed endoscope.

Biopsy processing and RNA extraction

All biopsy samples for the study were immediately stored in RNeasy[®] (Ambion, Austin, Texas, United States) as per the manufacturer's protocol at -20 °C until required. When required, the samples were thawed and RNeasy removed. Twenty-five percent of each tissue sample was fixed in formalin and embedded in paraffin for histopathology to confirm that the sample contained the required epithelium. This protocol has been described in detail previously^[21]. There were no buried sub-squamous columnar glands detected by histopathology in any of the biopsies of neosquamous mucosa used in this study. The remaining tissue was used for gene expression analysis. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, United States), and RNA concentration was determined using a Biophotometer (Eppendorf AG, Hamburg, Germany).

Taqman[®] open array[®] Micro-RNA profiling

TaqMan[®] Array Human MicroRNA Card Set (A and B) v3.0 was used to profile the expression of 754 microRNAs^[22]. The extracted RNA was reverse transcribed using Megaplex[™] RT Primers Pool A and B. Each reverse transcription reaction had a final volume of 7.5 µL, and contained 45 ng of total RNA in 3 µL, and 4.5 µL of RT reaction mix containing reverse transcriptase, Megaplex[™] RT Primers Pool A and B, and other reverse transcription agents. The recommended RT thermal cycling conditions were used: (16 °C, 2 min; 42 °C, 1 min; 50 °C, 1 s for 40 cycles); 85 °C, 5 min; 4 °C hold. 2.5 µL of RT product (cDNA) was added to 22.5 µL of PreAmp Reaction mix, containing Megaplex[™] RT Primers Pool A or B and TaqMan[®] PreAmp Master Mix to increase the quantity of cDNA prior to PCR on the Taqman[®] Open Array[®] Micro-RNA Panels. The final volume (25 µL) of preamplification reaction mix underwent the following thermo cycling conditions: 95 °C 10 min; 55 °C, 2 min; 72 °C, 2 min; (95 °C, 15 s, 60 °C, 4 min for 12 cycles), 99.9 °C, 10 min; 4 °C hold. Four microlitres of each preamplified product was diluted in ultrapure water (156 µL) to give a final dilution of 1:40 as per recommended protocol. For the real-time PCR 22.5 µL of Taqman[®] Open Array[®] Real-Time PCR Master Mix was added to 22.5 µL of preamplification product to give a total volume of 45 µL. Five microlitres of each PCR Reaction mix was added to 8 wells on an OpenArray[®] 384-Well-sample

plate. The Taqman[®] Open Array[®] Micro-RNA Panel was loaded with the samples from the 384-Well-sample plate using the standard AccuFill[™] method. Open Array[®] Real-Time qPCR was performed on the loaded Taqman[®] Open Array[®] Micro-RNA Panel using an Open Array[®] Real-Time PCR instrument and recommended software.

Analysis of microRNA expression data

Raw fluorescence data was exported from the Open-Array[®] Real-Time qPCR Analysis Software (BioTrove[™], version 1.0.4) to a comma delimited text file. A Ct (cycle threshold) value was determined for each individual qPCR assay by using the statistical software R (version 3.0.2) to fit a 3-parameter logistic curve, assuming an amplification efficiency of 2, to the raw fluorescence data of each microRNA, and the Ct of each qPCR was determined using the second derivative maximum of the fitted logistic curve. PCR reactions that did not amplify were assigned a Ct value of 40.

To investigate whether any of the samples had low quality data we used the "detector profiling across samples" module in the RealTime PCR Statminer[®] software analysis program (v4.5, Integromics) to examine the correlations of Ct values between samples from the same epithelial tissue type across all of the amplified microRNAs. Samples that had multiple outliers were excluded from further analysis.

For normalisation of the OpenArray[®] microRNA expression data we selected 14 microRNAs using the following criteria: (1) they were expressed in all samples and at high levels (median Ct < 30); (2) they were not statistically different in epithelial tissue type comparisons (Welch's *t*-test, *P* > 0.1); and (3) they were the least variable miRNAs (coefficient of variation < 1.0 for relative levels in each epithelial tissue type). The values for these selection criteria for each of the 14 microRNAs used for normalisation, plus mature nucleic acid sequences and Accession numbers, are presented in Supplementary Table 1.

The relative levels of the microRNAs were determined using the formula $2^{(40-Ct)}$, and were normalized using the geometric mean of the relative levels of the 14 House Keeping Genes. The data was pre-filtered using the following criteria to include microRNAs that were more likely to be informative: (1) each microRNA had to have at least 50% of samples amplified in one of the comparison groups; and (2) the differential expression between groups had to be greater than 1.4 fold. Mann Whitney *U* tests were then used to discover differentially expressed microRNAs in control squamous mucosa vs post-ablation neosquamous mucosa, and in post-ablation squamous mucosa vs post-ablation neosquamous mucosa. False discovery rates (the proportions of false positives) were estimated for each epithelial tissue type comparison. MicroRNAs that had *P* < 0.05 in both of these epithelial tissue type comparisons were termed "overlapping miRNAs".

Subset analyses were subsequently performed for these overlapping microRNAs in 2 sub-groups: (1) patients who were treated either medically or surgically for reflux; and (2) patients in whom complete ablation was achieved vs all patients. This was done by averaging the differential expression and the Mann Whitney *U* test *P* values in each patient subgroup for the overlapping microRNAs. Differences between the groups in (1) differential expression; and (2) Mann Whitney *U* test *P* values were tested using Welch's *t*-test. The statistical methods of this study were reviewed by Professor Richard Woodman from Flinders University.

The overlapping miRNAs were further investigated to compare the direction of differential expression of these microRNAs in post-ablation neosquamous mucosa vs control squamous mucosa and post-ablation squamous mucosa, to the direction of differential expression in Barrett's esophagus mucosa vs control squamous mucosa and post-ablation squamous mucosa. The potential roles of the overlapping miRNAs in regulating cellular processes were investigated using biological pathway enrichment analysis (described in next section).

Biological pathway enrichment analysis of overlapping microRNAs

To identify highly predicted mRNA targets of the differentially expressed microRNAs in neosquamous mucosa, we used the Predicted Target Module of miRWalk v2^[23] (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>). To generate the putative target genes list we used a minimum seed length of 7 and/or *P* value < 0.05, from position 1 of the 3' UTR, and included extra databases: RNA22 (<https://cm.jefferson.edu/rna22/>), miRanda (<http://www.microrna.org/microrna/home.do>) and Targetscan (http://www.targetscan.org/vert_71/). The predicted lists for each microRNA were then screened to identify mRNAs that were predicted to be the targets of at least two different microRNAs.

To identify pathways containing a statistically significant number of predicted targets, we used a publicly available, manually curated signalling pathway database^[24] (<http://www.innatedb.com/redirect.do?go=batchPw>). The target list was subjected to a Pathway Enrichment Analysis which groups target genes according to function, and identifies further components and associated networks. The target list (in REFSeq ID format) was analysed using InnateDB, which identifies statistically enriched pathways by testing for over-representation using the Hypergeometric distribution (by default; other distributions are available), and by using the Benjamini Hochberg correction for multiple tests (by default). InnateDB uses multiple curated databases for the pathway analysis: Reactome (<http://www.reactome.org/>), KEGG (<http://www.genome.jp/kegg/>), PID Biocarta and PID NCI (<http://www.home.ndexbio.org/>), NetPath (<http://www.netpath.org/>), INOH (only

available within InnateDB).

RESULTS

Post-ablation neosquamous vs control squamous mucosa comparison

Forty-four microRNAs were differentially expressed at *P* < 0.05 between control squamous mucosa and post-ablation neosquamous mucosa (Supplementary Table 2). Thirty-three of these microRNAs had higher expression in post-ablation neosquamous mucosa vs control squamous mucosa, and 25 of these had a fold difference greater than 2. There were 11 microRNAs that had lower expression in post-ablation neosquamous mucosa vs control squamous mucosa, although only 2 of these were expressed at levels of 50% or less.

Post-ablation neosquamous vs post-ablation squamous mucosa comparison

Nineteen microRNAs were differentially expressed at *P* < 0.05 between post-ablation neosquamous and post-ablation squamous mucosa (Supplementary Table 3). Fourteen microRNAs had higher expression and 5 microRNAs had lower expression in post-ablation neosquamous mucosa compared with post-ablation squamous mucosa.

MicroRNAs that were different in both control squamous vs post-ablation neosquamous mucosa and post-ablation neosquamous vs post-ablation squamous mucosa

Due to the large number of microRNAs that were assayed it is possible that some differentially expressed microRNAs occurred by chance alone and are thus false positives. We therefore estimated the false discovery rate (FDR) in each epithelial tissue type comparison: in control squamous vs post-ablation neosquamous mucosa the FDR was 11%, and in the post-ablation squamous vs post-ablation neosquamous mucosa the FDR was 19%. The post-ablation squamous vs post-ablation neosquamous comparison could also identify microRNAs associated with differential expression along the length of the esophagus^[25]. In order to address these issues we investigated whether there were microRNAs that were differentially expressed in both epithelial tissue type comparisons. We reasoned that because the control squamous mucosa samples were obtained from different patients to the post-ablation squamous mucosa, any differentially expressed microRNAs found in both mucosal comparisons were much less likely to be due to chance alone. This approach identified 12 microRNAs that were present in both control squamous vs post-ablation neosquamous mucosa and the post-ablation squamous vs post-ablation neosquamous mucosa groups (Table 1). Scatter plots for the 12 overlapping microRNAs are in Supplementary Figure 2. OpenArray assay identifiers, miRBase names and accession numbers, and mature nucleotide sequences for these overlapping microRNAs

Table 1 Fold differences in gene expression and Mann Whitney *U* test *P* values for the microRNAs that were differentially expressed in both post-NS *vs* control-S and post-NS *vs* post-S mucosa comparisons

Mature miRNA	Post-NS/control-S	<i>P</i> value	Post-NS/ Post-S	<i>P</i> value	Higher in pre-BE <i>vs</i> post-NS
miR-424-5p	485.2	0.00002	233.7	0.00053	Yes
miR-135b-5p	2.0	0.00071	1.7	0.00363	No
miR-376c-3p	6.4	0.00145	3.1	0.02673	Yes
miR-135a-5p	3.0	0.00224	2.5	0.00821	Yes
miR-187-3p	208.1	0.00414	2.7	0.01196	No
miR-409-3p	6.8	0.00502	4.6	0.00272	Yes
miR-214-5p	47.0	0.00502	46.1	0.02673	Yes
miR-31-5p	1.44	0.00869	1.5	0.00632	No
miR-199a-5p	396.3	0.01223	512.9	0.04478	Yes
miR-223-5p	230.9	0.02306	204.5	0.01350	Yes
miR-127-3p	4.8	0.02675	3.8	0.03305	Yes
miR-136-3p	7.2	0.02675	201.2	0.03305	Yes

Table 2 Predicted molecular pathways of the mRNA targets of differentially expressed microRNAs that are increased in neosquamous but not Barrett's esophagus mucosa

Pathway name	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway <i>P</i> value	Pathway <i>P</i> value (corrected)
Ion transport by P-type ATPases	5	43	1.57E-05	0.005
Transmembrane transport of small molecules	14	606	1.31E-04	0.022
Ion channel transport	7	169	2.56E-04	0.028
Calcium signaling pathway	7	183	4.15E-04	0.035

Predicted molecular pathways using miRWalk to generate predicted mRNA targets of the microRNAs differentially expressed in both Neosquamous *vs* healthy control and Neosquamous *vs* Proximal squamous comparisons, and InnateDB to identify pathways in which the mRNA targets are over-represented.

are in Supplementary Table 4. All of these microRNAs were more highly expressed in post-ablation neosquamous tissues, and 10 of the 12 overlapping microRNAs had similar fold differences in the two groups. In both comparisons miR-424-5p was the most significantly differentially expressed microRNA (Table 1). We further investigated these overlapping miRNAs in subsets of the data (patients with complete ablation *vs* all patients, and patients who were medically treated *vs* surgically treated for reflux) and did not find significant differences in differential expression between these subset groups (Supplementary Tables 7 and 8).

The 12 overlapping microRNAs were further investigated to determine their levels of expression in pre-ablation Barrett's esophagus mucosa. The expression levels of 9 of the 12 overlapping microRNAs were higher in Barrett's esophagus mucosa than in the squamous mucosa, and for all of these 9 microRNAs their expression in post-ablation neosquamous mucosa was in the same direction (*i.e.*, higher) as in the Barrett's esophagus mucosa (Table 1; Figure 1A for a representative example). For the remaining 3 microRNAs the levels in the Barrett's esophagus mucosa were not different to the non-neosquamous mucosa (Figure 1B for a representative example).

Target prediction and pathway analysis of the overlapping microRNAs

For the 3 overlapping microRNAs that were found not

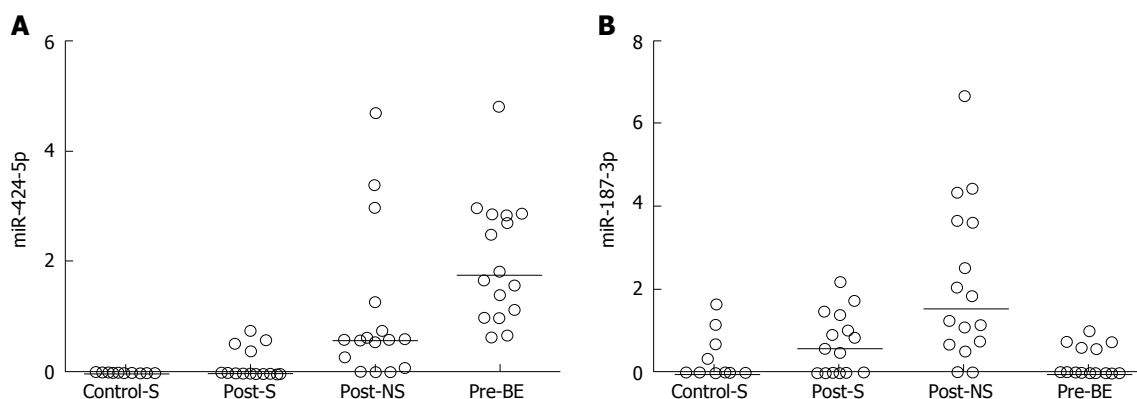
to be increased in Barrett's esophagus mucosa relative to non-neosquamous mucosa mirWalk predicted 1566 mRNA targets, and 163 mRNAs with 2 or more microRNAs targeting them (Supplementary Table 5). Pathway analysis using InnateDB indicated that the predicted target mRNAs are involved in active membrane transport and in calcium signalling (Table 2). For the 9 microRNAs that are increased in both neosquamous and Barrett's esophagus mucosa relative to squamous mucosa mirWalk predicted 3297 mRNA targets, and 839 mRNAs with 2 or more microRNAs targeting them (Supplementary Table 6). Pathway analysis using InnateDB indicated that the predicted target mRNAs are involved in hemostasis and in cell survival pathways (Table 3).

DISCUSSION

Over the last decade, endoscopic treatment of high grade dysplasia and early cancer arising in Barrett's esophagus has largely superseded surgical resection, because of the perception of reduced morbidity, and the lower risk of lymph node metastases when cancer stage is limited to no worse than stage T1a^[26,27]. Consensus guidelines for endoscopic therapy suggest that complete eradication of all Barrett's esophagus mucosa is required to eliminate the risk of metachronous and covert synchronous neoplasia^[28,29]. However, increasing evidence suggests that complete

Table 3 Predicted molecular pathways of the mRNA targets of differentially expressed microRNAs that are increased in both neosquamous and Barrett's esophagus mucosa

Pathway name	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway <i>P</i> value	Pathway <i>P</i> value (corrected)
JAK/STAT pathway and regulation	28	273	5.41E-06	0.005
Hemostasis	41	508	1.68E-05	0.005
Regulation of bad phosphorylation	7	23	2.36E-05	0.006

**Figure 1** Normalised relative expression levels in control squamous mucosa (control-S), post-ablation squamous mucosa (post-S), post-ablation neosquamous mucosa (post-NS) and pre-ablation Barrett's esophagus mucosa (pre-BE) of representative microRNAs, miR-424-5p (A) and miR-187-3p (B). Horizontal bars are medians.

eradication of Barrett's esophagus might not eliminate the risk of cancer in some patients. For example, Templeton *et al.*^[11] (2014) reported three patients who progressed to invasive adenocarcinoma despite prior complete eradication of Barrett's esophagus using endoscopic therapy. Even after re-treatment and complete endoscopic eradication of the post-ablation recurrences of Barrett's esophagus, Guthikonda *et al.*^[12] (2016) reported a progression rate to invasive cancer of 2.1% per year, and predicted that 5.1% (Kaplan Meier model estimate; 95%CI: 0.0-11.3) of re-treated patients would experience invasive cancer progression by 5 years after complete eradication of Barrett's esophagus^[12]. In a meta-analysis of 21 radiofrequency ablation studies that reported 603 cases of Barrett's esophagus recurrence from 3186 patients, pooled incidence ratios (IR's) of recurrent Barrett's esophagus, dysplastic Barrett's esophagus, and HGD/EAC were 9.5% (95%CI: 6.7-12.3), 2.0% (95%CI: 1.3-2.7), and 1.2% (95%CI: 0.8-1.6) per patient-year, respectively^[13]. Ongoing endoscopic surveillance has therefore been recommended after eradication of Barrett's esophagus to monitor for recurrence and disease progression.

It has been suggested that residual sub-squamous glandular tissue after ablation may contribute to the progression of Barrett's esophagus to high grade dysplasia and adenocarcinoma^[30,31]. For example, it has been reported that after argon plasma coagulation ablation buried glandular tissue underneath neosquamous mucosa had higher levels of cancer associated biomarkers (Ki67, COX-2, BCL-2) than

normal esophageal epithelium^[32]. However, Basu *et al.*^[8] (2002) reported that the presence of buried Barrett's glands was not associated with recurrence in patients with effective acid suppression after argon plasma coagulation ablation. Furthermore, neosquamous epithelium has been reported not to contain genetic abnormalities after radio frequency ablation in patients who had pre-ablation Barrett's esophagus containing early cancer or high-grade dysplasia^[9].

In an earlier study we investigated neo-squamous mucosa in patients who had undergone argon plasma coagulation ablation of non-dysplastic Barrett's esophagus, and observed that expression levels of miR-143 were elevated in neosquamous epithelium and biopsies from squamous epithelium above the metaplastic segment, compared to squamous epithelium from controls without Barrett's esophagus^[15]. miR-143 has also been shown to have increased expression in Barrett's esophagus mucosa. This study suggested that post-ablation neosquamous epithelium might not be normal, and might express persistent molecular markers consistent with the original Barrett's esophagus.

In our current study we used post-ablation mucosa from patients who did not have dysplasia or early cancer to further investigate the biology of the neosquamous epithelium, and it is unlikely that these biopsies would have contained residual dysplasia associated biomarkers^[32], or be effected by DNA mutations that are commonly found in dysplastic tissues^[6].

We sought to strengthen our approach by restricting the list of microRNAs to those that are differentially

expressed in both neosquamous vs independent squamous mucosa from control individuals, and in neosquamous vs paired proximal squamous mucosa comparisons. This approach should minimise the issues associated with inter-individual variation, epithelial repair, and tissue proximity to the gastro-esophageal junction as possible causes of differences in microRNA expression. This approach produced 12 microRNAs that were differentially expressed in both of the squamous mucosa comparisons.

To investigate the potential biological effects of the differential expression of these 12 microRNAs we utilized miRWalk to predict their mRNA targets, and InnateDB to assess which potential signalling pathways these mRNAs are involved in. This approach identified signalling pathways which the differentially expressed miRNAs might be regulating.

Nine microRNAs were expressed at higher levels in both neosquamous and pre-ablation Barrett's mucosa, vs both squamous mucosa from controls and proximal squamous mucosa collected after ablation from the ablation patients. The predicted mRNA targets of these microRNAs are involved in the JAK-STAT signalling pathway, and in the regulation of the anti-apoptotic family member, Bad. Three of the discovered microRNAs have been reported to have targets involved in hemostasis^[33-35].

The JAK-STAT signalling pathway transmits information from extracellular signals directly to the cell nucleus, and results in expression of genes involved in proliferation, differentiation, apoptosis and oncogenesis. The JAK-STAT3 pathway is activated by IL-6 in Barrett's esophagus, and this promotes survival of the metaplastic intestinal cells^[36,37]. However, activated JAK-STAT has been reported to be undetectable in normal esophageal squamous mucosa^[38], so the effect of altered regulation of this pathway in neosquamous mucosa is not clear.

Three microRNAs were increased in neosquamous, but not pre-ablation Barrett's esophagus mucosa, vs both healthy squamous and post-squamous mucosa. The predicted mRNA targets of these microRNAs are potentially involved in regulating pathways involved in transmembrane transport of small molecules, in ion channel transport, and in ion and lipid transport by P-type ATPases (which includes the calcium pump, Ca²⁺-ATPase). Jovov *et al*^[39] (2013) found that post-ablation neosquamous epithelium has decreased barrier function, measured as persistent paracellular permeability to ions and uncharged molecules^[39], so these active transporter targets are likely to be involved in the regulation of barrier protection. This is an important consideration because acid reflux injury has been implicated in the development of intestinal metaplasia, and decreased barrier function may therefore contribute to recurrence in patients with uncontrolled reflux following successful ablation.

The mucosal biopsies used in this study were

from patients treated with Argon Plasma Coagulation ablation. These were collected as part of clinical trials previously established in our institution and were therefore readily available to us^[20]. The use of biopsies from patients treated with argon plasma coagulation ablation is a potential limitation of our study, since radiofrequency ablation is now the standard technique for ablation of Barrett's esophagus due to its ease of use and consistent depth of tissue destruction^[40]. However, some patients in whom complete eradication has been achieved following radiofrequency ablation have still progressed to invasive cancer, which suggests that post radiofrequency ablation neosquamous epithelium may also not be normal. It is worth noting that the above described report of decreased barrier function in neosquamous mucosa was associated with radiofrequency ablation^[39]. Future studies of neosquamous epithelium miRNA profile should therefore include other endoscopic methods such as radiofrequency ablation and endomucosal resection, and validation in independent cohorts.

The observed decrease in barrier function in neosquamous mucosa is consistent with reports of an association between recurrence after ablation and persistent reflux, and with reduced proton pump inhibitor dosing. Kahaleh *et al*^[41] (2002) found that persistence of acid reflux and greater length of diseased segment were the major factors associated with a recurrence after successful initial reversal with argon plasma coagulation ablation. Basu *et al*^[8] (2002) reported that patients who reduced their dose of the proton pump inhibitor omeprazole to 20 mg once daily or less after argon plasma coagulation ablation had significantly greater recurrence of intestinal metaplasia^[8]. Conversely, in patients with complete squamous regeneration after argon plasma coagulation ablation who took a high dose of omeprazole (40 mg three times a day) there were no relapses or evidence of dysplasia under continuous acid suppression during a median follow-up of 12 mo (range 2 to 51 mo)^[42].

Three (miR-424-5p, miR-223-5p, miR-409-3p) of the microRNAs that are increased in neosquamous mucosa relative to post-squamous and control-squamous mucosa have been reported to be up-regulated in esophageal adenocarcinoma relative to Barrett's esophagus and normal squamous tissues. Wu *et al*^[18] (2013) observed progressively increased expression of miR-424-5p, miR-223-5p and miR-409-3p from normal squamous epithelium to Barrett's to adenocarcinoma. MiR-223-5p and miR-409-3p have also been reported to be overexpressed in serum exosomes from patients with esophageal adenocarcinoma^[43].

In conclusion, this study demonstrates that the miRNA expression profile in neosquamous mucosa, following argon plasma coagulation ablation of Barrett's esophagus, is significantly different from normal squamous mucosa. The main strength of this study is that the mucosal

biopsies were not from patients who were treated for dysplasia or cancer. Our results suggest that altered miRNA expression may contribute to the previously reported defective barrier function in neosquamous epithelium, and this may place the mucosa at increased risk of disease progression relative to normal esophageal squamous mucosa. Further research to explore the roles of miRNAs in the response to ablation of Barrett's esophagus, and the long term behaviour of neosquamous epithelium may lead to improvements in clinical management of this condition.

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COMMENTS

Background

Overall 5 year survival of patients with esophageal adenocarcinoma is around 15%, but survival can be improved via early detection using endoscopic surveillance of patients with Barrett's esophagus, and endoscopic ablative therapy for the treatment of early stage disease.

Research frontiers

Some patients still experience disease progression after complete ablation of Barrett's esophagus, which suggests that the neosquamous mucosa formed after ablation may be at increased risk of disease progression. Improved understanding of the biological properties of post-ablation neosquamous mucosa might improve the clinical management of patients who undergo ablative therapy.

Innovations and breakthroughs

Previous studies have investigated whether post ablation mucosa has genetic alterations in, or increased expression of cancer associated genes. Neither of these approaches directly addresses whether the non-cancer associated biology of neosquamous mucosa is different. Previous studies have reported reduced barrier function in neosquamous mucosa, and this may have implications for clinical management.

Applications

Altered microRNA expression in neosquamous mucosa might result in reduced barrier function, thereby placing the mucosa at increased susceptibility to reflux induced disease. MicroRNAs might therefore have the potential to be developed into a biomarker with clinical utility to improve the management of patients who have been treated endoscopically for early stage disease.

Terminology

Post-ablation neosquamous mucosa: post-treatment regenerated esophageal squamous mucosa that was Barrett's intestinal metaplasia prior to treatment, with biopsies taken 1 cm above the gastro-esophageal junction. Post-ablation squamous mucosa: post-treatment proximal normal non-treated esophageal mucosa, with biopsies collected 5 cm above the proximal margin of the pre-ablation Barrett's mucosa. Healthy squamous mucosa: squamous mucosa from patients without esophageal disease, with biopsies taken from the same level relative to the gastro-esophageal junction as the post-ablation neosquamous mucosa.

Peer-review

This manuscript evaluates a topic of real interest. The analysis of the subject has been done in an appropriate way. The background of the problem was evaluated in a comprehensive way, the hypothesis was clearly stated, and the

materials, methods and results are presented in an understandable way (I will add minor comments).

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