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In GERD patients, mucosal repair associated genes are upregulated in non-inflamed oesophageal epithelium

D. R. de Vries [a, *], J. J. M. Ter Linde [a], M. A. van Herwaarden [b], M. P. Schwartz [c], P. Shephard [d], M. M. Geng [d], A. J. P. M. Smout [a], M. Samsom [a]

[a] Department of Gastroenterology and Hepatology, University Medical Center, Utrecht, the Netherlands
[b] Department of Gastroenterology and Hepatology, Deventer Hospital, Deventer, the Netherlands
[c] Department of Gastroenterology and Hepatology, Meander Medical Center, Amersfoort, the Netherlands
[d] Nycomed Pharma AG, Konstanz, Germany

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Abstract

Previous studies addressing the effects of acid reflux and PPI therapy on gene expression in oesophageal epithelium concentrated on inflamed tissue. We aimed to determine changes in gene expression in non-inflamed oesophageal epithelium of GERD patients. Therefore, we included 20 GERD patients with pathological total 24-hr acid exposure of 6–12% and SAP $\geq$ 95%. Ten patients discontinued PPI treatment (PPI–), 10 took pantoprazole 40 mg bid (PPI+). Ten age/sex-matched healthy controls were recruited. Biopsies were taken from non-inflamed mucosa 6 cm and 16 cm proximal to the squamocolumnar junction (SCJ). Gene expression profiling of biopsies from 6 cm was performed on Human Genome U133 Plus 2.0 arrays (Affymetrix). Genes exhibiting a fold change >1.4 (t-test *P*-value $< 1^{E} - 4$) were considered differentially expressed. Results were confirmed by real-time RT-PCR. In PPI– patients, 92 microarray probesets were deregulated. The majority of the corresponding genes were associated with cell–cell contacts, cytoskeletal reorganization and cellular motility, suggesting facilitation of a migratory phenotype. Genes encoding proteins with anti-apoptotic or anti-proliferative functions or stress-protective functions were also deregulated. No probesets were deregulated in PPI+ patients. QPCR analysis of 20 selected genes confirmed most of the deregulations in PPI– patients, and showed several deregulated genes in PPI+ patients as well. In the biopsies taken at 16 cm QPCR revealed no deregulations of the selected genes. We conclude that upon acid exposure, oesophageal epithelial cells activate a process globally known as epithelial restitution: up-regulation of anti-apoptotic, anti-oxidant and migration associated genes. Possibly this process helps maintaining barrier function.

Keywords: gastroesophageal reflux disease • microarray expression profiling • epithelial restitution • oesophagus • oesophageal epithelial defense

Introduction

Gastrooesophageal reflux disease (GERD) is very common in the western world with 10–20% of the population suffering from reflux symptoms at least weekly [1, 2]. Visible mucosal breaks in the oesophageal lining, facilitating access of refluxate to submucosal nociceptive nerve endings provide an accepted explanation for symptom generation in GERD patients with oesophagitis. However, a substantial number of patients with typical GERD symptoms does not show any macroscopic signs of damage to the oesophageal lining and can be classified as non-erosive reflux disease (NERD) patients. A recent report, investigating 999 subjects from the general population, showed that in subjects with daily reflux symptoms, prevalence of oesophagitis was only 35.6% [3]. In GERD patients with and without oesophagitis, visceral hypersensitivity has been demonstrated in non-inflamed areas [4]. This could be explained by central sensitization of nociceptive pathways [5]. Furthermore, epithelial cells under acid stress may secrete neuroinflammatory substances that can sensitize nociceptive nerve endings peripherally, and cause visceral hypersensitivity in GERD patients with and without oesophagitis [6].

Despite the fact that only a minority of GERD patients have oesophagitis, studies involving molecular analysis of oesophageal epithelium in GERD have, to date, focused mainly on inflamed tissue.

*Correspondence to: D. R. DE VRIES,
Department of Gastroenterology and Hepatology,
University Medical Center, Utrecht, PO Box 85500,
3508 GA Utrecht, the Netherlands.
Tel.: +3188 7555555
E-mail: d.r.devries@umcutrecht.nl

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In inflamed mucosa, upregulation of cytokines and other pro-inflammatory gene products has been found, *e.g.* interleukin-8 (IL-8), cyclooxygenase-2 (Cox-2) and nuclear factor kappa B (NF-κB) [7–11]. Interestingly, Isomoto *et al.* found upregulated expression and increased content of IL-8 and NF-κB in non-inflamed oesophageal mucosal biopsies of NERD patients as well [12].

Inflammatory processes, however, are not the initial reaction of oesophageal epithelium to acid. Non-inflamed oesophageal epithelium possesses a number of defence mechanisms against acid. Inside the epithelial layer, structural and functional defences provide protection against damage by reflux, *e.g.* the different junctional complexes between cells (tight junctions (TJs), adhering junctions (AJs) and desmosomes), intercellular glycoconjugates with buffering properties and the epithelial transport proteins that regulate pH and buffering [13]. At a light microscopic level, macroscopically non-inflamed epithelium from GERD patients can display various characteristics, including submucosal papillary elongation, basal layer hyperplasia, infiltration of inflammatory cells, glycogenic acanthosis, hyperemia of the submucosa, thickening of the basement membrane and dilated intercellular spaces [14, 15].

The extent of acid exposure may affect the transcriptional response of the oesophageal epithelium. This assumption can be addressed by studying the effect of PPI therapy on transcription. So far, changes in mRNA expression resulting from PPI therapy have only been determined in oesophageal epithelium containing inflitrates of inflammatory cells [12, 11]. Furthermore, insight into the effect of the extent of acid exposure on mRNA expression may be gained by comparing proximal and distal transcription in the oesophagus. Weusten *et al.* showed that acid exposure to the oesophageal lining decreases dramatically when a pH-probe is positioned more proximally in the oesophagus. This was shown in healthy volunteers and GERD patients [16, 17].

This study aimed, therefore, to investigate the influence of acid reflux on gene expression in non-inflamed oesophageal mucosa of GERD patients with pathological oesophageal acid exposure, using genome-wide mRNA expression analysis.

Materials and methods

Patients

From the patients visiting the gastroenterology department at our hospital with recurrent heartburn, acid regurgitation and/or non cardiac chest pain, for at least 2 days per week, lasting 3 months or more, for whom diagnosis of GERD was established by 24-hrs oesophageal pH recording, 20 consecutive patients characterized by a total oesophageal acid exposure time between 6% and 12% were approached.

Patients with severe concomitant diseases, prior oesophageal or gastric surgery, oesophagitis C or D or Barrett's oesophagus, peptic ulcer disease and comorbid conditions that might interfere with oesophageal or gastric motility including diabetes mellitus, systemic sclerosis and neurological disorders were non-eligible.

Ten patients discontinued any acid suppressing drugs for the duration of 2 weeks prior to endoscopy and sampling (PPI–). These patients were permitted to take antacids to alleviate unbearable symptoms with the exception of the 24 hrs directly preceding endoscopy. They marked their antacid use on a diary card. The remaining 10 patients were prescribed a fixed PPI dose for 2 weeks prior to upper GI-endoscopy (PPI+) (pantoprazole 40 mg bid) to ensure maximum acid suppression in this group. These patients were randomly assigned to either of the groups, in order of inclusion.

Healthy controls

An advertisement was placed in a local newspaper, and from the people who reacted 10 age- and sex-matched healthy controls free of gastrointestinal symptoms or a history of gastrointestinal disease were included. In conformity with their medical history, none of these subjects had undergone endoscopy before. Should a hiatal hernia or any lesions in the oesophagus, stomach or duodenum be found during upper GI endoscopy healthy controls were to be excluded.

Questionnaires

All patients completed a questionnaire assessing reflux symptoms (heartburn, regurgitation, retrosternal pain and belching) in the 2 weeks prior to endoscopy, modeled after the validated Nepean symptom score [18].

Sample collection

All subjects underwent oesophago-gastro-duodenoscopy, all endoscopies were performed by the same gastroenterologist. Six mucosal biopsies were collected at 6 and 16 cm proximal, respectively, from the squamocolumnar junction (SCJ) (reusable biopsy forceps, 2.2 mm oval cup with spike, Fujinon Medical Holland b.v., Veenendaal, The Netherlands). The biopsy samples were lifted from the forceps with a sterile hypodermic needle. Two biopsies from each location were placed in a sterile 2 ml microcentrifuge tube (Eppendorf, Germany), snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. The remaining two biopsies were fixated in formaldehyde solution for histopathologic evaluation.

RNA extraction

Frozen biopsies were disrupted and homogenized with the Omni μH rotor-stator homogenizer in RLT buffer and subsequently total RNA was extracted using Qiagen RNeasy microkit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Integrity of the samples was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) for distinct 18S and 28S rRNA peaks.

Microarray hybridization

RNA amplification and labelling

The RNA extracted from the biopsies acquired at 6 cm proximal to the SCJ were used for Affymetrix GeneChip hybridization. (Specifications in Appendix S1.)

Table 1 Characteristics of healthy controls (HC), GERD patients off acid suppressive medication (PPI−) and GERD patients using maximum PPI dose (PPI+) 2 weeks prior to endoscopy

	N	Male	Age	Acid exposure (%)*	Esophagitis (N)	Symptoms (all)†	Heartburn, pain‡	Belching, regurgitation
HC	10	5	49 (37–63)	N/A	0	N/A	N/A	N/A
PPI−	10	6	46 (25–67)	7.1 (5.7–9.9)	7	37 (12–57)	21 (4–32)	16 (0–27)
PPI+	10	4	56 (36–75)	9.3 (6.1–11.5)	4	21 (0–44)	10 (0–23)	11 (0–23)

Age, acid exposure and symptoms are displayed as mean (range). Symptom scores cover the 2 weeks prior to endoscopy and sample collection. N/A: not applicable. *$P = 0.005$; †$P = 0.042$; ‡$P = 0.017$.

Quantitative real-time reverse transcriptase polymerase chain reaction (QPCR)

RNA extracted from biopsies taken at both locations was used for QPCR expression analysis. QPCR reactions were performed using TaqMan® low-density arrays (LDAs) (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM® 7900HT Sequence Detection System (AME Bioscience A/S, Norway). (Specifications in Appendix S1.)

Reference sample

A pooled reference cDNA sample was synthesized using 200 ng of RNA from five healthy controls for use in the normalization calculations, and profiled on the LDAs in the same session of runs as the rest of the samples.

Normalization of PCR data using housekeeping genes

To permit comparison between samples, several housekeeping genes were included in the low-density array to correct for variations in mRNA quality and quantity. These housekeeping genes were chosen after reviewing respective expression values as derived from the microarrays for several well-described housekeeping genes. The housekeeping genes we used were ACTB (beta actin), HMBS (hydroxymethylbilane synthase) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). These genes displayed a stepwise difference in expression level and were not differentially expressed between any of the groups. Housekeeping gene performance was further characterized using the M-value method with the Genorm software package (medgen.ugent.be/genorm) described by Vandesompele *et al.* [19].

Statistical analysis

Comparison of subject characteristics

Patient characteristics and questionnaire scores were compared between the two groups using Student's t-test or Chi-square tests as appropriate, considering a *P*-value of <0.05 statistically significant.

Computational analysis of microarray data

Affymetrix raw data (CEL files) were analyzed using the Gene Data Expressionist Pro software package in version 2.0 (Gene Data, Basel, Switzerland). (Specifications in Appendix S1.)

Analysis of low-density array data

TaqMan® LDA results were analyzed with the SDS 2.2.1 software package using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [20]. The resulting relative quantities (RQs) were compared between groups using one-way analysis of variance (ANOVA) with Dunnett's post-hoc tests. In comparing RQs a *P*-value of <0.05 was considered statistically significant and a *P*-value of <0.10 was considered a trend towards significance.

Results

Subjects

The characteristics of the patients and controls are shown in Table 1. A total of 14 healthy controls underwent endoscopy, four healthy controls were excluded upon finding abnormalities (2 grade A oesophagitis, 1 grade B oesophagitis, 1 Barrett's epithelium).

Age and sex did not differ significantly between the healthy controls and both patient groups and between the patient groups. As expected, PPI– patients had more symptoms in total and especially more heartburn and retrosternal pain than the PPI+ group during the 2 weeks prior to biopsy collection. Regurgitation and belching, symptoms that are not treated by acid suppression, were equally prevalent in both patient groups. Oesophagitis prevalence did not differ between the PPI– and PPI+ groups. Histologic evaluation confirmed the absence of inflammation in all biopsy locations in all subjects. In three subjects from both patient groups, some infiltrating inflammatory cells were observed, however, in insufficient numbers to qualify as inflammation. Furthermore in some biopsy specimens stromal papillae were close to the mucosal surface, or mild glycogenic acanthosis was present. These findings were equally dispersed between the PPI– and the PPI+ groups. In the healthy controls group one biopsy showed mild glycogenic acanthosis and some lymphocytes.

Differentially expressed genes

Microarray mRNA expression profiling

The results from the microarray mRNA expression profiling are summarized in Table 2. The raw data are available as CEL files

Table 2 Microarray results, GERD patients off PPI compared to healthy controls

Gene	Description of gene function	Affymetrix probe set	Fold change
	Genes with functions in cytoskeletal rearrangements and cellular motility		
ARF6	ADP-ribosylation factor 6	214182_at	1.66
ARFGEF1	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)	216266_s_at	1.42
MTPN	Myotrophin	223925_s_at	1.55
NCKAP1	NCK-associated protein 1	217465_at	1.62
PLD1	phospholipase D1, phosphatidylcholine-specific	215723_s_at	1.40
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	203736_s_at	1.45
RPS6KB1	ribosomal protein S6 kinase, 70 kDa, polypeptide 1		
LOC729334	similar to ribosomal protein S6 kinase, polypeptide 1	211578_s_at	1.46
LOC731896			
SLC39A6	solute carrier family 39 (zinc transporter), member 6	1556551_s_at	1.42
TUBGCP3	tubulin, gamma complex associated protein 3	203690_at	1.71
WASF2	WAS protein family, member 2		
WASF4	WAS protein family, member 4	224563_at	–1.43
LOC647909	similar to WASP-family protein member 4		
ZNF655	zinc finger protein 655	1554726_at	1.49
	Genes with functions in cell–cell contacts		
BRMS1L	breast cancer metastasis-suppressor 1-like	224484_s_at	1.72
MPP7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	1564308_a_at	1.60
MYCBP2	MYC binding protein 2	201960_s_at	1.56
PVRL4	poliovirus receptor-related 4	223540_at	1.44
SPHK2	sphingosine kinase 2	40273_at	1.67
DBP	D site of albumin promoter (albumin D-box) binding protein		
	Genes with generic functions in epithelial restitution		
ADSS	adenylosuccinate synthase	221761_at	1.55
CSNK1A1	casein kinase 1, alpha 1	208866_at	1.49
MAML3	mastermind-like 3 (Drosophila)	242794_at	1.84
	Genes with anti-apoptotic functions		
AKAP1	A kinase (PRKA) anchor protein 1	210625_s_at	1.51
		201674_s_at	1.41
CSNK1A1	casein kinase 1, alpha 1	208866_at	1.49
ETFDH	electron-transferring-flavoprotein dehydrogenase	33494_at	1.49
PPID	peptidylprolyl isomerase D (cyclophilin D)	205530_at	1.44
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	214056_at	1.68
RBMS1	RNA binding motif, single stranded interacting protein 1	207266_x_at	–1.48
		209868_s_at	–1.50
TANK	TRAF family member-associated NFKB activator	210458_s_at	1.65
		207616_s_at	1.46
	Genes with anti-proliferative functions		
BRMS1L	breast cancer metastasis-suppressor 1-like	224484_s_at	1.72
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	212515_s_at	1.43

Continued

Table 2 Continued

Gene	Description of gene function	Affymetrix probe set	Fold change
IRF6	interferon regulatory factor 6	1552478_a_at	1.49
KPNB1	karyopherin (importin) beta 1	208975_s_at	1.65
		208974_x_at	1.64
		213507_s_at	1.45
RPL29	ribosomal protein L29	213969_x_at	–1.44
		200823_x_at	–1.46
	Genes with functions protective against oxidative stress		
ALDH4A1	aldehyde dehydrogenase 4 family, member A1	203722_at	1.51
ALDH7A1	Antiquitin; aldehyde dehydrogenase 7 family, member A1	208951_at	1.49
FAHD1	fumarylacetoacetate hydrolase domain containing 1	227960_s_at	1.44
HAGH	hydroxyacylglutathione hydrolase		
	Genes with aspecific or unknown functions		
AGTPBP1	ATP/GTP binding protein 1	204500_s_at	1.47
ASNSD1	asparagine synthetase domain containing 1	217987_at	1.54
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	207573_x_at	–1.40
UBE4A	ubiquitination factor E4A (UFD2 homolog, yeast)	210453_x_at	–1.47
		208746_x_at	–1.49
ATP8B1	ATPase, Class I, type 8B, member 1	214594_x_at	1.42
ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)	208859_s_at	1.64
LOC728849	similar to transcriptional regulator ATRX isoform 1		
C10orf119	chromosome 10 open reading frame 119	222464_s_at	1.66
C12orf29	chromosome 12 open reading frame 29	213701_at	1.85
C14orf2	chromosome 14 open reading frame 2	210532_s_at	–1.45
CCDC47	coiled-coil domain containing 47	217814_at	1.54
CDV3	CDV3 homolog (mouse)	213548_s_at	1.50
COPA	coatomer protein complex, subunit alpha	208684_at	1.56
CTSB	cathepsin B	227961_at	1.44
DBT	dihydrolipoamide branched chain transacylase E2	205371_s_at	1.59
EIF3J	eukaryotic translation initiation factor 3, subunit J	208985_s_at	1.46
EIF4A2	eukaryotic translation initiation factor 4A, isoform 2	1555996_s_at	1.70
ELF1	E74-like factor 1 (ets domain transcription factor)	233931_at	1.47
FASTKD5	FAST kinase domains 5	219016_at	1.46
UBOX5	U-box domain containing 5		
GNG5	guanine nucleotide binding protein (G protein), gamma 5	207157_s_at	–1.43
CTBS	chitobiase, di-N-acetyl-		
GNL3L	guanine nucleotide binding protein-like 3 (nucleolar)-like	205010_at	2.30
KIAA0256	KIAA0256 gene product	212451_at	–1.58
LOC400506	similar to TSG118.1	213237_at	1.44
MOV10	Mov10, Moloney leukemia virus 10, homolog (mouse)	223849_s_at	1.40
NIPBL	Nipped-B homolog (Drosophila)	207108_s_at	1.46

Table 2 Continued

Gene	Description of gene function	Affymetrix probe set	Fold change
PDE4DIP	phosphodiesterase 4D interacting protein (myomegalin)	214130_s_at	1.59
LOC727942	similar to phosphodiesterase 4D interacting protein isoform 2		
PITPNA	phosphotidylinositol transfer protein, alpha	201191_at	1.48
PRPF39	PRP39 pre-mRNA processing factor 39 homolog (yeast)	220553_s_at	1.41
PURB	purine-rich element binding protein B	225120_at	1.52
RETSAT	retinol saturase (all-transretinol 13,14-reductase)	1566472_s_at	1.40
RNF10	ring finger protein 10	237062_at	1.68
RNMTL1	RNA methyltransferase like 1	218993_at	1.46
RPE	ribulose-5-phosphate-3-epimerase	216574_s_at	1.40
LOC649755	similar to Ribulose-5-phosphate 3-epimerase (HUSSY-17)		
RPL30	ribosomal protein L30	200062_s_at	–1.44
SNORA72	small nucleolar RNA, H/ACA box 72		
RPL37A	ribosomal protein L37a	201429_s_at	–1.41
RPS13	ribosomal protein S13		
SNORD14A	small nucleolar RNA, C/D box 14A	200018_at	–1.42
SNORD14B	small nucleolar RNA, C/D box 14B		
RPS18	ribosomal protein S18	201049_s_at	–1.40
RP5-1033B10.18	similar to ribosomal protein S18		
RPS24	ribosomal protein S24	200061_s_at	–1.46
RTF1	Rtf1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	212302_at	1.56
SAMD8	sterile alpha motif domain containing 8	225950_at	1.87
SAPS3	SAPS domain family, member 3	228105_at	1.62
SART3	squamous cell carcinoma antigen recognised by T cells 3	209127_s_at	1.68
SH3RF2	SH3 domain containing ring finger 2	228892_at	1.47
SLAIN2	SLAIN motif family, member 2	233230_s_at	1.47
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	223197_s_at	1.45
TCF25	transcription factor 25 (basic helix-loop-helix)	221495_s_at	1.44
TIMM8B	translocase inner mitochondrial membr. 8 homolog B (yeast)	218357_s_at	–1.41
TTF1	transcription termination factor, RNA polymerase I	204772_s_at	1.53
USP24	ubiquitin specific protease 24	212381_at	1.47
ZFYVE16	zinc finger, FYVE domain containing 16	203651_at	1.58
ZKSCAN1	zinc finger with KRAB and SCAN domains 1	1557953_at	1.50
ZNF33A	zinc finger protein 33a (KOX 31)	231864_at	1.45
AK096729		1553979_at	1.45
AK092090			
BC015866		1555461_at	1.65

Results are arranged according to function, with the gene names in alphabetical order. For the sake of completeness, genes that have more than one function as represented in this table are mentioned more than once. Grey and white lines belong to different sets of results: either one gene was recognized by multiple probe sets, *e.g.* AKAP1, or one probe set could have recognized more than one gene, *e.g.* ATRX/LOC728849. In the case of ATP5L/UBE4A, two genes could both have been recognized by three probesets.

online at the NCBI Gene Expression Omnibus (GEO) page. Genes differentially expressed relative to healthy controls were found in the biopsies from PPI– patients, but no genes were significantly differentially expressed in the PPI+ patients. Among the differentially expressed genes, groups were discerned with functions that could be tied to cytoskeletal rearrangements and cellular motility or cell–cell contacts.

Cytoskeletal rearrangements and cellular motility

The cytoskeleton, consisting of actin, intermediate and tubulin filaments anchored to cell membrane and organelles, is a dynamic structure that enables cellular motion. Several genes with functions in modulating actin or tubulin towards increased cellular motility were found to be differentially expressed. The functions of these genes are specified in Appendix S1.

Cell–cell contacts

Migration is accompanied by changes in cell–cell contacts. Intercellular adhesion is mediated by junctional complexes, consisting of the apical tight junctions (TJs), essential for epithelial barrier function, the subapical adherens junctions (AJs) and the basolateral desmosomes. Junctional complexes are linked to cytoskeletal filaments. AJs have a critical role both as sensors of extracellular stimuli and in regulating the dynamics of epithelial cell layers. Several genes with functions in cell–cell contacts were deregulated, they are specified in Appendix S1.

Epithelial restitution

Cytoskeletal rearrangements, migration and regulation of intercellular adhesion can be linked to a process known as epithelial restitution. This is a repair mechanism whereby epithelial continuity is restored and barrier function maintained after superficial injury to the mucosa [21, 22]. During the process of restitution, cells bordering the zone of injury undergo junctional disassembly and flatten. This is viewed as a shift to a migratory phenotype driven by cytoskeletal rearrangements. The flattened migratory epithelial cells spread forward by extending pseudopod-like structures known as lamellipodia, thereby covering the defect in the epithelial layer. Subsequently, cell–cell contacts are re-established and normal cell shape and phenotype is retained. It has been shown that gap junctional intercellular communication is involved in gastric mucosal restitution following acid-induced injury [23]. Gap junctions are membrane-spanning channels composed of connexins that allow small signalling molecules to pass from cell to cell. Phosphorylation of connexins, mediated by one of the upregulated genes (CSNK1A1) [24], has been implicated in the regulation of gap junctional communication.

The MAML3 gene (mastermind-like 3) codes for a positive regulator of the Notch signalling pathway, which mediates cell-cell communications required for cell fate decisions [25].

Epithelial restitution is a highly energy-dependent process. This might explain the upregulation of adenylosuccinate synthetase (ADSS), an enzyme essential in ATP production. Accordingly, this enzyme was reported to be upregulated in the healing edges of epithelial wounds [26].

Anti-apoptotic genes

Epithelial restitution is independent of cell proliferation, cell survival however is essential. Several genes with anti-apoptotic functions were upregulated. This may ensure the survival of cells until epithelial restitution is completed.

The upregulated CSNK1A1 [27] and PPID [28] have anti-apoptotic effects, whereas the pro-apoptotic gene RBMS1 was downregulated [29]. AKAP1 targets protein kinase A to mitochondria, consequently PKA-dependent phosphorylation and thereby inactivation of proapoptotic protein BAD is increased and cell survival enhanced [30]. TANK has anti-apoptotic effects by modulating NF-κB activation [31, 32]. MCL1 is a gene of which the N-terminus determines its function: the protein product with the extended N-terminus has strong anti-apoptotic properties and a mild anti-proliferative effect, whereas the protein product with the N-terminal part deleted has a potent anti-proliferative effect whereas the anti-apoptotic function is less pronounced [33].

Anti-proliferative genes

Several genes with anti-proliferative and/or pro-differentiation properties were found. BRMS1L and DDX3X inhibit cell growth [34, 35], KPNB1 negatively regulates mitotic spindle formation [36, 37] and IRF6 keeps the cell in the G_0 stage of the cell cycle, thus simultaneously promoting differentiation of the cell instead of proliferation [38]. In addition, downregulation of RPL29/HIP is associated with differentiation [39].

Genes with protective roles against oxidative stress

Excessive gastrooesophageal reflux is associated with enhanced production of reactive oxygen species (ROS) [40]. Upregulation of ALDH4A1, which has the capacity to reduce ROS generation [41], may protect against reflux-induced oxidative stress. Peroxidation of membrane lipids is one of the mechanisms by which ROS lead to cell damage. Upregulation of ALDH7A1 and HAGH, which function in detoxification including lipid peroxidation products [42, 43], is expected to increase the survival chance of damaged cells.

Two other potentially interesting genes were upregulated with functions independent from epithelial restitution.

COPA encodes the α-subunit of the coatomer protein complex, involved in intracellular protein transport [44]. The N-terminal amino acids of α-COP are identical to xenin, a neurotensin receptor agonist. Xenin can be cleaved from α-COP by aspartic proteinases such as pepsin [45]. Interestingly, generation of xenin from its large precursor α-COP increases with acidic pH and exogenous administration of neurotensin reduces LES

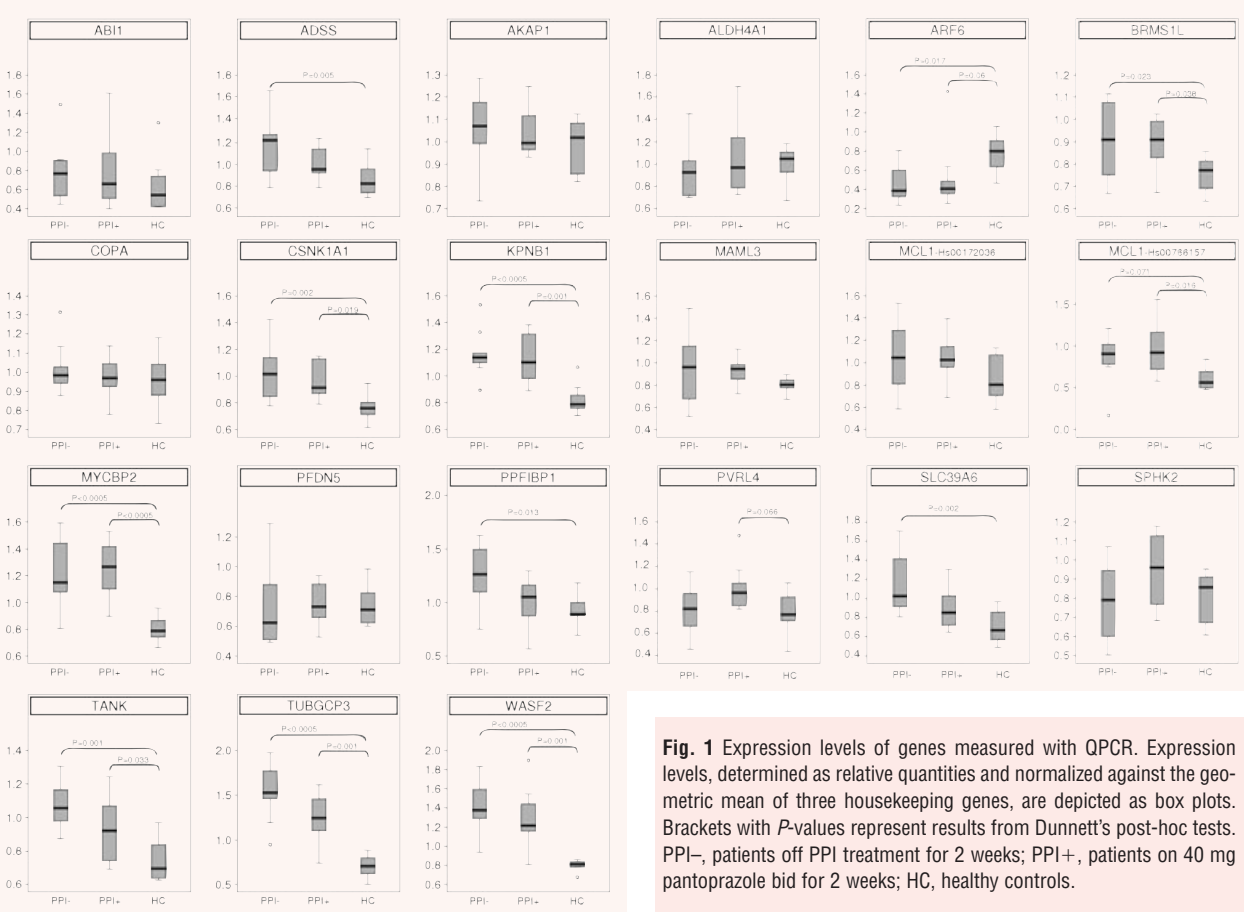

Fig. 1 Expression levels of genes measured with QPCR. Expression levels, determined as relative quantities and normalized against the geometric mean of three housekeeping genes, are depicted as box plots. Brackets with *P*-values represent results from Dunnett's post-hoc tests. PPI–, patients off PPI treatment for 2 weeks; PPI+, patients on 40 mg pantoprazole bid for 2 weeks; HC, healthy controls.

pressure [46]. Xenin levels have not been quantified in the oesophagus to date.

ATP8B1 is a flippase, an enzyme active in restoring membrane symmetry [47], a process that may be involved in maintaining membrane integrity following superficial damage.

TaqMan® low-density array validation

Differential expression of 18 genes with putative functions in epithelial restitution was verified using QPCR. The selection of genes covers each of the different aspects of this process described above: cell–cell contacts (CSNK1A1, MAML3, PVRL4), cytoskeletal alterations and cellular motility (Abi-1, ARF6, MYCBP2, PFDN5, PPFIBP1, SLC39A6, SPHK2, TUBGCP3, WASF2), anti-apoptosis (AKAP1, CSNK1A1, MCL1, TANK), anti-proliferation (BRMS1L, KPNB1), and energy supply (ADSS). In addition, ALDH4A1 and COPA were included in the LDA setup.

Relative quantities of the transcripts are depicted as box plots with post-hoc test *P*-values in Fig. 1. Eleven genes showed significantly different mRNA expression levels between GERD patients off PPI relative to healthy controls and two genes showed a trend towards significance.

In contrast to the microarray results, in the PPI+ group nine genes were found to be differentially expressed as well, and one gene showed a trend towards significance. Most of the differences, however, were smaller than those in the PPI– group.

The gene MCL1 was represented by two assays on the LDA, because the affymetrix probe set for MCL1, which was detected as significantly different, recognizes two alternative gene products with different functions. Hs00172036_m1 measures the predominantly antiproliferative transcript without the N-terminal end, and Hs00766187_m1 measures the predominantly anti-apoptotic transcript with the N-terminal end intact. Only the latter was differentially expressed in the PPI+ group and showed a trend towards differential expression in the PPI– group.

In addition mRNA from the higher biopsy location, at 16 cm proximal to the SCJ, was subjected to LDA analysis, thereby investigating the expression levels of the selected genes in mucosa that had been exposed to substantially lower amounts of acid [16]. No differential expression of any of the 20 genes was, however, detected at this location in the oesophagus.

Discussion

The most important finding of the current study is that in GERD patients on and off PPI treatment, upregulated and downregulated genes were found with functions that inhibit apoptosis, prolong survival, promote differentiation and facilitate the loosening of intercellular contacts, thereby allowing the transformation into a migratory phenotype. This implies, that potential to counter damaging effects of excessive reflux is available in epithelial cells.

The findings of the current study suggest that the first defence of the oesophageal epithelium against excess acid reflux consists of only ultrastructural alterations and changes leading to an increased chance of cellular survival. No modifications of the mucosal defence that is already in place (*i.e.* the mucous layer, the intercellular matrix and the buffer capacity of cells and matrix components) could be deduced from the differentially expressed genes that were found. This does not necessarily mean that humoral defence mechanisms do not play a role in the first response to acid reflux, because the cells that secrete mucus and other buffering substances represent a minority of the epithelial layer. The underrepresentation of cells that secrete matrix components renders differences in mRNA levels in these cells between the investigated groups less likely to be detected.

Despite random assignment, the PPI– group had an average 2.2% less acid exposure than the PPI+ group, however, this difference is clinically irrelevant because both means are well above the 6% threshold that defines pathological reflux.

No significantly deregulated genes were found in the comparison of microarray results between healthy controls and PPI+ patients. In contrast, several of the genes selected for QPCR because of differential expression in PPI– patients relative to healthy controls turned out to be significantly deregulated in PPI+ patients as well. These changes in gene expression were, generally, less pronounced than those seen in the PPI– group. This result makes sense, biologically, because acid exposure is not reduced to zero in PPI– treated patients and other caustic components of the refluxate are not targeted by PPIs.

No differences in the transcript levels of the genes selected for validation by QPCR were found in the biopsy specimens from the location that was 10 cm more proximal, 16 cm above the SCJ. Because acid exposure can be assumed to be approximately four times less at the proximal relative to the distal site [16], this suggests that the changes in mRNA expression we found are triggered when acid exposure exceeds a certain threshold. The findings of several studies in GERD patients with and without oesophagitis (erosive reflux disease (ERD) and non-erosive reflux disease (NERD)) show that reflux episodes that have a larger proximal extent are more likely to be perceived by the patient [48, 49]. Thus, the mechanisms for perception of these extensive reflux episodes appear to be independent of the gene expression response to acid we describe in the present study. A possible explanation is, that acid perception is primarily a neural response, and because the cell body of the primary afferent neuron is located outside the oesophagus, expression differences in these cells would be impossible to detect in the biopsies that were collected in the present study.

The biopsy specimens showed no signs of inflammation on histological evaluation, in patients with and without oesophagitis as well as in healthy controls. The explanation for this is that reflux oesophagitis is a well-demarcated condition, occurring just proximal to the oesophagogastric junction, rarely extending more than a few centimetres in the most severe cases, and these biopsies were taken at 6 and 16 cm proximal to the oesophagogastric junction.

In the RT-PCR validation results, ARF6 is downregulated and WASF2 is upregulated, opposite to the results of the microarray analysis. The TaqMan® probe for ARF6 has the extension _s1, meaning that this probe recognizes a single-exon transcript, making it possible that part of the signal is generated by genomic DNA contaminating the original RNA sample [50]. The probe for WASF2 has the extension _gH, signifying that this also potentially recognizes genomic DNA [50]. The original samples have been treated with DNase to remove any genomic DNA, minimizing the chance of DNA contamination, however, further validating experiments may be necessary to interpret the findings of the current study.

The epithelial restitution process has been described in oesophageal cells *in vitro* by Jimenez *et al.* [51, 52], however, despite attempts in several studies, it has not been observed *in vivo* in oesophageal epithelium to date [53]. The results from the present study, however, do indicate a role for epithelial restitution in the first response to excessive acid exposure of the oesophageal epithelium. The finding that so many genes that have anti-proliferative and anti-apoptotic functions were deregulated, in combination with the deregulation of genes that facilitate transition into a migratory phenotype are concordant with the two key events of the epithelial restitution process: cell survival and subsequent migration, in reaction to minor breaches in the epithelial continuity. The epithelial restitution process has been well characterized in intestinal columnar epithelium, however no events resembling epithelial restitution have been described in oesophageal epithelium to date. A possible explanation is that the oesophagus is lined with pseudostratified squamous epithelium, in which minor migratory movements are hard to discern, especially when no such concept has been postulated yet. Further studies *in vitro* and *in vivo* could clarify the phenotypic correlate of the gene expression changes we found.

No genes encoding cytokines, other pro-inflammatory substances or upregulators thereof were found to be differentially expressed in our patients. On the contrary, upregulation of the TANK gene which encodes a protein that inhibits NF-κB activation may exert an anti-inflammatory effect. At the AGA Digestive Disease Week of 2005, Yoshida *et al.* presented results of a microarray study conducted in NERD patients. They did report up-regulation of a wide variety of pro-inflammatory substances in the epithelial cells they had selectively studied with the help of laser capture microdissection [54]. However, this group did not confirm results with RT-PCR, and furthermore the biopsies studied by Yoshida *et al.* were taken closer to the squamocolumnar junction. Inflammatory infiltrates are present in a large portion of NERD patients' oesophagus near the SCJ [55], and these infiltrating cells could have exerted an influence

on gene expression in the epithelial cells. In another study using microarray expression profiling with the same arrays as were used in the present study, Ostrowski *et al.* compared biopsy specimens from non-inflamed oesophageal epithelium of NERD patients, patients with oesophagitis and patients with Barrett's oesophagus. They found a distinct genetic signature for each of these patient groups. Unfortunately, no GERD-free controls were included in that study, which makes comparing their work to the current study difficult [56]. Furthermore, no 24-hrs pH-monitoring was included in the diagnostic work-up of the included subjects, possibly introducing phenotypical heterogeneity.

No upregulation of neuroinflammatory substances was detected. It can be hypothesized that protons, that are able to reach the nerve endings in the intra- and subcellular layer in larger amounts because of increased paracellular permeability, are the main mechanism for the heightened visceral sensitivity that has been found in GERD patients.

The COPA gene could not be confirmed by QPCR, however, this does not rule out xenin expression in the oesophagus, and a study design incorporating immunohistochemistry is needed to clarify this. It would also be interesting to further investigate the ATP8B1 gene, because no expression in the oesophagus of this important gene has been described thus far. The CEL files that have been made available online provide an excellent resource for researchers to verify the oesophageal expression of genes of their interest, and could instigate numerous new studies.

One could argue that the groups in the present study were relatively small, and that the results should be interpreted with caution. Two important preventive measures were taken, however, to maximize reliability: first, the detection thresholds for the microarray results were set to very strict levels, in order to ascertain that only significant expression differences would be detected. Second, RT-PCR analysis confirmed the majority of the found differences, providing a second measure of validity of the results. The current design was chosen to maximize results within the ethical and financial boundaries we faced, and was meant as a pilot work for future investigations.

Further studies are needed to investigate the epithelial restitution process in the oesophagus to clarify, for instance, the epithelial response in different groups of GERD patients, *e.g.* NERD patients or patients with severe oesophagitis or Barrett's oesophagus, and to investigate whether these genetic changes can be tied to a morphologic correlate.

In conclusion, this study is the first to provide validated genome-wide expression data about non-inflamed oesophageal epithelium in GERD patients compared to healthy controls. The results point towards activation by acid reflux of the process we know as epithelial restitution, which has not been described before in the oesophagus.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1 Methods and results

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2008.00626.x
(This link will take you to the article abstract).

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Conflict of Interest

This work was partially funded by Nycomed Pharma AG, Konstanz, Germany. P Shephard and MM Geng were members of the research staff at Nycomed Pharma AG at the time of the conceptualization and data collection of the study. No conflicts of interest exist for DR de Vries, JJM ter Linde, MA van Herwaarden, MP Schwartz, AJPM Smout and M Samsom.

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