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Original Articles

Examining the connectivity between different cellular processes in the Barrett tissue microenvironment

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ABSTRACT

In Barrett associated tumorigenesis, oxidative phosphorylation and glycolysis are reprogrammed early in the disease sequence and act mutually to promote disease progression. However, the link between energy metabolism and its connection with other central cellular processes within the Barrett microenvironment is unknown. The aim of this study was to examine the relationship between metabolism (ATP5B/GAPDH), hypoxia (HIF1α), inflammation (IL1β/SERPINA3), p53 and obesity status using *in-vivo* and *ex-vivo* models of Barrett oesophagus. At the protein level, ATP5B ($r = 0.71$, $P < 0.0001$) and p53 ($r = 0.455$, $P = 0.015$) were found to be strongly associated with hypoxia. In addition, levels of ATP5B ($r = 0.53$, $P = 0.0031$) and GAPDH ($r = -0.39$, $P = 0.0357$) were positively associated with p53 expression. Moreover, we demonstrate that ATP5B ($r = 0.8$, $P < 0.0001$) and GAPDH ($r = 0.43$, $P = 0.022$) were positively associated with IL1β expression. Interestingly, obesity was negatively associated with oxidative phosphorylation ($r = -0.6016$, $P = 0.0177$) but positively associated with glycolysis ($r = 0.743$, $P = 0.0015$). Comparable correlations were exhibited in the *ex-vivo* explant tissue between metabolism, *p53*, hypoxia, inflammation and angiogenesis ($P < 0.05$). We have shown that metabolism is closely linked with many cellular processes in the Barrett tissue microenvironment.

Introduction

Barrett oesophagus is an inflammatory preneoplastic lesion defined as a condition in which the normal stratified squamous epithelium of the oesophagus is replaced by metaplastic columnar epithelium, specifically specialised intestinal metaplasia (SIM) [1]. Barrett oesophagus arises from chronic gastroesophageal reflux disease (GORD) of acid and bile, and can progress to oesophageal adenocarcinoma (OAC) through low-grade dysplasia (LGD) and high-grade dysplasia (HGD) [2,3]. The annual risk of OAC in individuals with Barrett oesophagus is approximately 0.12% [2]. At this time, no established biomarker has been uncovered to establish risk and patients undergo regular surveillance endoscopy with multiple biopsies to assess LGD or HGD. The medical management is predominantly acid suppression therapy with proton pump inhibitors, and no therapies that target the immune or inflammatory environment have been developed [4,5].

It is established that key cellular processes, with distinctive functional roles, are involved in promoting disease progression in Barrett oesophagus. Some of these processes include inflammation, hypoxia and angiogenesis [6–12]. Hypoxia, mediated through hypoxia-inducible factor-1 alpha (HIF1α), is associated with the inflammatory reaction in Barrett oesophagus, however, little is known on how it links with other cellular mediators [7]. Increased HIF1α and HIF2α expression has been demonstrated across the metaplastic-dysplastic-OAC sequence [6]. Barrett-associated inflammation, through canonical pro-inflammatory mediators such as interleukin-6 (IL6), IL1β and signal transducer and activator of transcription 3 (STAT3), has additionally been shown to exacerbate inflammation and promote OAC [9,11]. A recent study showed that neoplastic and non-neoplastic Barrett cells expressing vascular endothelial growth factor (VEGF) and VEGFR2 mRNA and protein, promoted cell proliferation through a phospholipase C gamma1-protein kinase C-ERK pathway subsequent to VEGF-VEGFR2 activation [12]. Moreover, sunitinib-induced VEGF inhibition reduced the weight and volume in mouse xenografts tumours from transformed Barrett cells [12].

Obesity has further been implicated in disease progression in Barrett oesophagus [13,14]. Individuals with central or visceral

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obesity, measured by waist circumferences, are at an increased risk of developing Barrett oesophagus [13]. Furthermore, individuals with a greater propensity to obesity have higher risks of intestinal metaplasia, thereby showing that obesity is independently associated with Barrett oesophagus and its subsequent progression to OAC [14]. Epidemiological studies have also shown a link between leptin and reduced adiponectin, both characteristic of obesity and Barrett oesophagus [15–18]. However, how obesity and associated changes in adipokines, cytokines, insulin and IGF1-axis, immune mechanisms and their downstream signalling pathways influences Barrett oesophagus and Barrett tumorigenesis at the cellular level is currently unknown.

Alterations in energy metabolism is one of the new emerging hallmarks of cancer and disease progression [19,20]. Previous research from our group has demonstrated that both oxidative phosphorylation and glycolysis are reprogrammed early in the inflamed Barrett disease sequence and may act mutually to promote disease progression in Barrett oesophagus [21]. In addition, levels of ATP5B, a marker of oxidative phosphorylation, in first time surveillance biopsy material, segregated Barrett patients who progressed to HGD/OAC from non-progressors, highlighting the possible prognostic advantage of assessing metabolic profiles in these preneoplastic patients [21]. Moreover, Barrett cells were shown to favour the more detrimental oxidative phenotype that may be selected for during the early stages prior to disease progression [21]. Various studies linking energy metabolism with inflammation, hypoxia and angiogenesis highlight how reciprocal processes act jointly to significantly alter the local microenvironment and attenuate disease progression [22–25]. However, despite recent insight into energy metabolism profiles in Barrett oesophagus, less is known about how energy metabolism and other key cellular processes cooperate in the Barrett microenvironment. Interestingly, p53 overexpression is associated with an increased risk of neoplastic progression in patients with Barrett oesophagus, however, the risk is greater with loss of p53 expression [26]. Moreover, in inflamed Barrett oesophagus patients with progressive disease, oxidative-induced damage results in telomere shortening and mutations in the p53 gene abrogate p53's role as the checkpoint of proliferation and apoptosis [27]. In addition to obesity and p53 status, the length of the Barrett segment has been shown to be a risk factor in the development of Barrett associated OAC [28–30].

The aim of this study was to examine the link between energy metabolism, hypoxia, inflammation, p53 and obesity in the Barrett tissue microenvironment *in-vivo* and *ex-vivo*. We report herein that oxidative phosphorylation and p53 status positively correlated with hypoxia. Moreover, levels of oxidative phosphorylation are positively linked to p53 expression whereas levels of glycolysis are negatively associated with p53 expression. In addition, oxidative phosphorylation and glycolysis are positively associated with inflammation. Interestingly, we demonstrated that obesity was negatively associated with oxidative phosphorylation but positively associated with glycolysis. Analogous correlations were exhibited in *ex-vivo* explant tissue between metabolism, p53, hypoxia, inflammation and angiogenesis.

Materials and methods

ATP5B, GAPDH, p53, IL1β, SERPINA3 and HIF1α immunohistochemical analysis using tissue microarrays

Ethical approval to conduct all aspects of this work was granted by the Adelaide and Meath Hospital (AMNCH), Tallaght, Dublin (REC 200110405). All cases were prospectively recruited at our national referral centre for upper GI malignancy and written informed consent was obtained in accordance with local institutional ethical guidelines. All patients attending with histologically confirmed Barrett oesophagus were considered for inclusion. Patients with a prior history of dysplasia or carcinoma, other malignancy of any type, or ablative therapy (radiofrequency ablation, argon plasma coagulation, cryoablation) were excluded. Endoscopic examination consisting of white light and chromoendoscopy (FICE (fujinon) or NBI (olympus)) was performed in all cases (DOT and FMC). The Barrett segment was assessed and measured as per the Prague classification system and biopsied using large capacity forceps.

Patients with intestinal metaplasia were identified from our Barrett tissue database and immunohistochemistry was performed. The median age of patients ($n = 29$) with intestinal metaplasia was 65 years, there was a 2.22-fold male predominance and all patients were followed for a median of 7.5 years. The areas of interest on the diagnostic biopsy blocks were marked by a pathologist. 0.6 mm cores were taken from the blocks and tissue microarrays (TMAs) were constructed and pathology of the tissue re-evaluated prior to antibody staining. Immunohistochemistry was performed utilising the Vectastain Kit (Elite) as per manufacturer's instructions. All tissue microarray sections were processed and stained on the same day at room temperature. Endogenous peroxidases were quenched in 3% hydrogen peroxide (in methanol) for 30 mins and slides blocked with serum for 30 mins. Primary antibodies used were a mouse anti-ATP5B IgG (SantaCruz Biotechnology) (1:1000), a rabbit anti-GAPDH IgG (AbDserotec Division of MorphoSys) (1:300), a rabbit anti-p53 IgG (Abcam) (1:150), a rabbit anti-IL1β IgG (Abcam) (1:200), a rabbit anti-SERPINA3 IgG (Abcam) (1:200) and a rabbit anti-HIF1α IgG (Abcam) (1:200) diluted in PBS. Staining was undertaken with adequate negative controls (PBS only without primary antibody). Slides were incubated with primary antibody for 1 hour, then biotinylated antibody for 30 minutes, avidin–biotin complex for 30 minutes and DAB substrate for 2–15 minutes. DAB was rinsed off slides upon colour development and haematoxylin added for 30 seconds. Lastly, slides were dehydrated and mounted using DPX. Slides were scanned (Philips Digital Pathology Solutions) and immunoreactivity was assessed digitally under 40× magnification in a semi-quantitative manner for each protein by observers who were blinded to the pathology of all patients in the study. For each protein, both epithelial and stromal cells were evaluated for both percentage positivity and intensity of cytoplasmic staining. A third score, designated I × P, was obtained by multiplying intensity by positivity. Intensity was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) and positivity was evaluated as 0%, 10%, 25%, 50%, 75%, 90% or 100%. The scoring was undertaken by consensus evaluation (JP and JOS) and the average value was calculated.

Expression of all protein markers was subsequently correlated to waist circumference and to the length of the Barrett segment. Waist circumference and Barrett segment length demographics were available on 15 and 22 patients respectively from the 29 patients who participated in the study. 15 patients were used to assess the link between ATP5B, GAPDH and waist circumference; the median age of patients with intestinal metaplasia was 65 years, there was a 2.75-fold male predominance and patients had a median waist circumference of 104 cm. 22 patients were used to assess the link between ATP5B, GAPDH and the length of the Barrett segment; the median age of patients ($n = 22$) with intestinal metaplasia was 61.5 years, there was a 2.14-fold male predominance and patients had a median Barrett segment length of 5 cm.

Assessing the effect of hypoxia in an in-vitro *model of Barrett oesophagus*

Cell culture

The QH (Barrett metaplasia) cell line was sourced from American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK). QH cells were grown to 70% confluency in BEBM medium supplemented with BEBM SingleQuots (2 mL BPE, 0.5 mL insulin, 0.5 mL HC, 0.5 mL GA-1000, 0.5 mL retinoic acid, 0.5 mL transferring, 0.5 mL triiodothyronine, 0.5 mL adrenaline and 0.5 mL hEGF per 500 ml media). QH cells were seeded 50,000 per well in 12 well plates and subsequently cultured under normoxia (21% oxygen) and hypoxia (0.5%) conditions for 24 hours. RNA extraction was subsequently performed using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA content and quality was quantified and assayed respectively (Nanodrop®, 8-Sample Spectrophometer, ND-800) and RNA reverse transcribed using Bioscript enzyme (Bioline).

Gene expression analysis

Gene primer probes for *VEGFA*, *ATP5B*, *p53*, *PKM2*, *IL1β*, *AMPK*, *GAPDH*, *HSP60*, *mTOR* and *18S* (Applied Biosystems) were purchased and realtime PCR was performed using Taqman mastermix following manufacturer's instructions on a 7900HT Fast Realtime PCR Light-Cycler System (Applied Biosystems). PCR data was analysed utilising the $2^{-\Delta\Delta Ct}$ method [21]. First, threshold cycle (Ct) values were converted to 2^{-Ct} in order to be proportional to the amount of transcripts in cell line samples. Next, $2^{-\Delta Ct}$ values were calculated by normalising the data to a housekeeping gene, 18S, as follows; $2^{-\Delta Ct} = 2^{-Ct}$ (QH 21%)/2^{-Ct} (18S). In order to compare relative gene expression between cells exposed to normoxia and hypoxia, $2^{-\Delta\Delta Ct}$ values were calculated by normalising the experimental data by reference data. For example, data from one sample was normalised to another sample as follows: $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct}$ (QH 21%)/$2^{-\Delta Ct}$ (QH 0.5%).

Assessment of secreted inflammatory and angiogenic markers

QH cell supernatant, previously exposed to normoxia and hypoxia conditions, was screened for a panel of secreted inflammatory and angiogenic markers. The inflammatory markers assessed were interleukin-10 (IL10), IL12p70, IL13, IL2, IL6, IL4, IL8 and tumour necrosis factor alpha (TNFα). The angiogenic markers assessed were angiopoietin 2 (ANG2), basic fibroblast growth factor (bFGF), intracellular cell ad-