

# Comparison of Kinome Profiles of Barrett's Esophagus with Normal Squamous Esophagus and Normal Gastric Cardia

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## Abstract

The precursor metaplastic mucosal lesion that predisposes for esophageal adenocarcinoma is Barrett's esophagus. Because the signal transduction events that occur in Barrett's esophagus are poorly understood, this study aimed at generating a comprehensive description of cellular kinase activity in Barrett's esophagus, normal squamous esophagus, and gastric cardia to gain more insight into the pathogenesis of Barrett's esophagus. Peptide arrays, exhibiting 1,176 specific consensus sequences for protein kinases, were used to produce a global analysis of cellular kinase activity in biopsies of Barrett's esophagus, and results were compared with the neighboring cardia and squamous epithelia. Several differences in kinase activity using immunoblot analysis and enzyme activity assays were validated in biopsies of 27 Barrett's esophagus patients. Three unique kinome profiles are described and compared. We identified cascades of activated kinases showing that mitogen-activated protein kinase and epidermal growth factor receptor activity are both significantly altered in Barrett's esophagus compared with squamous and gastric cardia epithelia. Another novel finding is that the glycolysis pathway is significantly up-regulated in Barrett's esophagus, which is illustrated by an up-regulated pyruvate kinase activity. Here, the unique kinome profile of Barrett's esophagus is made available as a comprehensive database. Several signaling pathways are revealed as specifically expressed in Barrett's esophagus when compared with the adjacent normal epithelia. These unique findings provide novel insight in the pathogenesis of Barrett's esophagus that will ultimately help to resolve the increasing problem of Barrett's esophagus and prevention of esophageal adenocarcinoma. (Cancer Res 2006; 66(24): 11605-12)

## Introduction

Barrett's esophagus is widely recognized as a premalignant condition in which the normal squamous mucosa of the distal esophagus is replaced by a metaplastic mucosa, defined as an incompletely differentiated intestinal type of epithelium (1-3).

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Barrett's esophagus is thought to be a complication of long standing gastroesophageal reflux disease (GERD) and can be found in 6% to 12% of patients with GERD (4, 5). Barrett's esophagus is further associated with the highly malignant esophageal adenocarcinoma with an estimated annual incidence of ~0.5% (6-8). Over the last 3 decades, the prevalence of Barrett's esophagus and Barrett's esophagus adenocarcinoma has been rising rapidly in Western countries (9-11). The phenotypic changes during the development of Barrett's esophagus have been described in several studies (12-15). Recently, microarray and other gene expression profile studies have been done showing that, also at the gene expression level, Barrett's esophagus has strong similarities with the anatomic surrounding epithelia (16, 17). Nevertheless, at the level of cellular functions and processes, the pathophysiology of Barrett's esophagus is hardly understood.

Analysis studies of genomes and transcriptomes have led to the notion that the greater part of the transcripts expressed in a cell is required to maintain a basal level of cell functioning and that only a small proportion of the transcriptome characterizes the more specific functions of a cell. This small part of the transcriptome can lead to enormous differences in enzymatic activity and as a result change the cell characteristics (18). Dysregulation and mutations of these enzymes play central roles in several human diseases, such as Barrett's esophagus, providing the opportunity of developing agonists and antagonists of these protein kinases that could be used in disease therapy (19-22). Here, we hypothesized that comparison of the kinomes of Barrett's esophagus with the surrounding normal squamous and gastric cardia epithelia would generate profound insight in the biological processes that are specifically activated in Barrett's esophagus. In the present study, kinome profiles were obtained using a peptide array containing 1,176 different kinase-specific consensus sequences. The method, as first described by Diks et al. (23), allows a comprehensive detection of the cellular metabolism in lysates. Up-regulation or down-regulation of a particular kinase activity may lead to a cascade of cellular events. These can be fit into specific cell signaling pathways or cellular functions and as such assign specific characteristics to certain cells. This methodology has not yet been applied to clinical samples, but as peptide arrays have the capacity to produce comprehensive descriptions of cellular signal transduction, it is expected that these arrays will provide new insights into poorly understood pathologic processes, such as a metaplastic lesion, such as Barrett's esophagus. In this study, we have attempted to obtain further insight into the molecular processes by applying peptide arrays to clinical samples of Barrett's esophagus, and using this technology, we delineate active cell signaling pathways in Barrett's esophagus and the anatomically adjacent squamous and gastric cardia epithelium from three

individual Barrett's esophagus patients. The three unique kinome patterns, as described in the present study, provide important information on kinase activity of Barrett's esophagus compared with its surrounding epithelia. Important differences in kinase activity were confirmed by conventional technology in tissue samples of 27 Barrett's esophagus patients. It was found that, at kinome level, Barrett's esophagus does have similarities with both adjacent epithelia, yet several unique kinase patterns were found as well. In summary, the present study contributes to a better understanding of Barrett's esophagus by providing a comprehensive description of kinase activity specific to this disease.

## Materials and Methods

**Patients and tissue specimens.** Tissue samples of 30 Barrett's esophagus patients (21 males, 9 females; mean age, 61 years; age range, 44–86; median length of Barrett's esophagus segment measured endoscopically, 5 cm; range 2–11 cm) taken during routine surveillance endoscopy were used. All patients had known Barrett's esophagus without dysplasia and were on long-term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis (patient self-report and medical records). Endoscopically, none of the patients had reflux esophagitis. Paired biopsies, taken next to each other, were obtained of the Barrett's segment, normal squamous esophagus, and gastric cardia. The Barrett's segment was biopsied at least 2 cm above the gastroesophageal junction yet within the Barrett's segment, recognized endoscopically as typically pink-colored mucosa. Normal squamous epithelium was biopsied at least 2 cm proximal of the Barrett's segment, and gastric cardia was taken within 2 cm below the gastroesophageal junction. Of each set of biopsies, one biopsy was used for histopathologic confirmation whereas the other was snap frozen for subsequent kinome analysis. All patients signed informed consent for the use of their biopsy material and did not have a history of any severe systemic diseases or recently diagnosed malignancies.

All patients had proven incompletely differentiated intestinal type of columnar epithelium without dysplasia in the histologic control biopsies with no signs of active or acute inflammation. Normal gastric cardia and normal esophageal squamous epithelia were also confirmed histologically in all the pairwise taken control biopsies with no signs of intestinal metaplasia. The first three consecutive patients with histopathologically confirmed intestinal metaplasia were selected for kinome analysis, whereas the remainder was used for confirmation of results using immunoblot analysis and enzyme activity assays.

**Kinome array analysis.** Kinome array analysis was done as described by Diks et al. and Löwenberg et al. (23–25). Furthermore, the protocol of the kinome array is described in detail on the Web site.<sup>6</sup> Full biopsies were weighted and lysed with lysis buffer (Cell Signaling, Beverly, MA) in a final concentration of 0.4 mg biopsy/ $\mu$ L lysis buffer (average biopsy weight was 20 mg) with the addition of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride, 1  $\mu$ g/mL leupeptin, and 1  $\mu$ g/mL aprotinin. The lysates were centrifuged at 20,000  $\times$  *g* for 10 minutes at 4°C, and the pellet was discarded. To study kinase activity, 50  $\mu$ L lysate was added to 12  $\mu$ L activation mix containing 50% glycerol, 250  $\mu$ mol/L ATP, 60 mmol/L MgCl<sub>2</sub>, 0.05% (v/v) Brij-35, 0.25 mg/mL bovine serum albumin, and 2,000  $\mu$ Ci/mL [ $\gamma$ -<sup>33</sup>P]ATP. The peptide arrays (Pepscan, Lelystad, the Netherlands) containing 1,164 different kinase pseudosubstrates and 12 control sequences, each spotted twice to confirm reproducibility of the results, were incubated with lysates for 90 minutes in a humidified stove at 37°C. Subsequently, the array was washed twice in PBS containing 0.1% Triton X-100, twice with 2 mol/L NaCl containing 0.1% Tween 20, and twice in distilled water. Slides were air dried and exposed to a phosphoimaging screen for 72 hours and scanned on a STORM apparatus (Molecular

Dynamics, GE Healthcare, Roosendaal, the Netherlands). As a control for a specific binding of [ $\gamma$ -<sup>33</sup>P]ATP to peptide motifs, [ $\alpha$ -<sup>33</sup>P]ATP was used: no radioactivity was detected. Furthermore, as a control, we have analyzed frozen versus fresh material using the PepChip because snap freezing could cause degradation in kinase activity. Results indicated that the kinase activity is not influenced by snap freezing of samples.

**Peptide array imaging and statistical data analysis.** The peptide array data analysis was done as described by Diks et al. and Löwenberg et al. (23–25). Briefly, ScanAlyze software<sup>7</sup> was used. Using grid tools, spot density and individual background were corrected and spot intensities and background intensities were analyzed. Data from three individual experiments were exported to an excel sheet for further analysis. Control spots on the array were analyzed for validation of spot intensities between the different samples. Inconsistent data (i.e., SD between the different data points >1.96 of the mean value) were excluded from further analysis. Spots were averaged and included for dissimilarity measurement to extract kinases of which activity was either significantly induced or reduced. Different kinase activities in lysates from Barrett's esophagus, squamous, and cardia biopsies were determined by significant fold change ratios of the combined values of phosphorylated peptides resembling a substrate for kinase activity. Significance analysis was done as described by Löwenberg et al., briefly; a minimal modification for the algorithm originally developed for microarray analysis<sup>8</sup> was used (24).

**Immunoblotting.** Immunoblotting was done as described by van Baal et al. (16) and Hardwick et al. (26). Blots were incubated with the primary antibody overnight at 4°C. Antibodies used were phosphorylated epidermal growth factor (EGF) receptor (EGFR) Tyr<sup>845</sup> (1:1,000; Cell Signaling), phosphorylated EGFR Tyr<sup>992</sup> (Cell Signaling), phosphorylated EGFR Tyr<sup>1068</sup> (Cell Signaling), phosphorylated HER2 Tyr<sup>1248</sup> (Cell Signaling), phosphorylated Src Tyr<sup>416</sup> (Cell Signaling), phosphorylated extracellular signal-regulated kinase (ERK) Tyr<sup>204</sup> (Santa Cruz Biotechnology, Santa Cruz, CA), protein kinase C (PKC)- $\beta$ 1 (Santa Cruz Biotechnology), phosphorylated phosphoinositide-dependent kinase (PKC) Ser<sup>241</sup> (Cell Signaling), and  $\beta$ -actin I-19 (Santa Cruz Biotechnology). After a final wash, blots were incubated for 5 minutes in Lumilite plus (Boehringer Mannheim, Mannheim, Germany) and then chemiluminescence was detected using a Fuji (Raytek, Sheffield, United Kingdom) LAS3000 illuminator. The phosphorylation level was determined by the ratio of signal intensity of the protein to that of the  $\beta$ -actin. Statistical analysis of phosphorylation levels was conducted using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Data are expressed as mean  $\pm$  SE. Comparison between two groups was analyzed using paired *t* tests (\*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001).

**Pyruvate kinase activity assay.** Biopsies were homogenized using 200  $\mu$ L homogenization mix containing 10 mmol/L MOPS buffer (pH 7.4), 150 mmol/L NaCl, and 0.1% Triton X-100 and sonicated. Pyruvate kinase activity was measured using a coupled enzyme assay based on the use of lactate dehydrogenase (LDH) to convert the pyruvate generated in the pyruvate kinase reaction into lactate with the concomitant oxidation of NADH to NAD. To this end, 15  $\mu$ L homogenate was added to a medium containing 86 mmol/L TRA buffer (pH 7.6), 10 mmol/L KCl, 2.5 mmol/L MgSO<sub>4</sub>, 4.7 mmol/L ADP, 0.2 mmol/L NADH, 9 units/mL LDH, and 0.1% Triton X-100. Reactions were initiated by adding phosphoenolpyruvate (PEP) at the final concentration of 0.53 mmol/L. The absorbance at 350 nm was measured using a Cobas Fara centrifugal analyzer (Roche, Basel, Switzerland). Data are expressed as mean  $\pm$  SE. Comparison between two groups was analyzed using paired *t* tests.

## Results

**Kinome profile comparison of Barrett's esophagus, normal squamous esophagus, and gastric cardia.** *In vitro* phosphorylation of peptide arrays by biopsy lysates revealed that Barrett's

<sup>6</sup> <http://www.pepscan.nl/pdf/Manual%20PepChip%20Kinase%200203.pdf>.

<sup>7</sup> <http://www.bio.davidson.edu/Biology/GCAT/protocols/scanalyze.html>.

<sup>8</sup> <http://www-stat.stanford.edu/~tibs/SAM/>.

esophagus, normal squamous esophagus, and gastric cardia contain substantial kinase activity; almost all substrate peptides incorporate [ $\gamma$ - $^{33}\text{P}$ ]ATP (Fig. 1). Subsequent analysis of the kinome profiles of three individuals revealed 130 kinase substrates showing a significantly differential expression profile when comparing Barrett's esophagus with normal squamous epithelium ( $P < 0.05$ ; Supplementary Data). Comparison of the kinase activity patterns of three individuals of Barrett's esophagus with gastric cardia identified 274 kinase substrates that were significantly differentially phosphorylated ( $P < 0.05$ ; Supplementary Data). Figure 2 shows scatter plots comparing substrate phosphorylation in Barrett's esophagus with normal squamous esophagus and normal gastric cardia, revealing the kinase signature of Barrett's esophagus to be an intermediate between the surrounding epithelia ( $R^2 = 0.80$  and  $0.80$ , respectively;  $P < 0.0001$ ; Fig. 2A and B). Accordingly, a scatter plot comparing substrate phosphorylation of normal esophagus with normal cardia shows poor correlation ( $R^2 = 0.66$ ;  $P < 0.0001$ ; Fig. 2C). Thus, overall kinome profiling suggests that Barrett's esophagus has similarities with both squamous and cardia epithelium.

#### Barrett's esophagus associated changes in kinase activity.

One of the most prominent effects in Barrett's esophagus compared with normal squamous epithelium was significantly decreased phosphorylation of Src consensus substrates. Comparing Barrett's esophagus with gastric cardia, phosphorylation of glycogen synthase kinase-3 $\alpha$  (GSK-3 $\alpha$ ) consensus substrates was one of the major effects that was significantly decreased in Barrett's

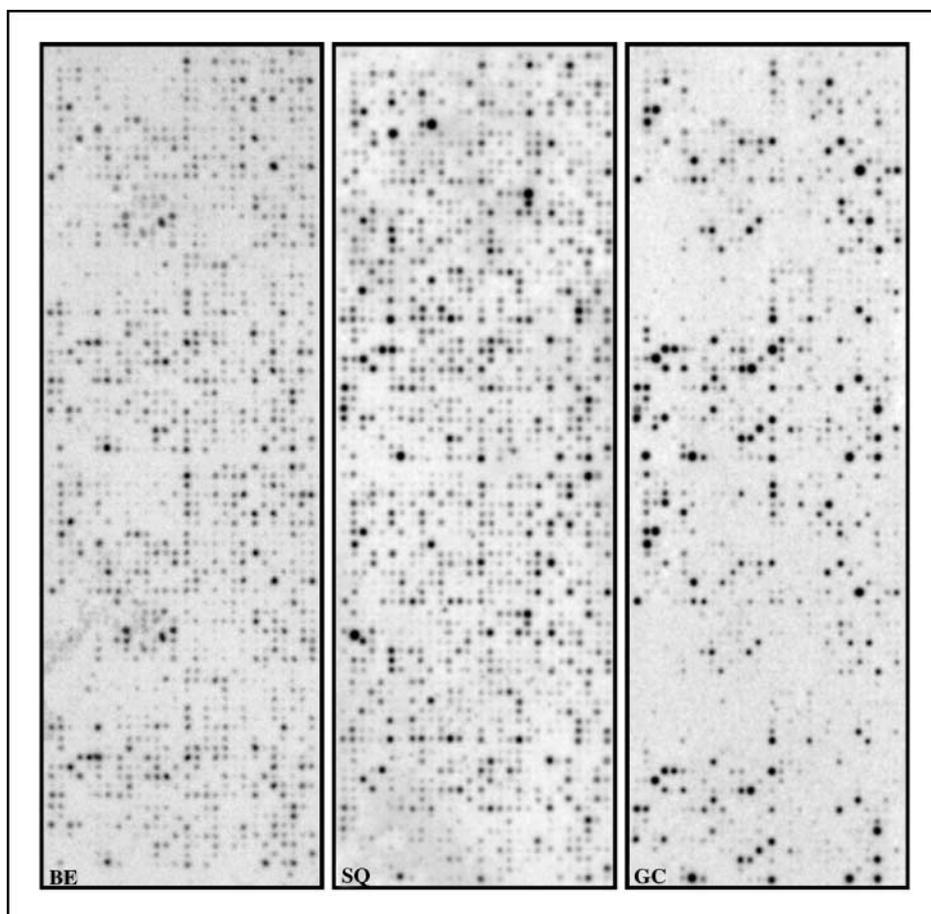
esophagus. A complete list of the peptide substrates with significantly altered phosphorylation in Barrett's esophagus versus normal squamous and in Barrett's esophagus versus gastric cardia can be found as Supplementary Data with corresponding fold induction.

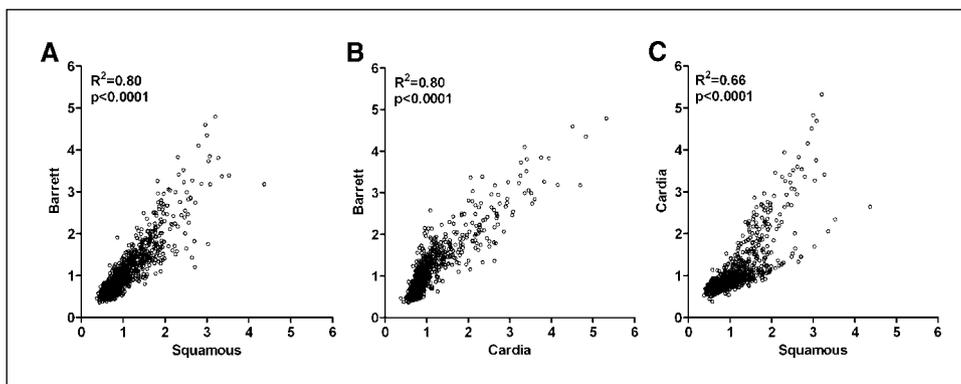
**Delineation of cell signaling pathways and functions in Barrett's esophagus.** The results obtained were used to construct provisional signal transduction schemes showing the differences in cellular signaling between Barrett's esophagus and the surrounding epithelia (Fig. 3). The results show a strong increase in glycolytic metabolism in Barrett's esophagus compared with cardia epithelium, which was associated with down-regulated signaling through the insulin receptor and its downstream mediators phosphatidylinositol 3-kinase, protein kinase B, and GSK3 (Fig. 3B).

The EGFR signaling was increased when Barrett's esophagus and cardia were compared (Fig. 3B) and decreased in Barrett's esophagus when compared with normal squamous (Fig. 3A). The EGF signal in Barrett's esophagus resulted in enhanced activation of phospholipase C- $\gamma$  and PKC (Fig. 3).

At the same time, signaling through the mitogen-activated protein kinase (MAPK) signaling cassette is decreased in Barrett's esophagus, probably partly due to increased inhibition of c-Raf through Rab (Fig. 3) and in case of the comparison between Barrett's esophagus and normal squamous epithelium also because of diminished activation of c-Raf through the down-regulation of EGFR signaling (Fig. 3A).

**Figure 1.** Scans of the peptide arrays after incubation with lysates of Barrett's esophagus (BE), normal squamous esophagus (SQ), or normal gastric cardia (GC) in the presence of [ $\gamma$ - $^{33}\text{P}$ ]ATP. Each spot represents phosphorylation of a specific substrate through kinase activity as present in the different lysates. Notice the specific patterns for the three different epithelia.



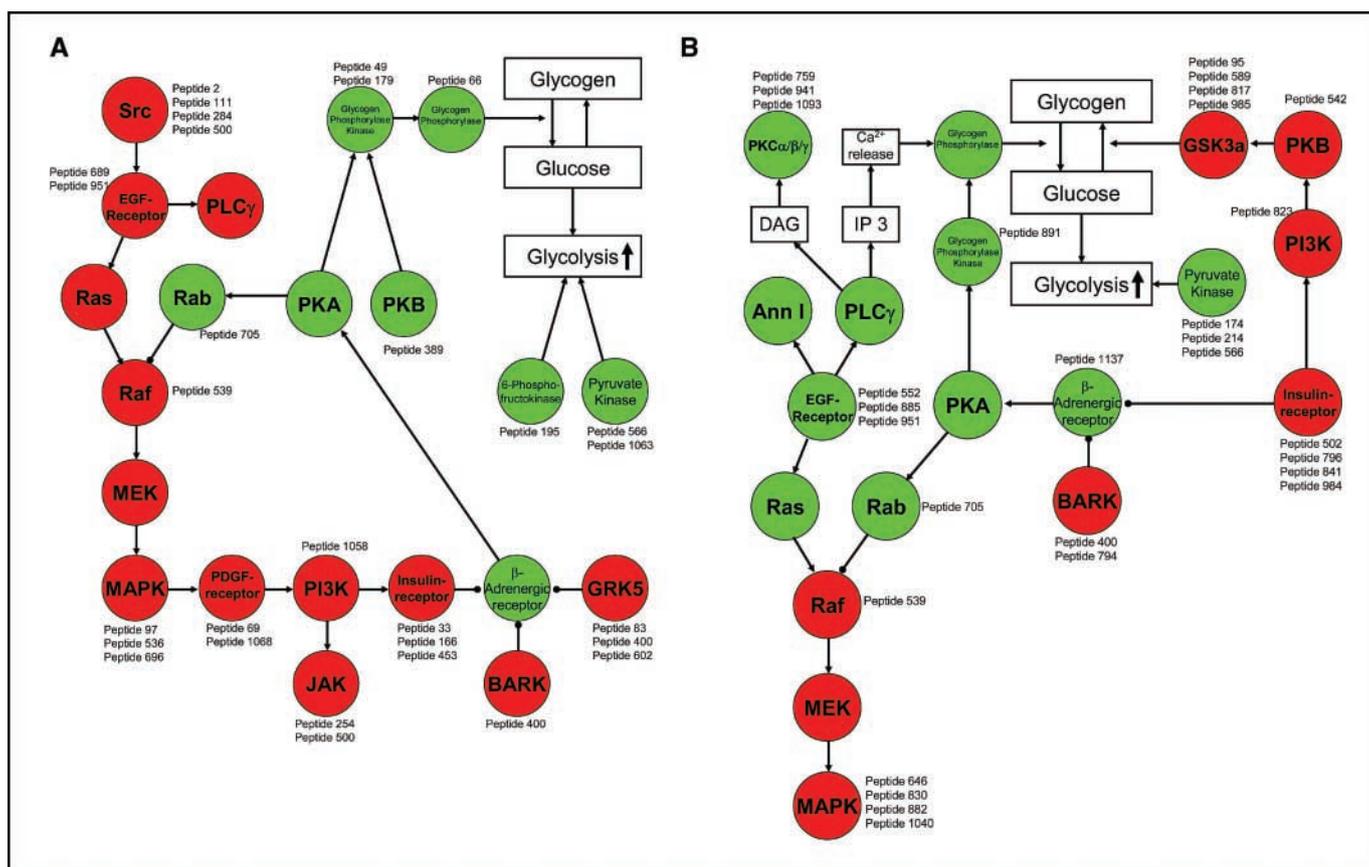


**Figure 2.** Scatter plots showing the overall distribution of all different kinase consensus substrates from three individual experiments in Barrett's esophagus versus normal squamous esophagus (A), Barrett's esophagus versus normal gastric cardia (B), and normal squamous esophagus versus normal gastric cardia (C) with the corresponding correlation in  $R^2$  and  $P_s$ .

Surprisingly, strong deactivation of the  $\beta$ -adrenergic receptor kinase, of which activation is associated with inhibition of the  $\beta$ -adrenergic receptor, was seen in Barrett's esophagus when compared with normal squamous and cardia epithelium, suggesting activation of  $\beta$ -adrenergic receptors in Barrett's esophagus (Fig. 3).

**Validation of kinome profile results.** Biopsies of a panel of 20 Barrett's esophagus patients were subjected to immunoblotting as to establish the validity of the findings described above. The most prominent effect seen in the peptide arrays, the differential

activity of the EGFR (normal squamous esophagus > Barrett's esophagus > normal gastric cardia), is suitable for such analysis, as various different phosphorylated-specific antibodies are available of which the immunoreactivity correlates well with EGFR activation. Figure 4A shows that, in all patients investigated, the differential activation of the EGFR was confirmed when three different tissues were compared for EGFR activation. EGFR is significantly more activated in normal squamous esophagus compared with both Barrett's esophagus and gastric cardia tissue ( $P < 0.05$ , paired  $t$  test). For all different tyrosine sites for the EGFR



**Figure 3.** Provisional signal transduction schemes of several cellular signaling pathways comparing Barrett's esophagus and normal squamous epithelium (A) and Barrett's esophagus with gastric cardia (B). *Green*, significantly higher activated proteins in Barrett's esophagus; *red*, significantly less activated proteins in Barrett's esophagus. Important in Barrett's esophagus are decreased activity of the MAPK signaling cassette and the increased glycolytic metabolism. The EGFR signaling is decreased in Barrett's esophagus compared with normal squamous tissue, whereas EGFR activity in Barrett's esophagus is increased compared with gastric cardia. Peptide numbers provided correspond to the substrates on the peptide arrays.

investigated in this study, the EGFR was significantly more activated in Barrett's esophagus compared with gastric cardia ( $P < 0.05$ , paired  $t$  test). In addition, other key signaling elements were according to expectation. Figure 5 shows significant reduced Src activation in Barrett's esophagus, significant diminished activation of the MAPK signaling cassette (phosphorylated ERK Tyr<sup>204</sup>) as expected from Rab activation in Barrett's esophagus, and, to a certain extent, the expected enhanced activation of PKC- $\beta$ 1 in Barrett's esophagus versus squamous epithelium. The kinome results suggest diminished activation of insulin receptor signaling in Barrett's esophagus, and we confirmed this using a phosphorylated PDK1 antibody immunoreactivity as a read out. Figure 5 shows that Barrett's esophagus is indeed associated with reduced activity of this pathway. Significantly decreased activity of PDK1 was seen in Barrett's esophagus compared with normal squamous esophagus and gastric cardia ( $P < 0.01$ , paired  $t$  test, for Barrett's esophagus versus squamous esophagus;  $P < 0.05$ , paired  $t$  test, for Barrett's esophagus versus gastric cardia; Fig. 5B).

For validation of the glycolytic metabolism, we did an enzyme activity assay for pyruvate kinase. Results show that pyruvate kinase was significantly up-regulated in Barrett's esophagus compared with normal squamous esophagus and gastric cardia ( $P < 0.05$ , paired  $t$  test; Fig. 6). Thus, assaying signal transduction in Barrett's esophagus and its surrounding epithelia by conventional technology corresponds to the results obtained with the peptide arrays.

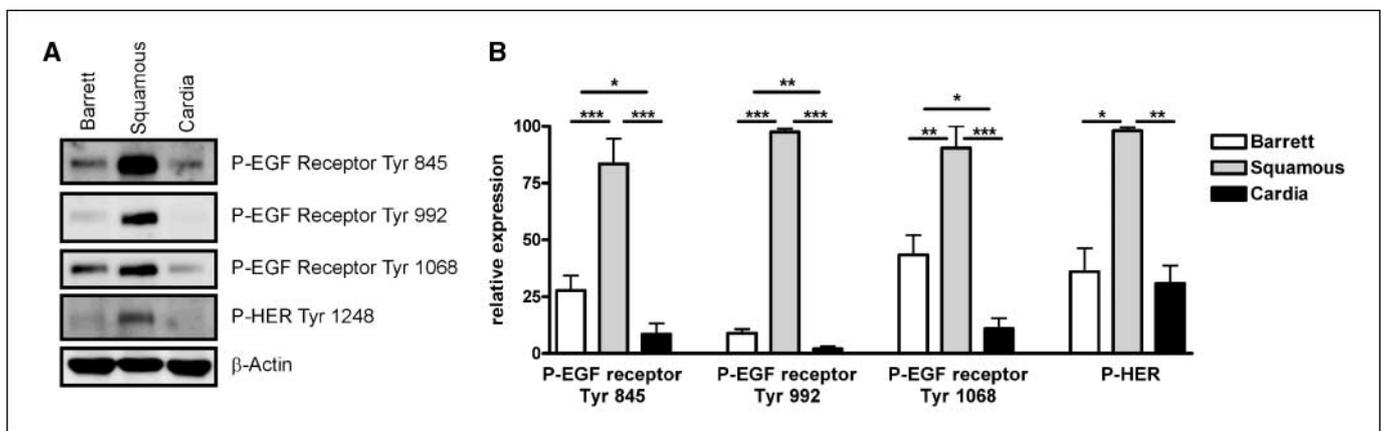
## Discussion

Over the last years, array and mass spectrometry technologies have enabled analysis of the transcriptome and proteome of Barrett's esophagus compared with its surrounding epithelia (16, 17, 27, 28). This information will be of significant value to the elucidation of molecular mechanisms that govern esophageal cell physiology and differentiation. However, an equally, if not more, important goal is to define those proteins that contribute in signaling pathways that participate in the development of Barrett's esophagus and provides critical information for understanding this

pre-malignant condition (19). Enzymes that phosphorylate tyrosine, serine, and threonine residues on other proteins play a major role in signaling cascades that determine cell cycle entry, survival, and the differentiation fate of cells in the mammalian body, including the gastrointestinal tract. Traditional genetic and biochemical approaches can provide some of these answers; however, for technical and practical reasons, these typically pursue one gene or pathway at a time. The technical restraints of conventional technology have led to a situation that molecular understanding of signaling is now largely dependent on findings from studies of cell lines and as such is not adequately representative of the signaling phenotypes of a complex population of cells as Barrett's esophagus in the gastrointestinal tract (18). In the present study, we have chosen to use full biopsies, providing more accurate insight into the signal transduction occurring in this important pre-malignant condition. A Barrett's esophagus biopsy, however, is a heterogeneous cell population containing not only epithelial cells but also stromal tissue and inflammatory cells. For future kinome analysis, the use of epithelial cell populations would be of interest. In this study, Barrett's esophagus, normal squamous esophagus, and gastric cardia were used for the analysis because recently microarray and serial analysis of gene expression studies reported that, at transcriptome level, Barrett's esophagus has strong similarities with the anatomic surrounding epithelia (16, 17). However, we should take in consideration that analyzing the kinomes of duodenum and colon epithelium could further improve our insight in the process of metaplasia.

Unlike the genome, the transcriptome and the kinome are variable and depend on gene function, developmental, and disease state of the individual. We only included patients with long-term acid suppression and without active reflux esophagitis to prevent confounding of the results by inflammatory factors. None of the patients had severe or active inflammation in the pairwise taken control biopsies. Furthermore, we used patients who had no history of any severe systemic disease or untreated malignancies because differences in metabolic conditions could influence the results analyzed with the PepChip.

Here, we choose to use biopsies of three male individuals known with nondysplastic Barrett's esophagus for analyzing the cellular



**Figure 4.** Immunoblot analysis of expression levels of several phosphorylation sites of the EGFR (A) in Barrett's esophagus, normal squamous esophagus, and gastric cardia. A, high expression levels of phosphorylated EGFR (P-EGF Receptor) in squamous epithelium, lower expression in Barrett's esophagus, and lowest in gastric cardia.  $\beta$ -Actin was used as a control. B, quantification of immunoblot results of phosphorylated EGFR Tyr<sup>845</sup>, Tyr<sup>992</sup>, and Tyr<sup>1068</sup> and phosphorylated HER2 (P-HER) Tyr<sup>1245</sup> in Barrett's esophagus, squamous esophagus, and normal gastric cardia of 20 patients shows that these are significantly higher expressed in the normal squamous esophagus biopsies. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Phosphorylation levels of EGFR were also significantly higher in Barrett's esophagus compared with gastric cardia. The phosphorylation levels were determined by the ratio of signal intensity of the protein to that of the  $\beta$ -actin. Columns, mean; bars, SE.

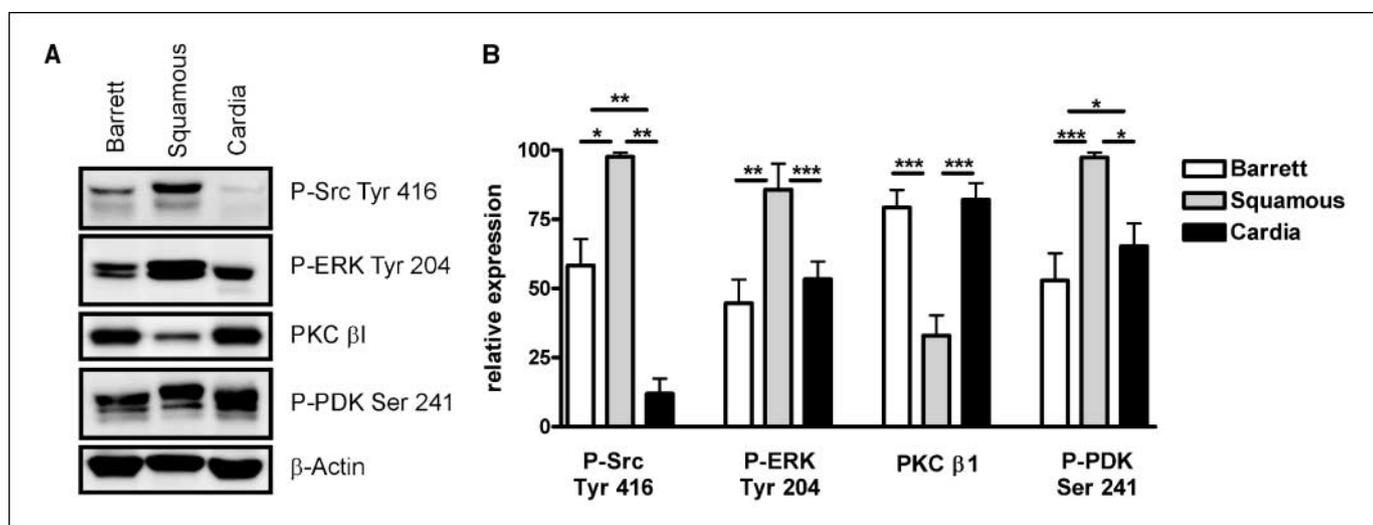
metabolisms with the peptide array technology. Using the results of the kinase arrays, we further unraveled several of the active cell signaling pathways and cellular functions in the three epithelia. Hereon, conventional technologies were used to validate these pathways in biopsy specimens of another 27 Barrett's esophagus patients. The results unambiguously show that the kinome signature of Barrett's esophagus has strong similarities with both the kinome profiles of normal squamous and normal cardia epithelium. Confirming that Barrett's esophagus does not represent a true transdifferentiation but is indeed an incompletely differentiated type of epithelium that has strong similarities with the two different surrounding types of tissue. More importantly, we identified several kinases that seem to be highly activated in Barrett's esophagus. Of particular interest is the kinase activity of the phosphorylated EGFR, which is a transmembrane protein receptor that may trigger numerous signaling pathways (20). Previously, Jankowski et al. (29) reported expression of EGFR in Barrett's esophagus; however, to our knowledge, no research has been done on EGFR activation in Barrett's esophagus. The immunoblot results show that EGFR is significantly more activated in normal squamous esophagus compared with Barrett's esophagus. Yet, the EGFR activity is significantly higher in Barrett's esophagus compared with normal gastric cardia. From these results, we may speculate that EGF is an important normal growth factor in squamous tissue regulating growth and differentiation but is only of minor significance for growth regulation in normal columnar type of epithelia, such as the gastric cardia. The significantly increased level of EGFR activation in Barrett's esophagus compared with cardia epithelium suggests that, although Barrett's esophagus has a columnar phenotype, there may be aberrant up-regulation of cell growth and differentiation that at least partly is induced through the EGFR activation.

We found a decreased activity of Src kinase consensus sequences in Barrett's esophagus compared with normal

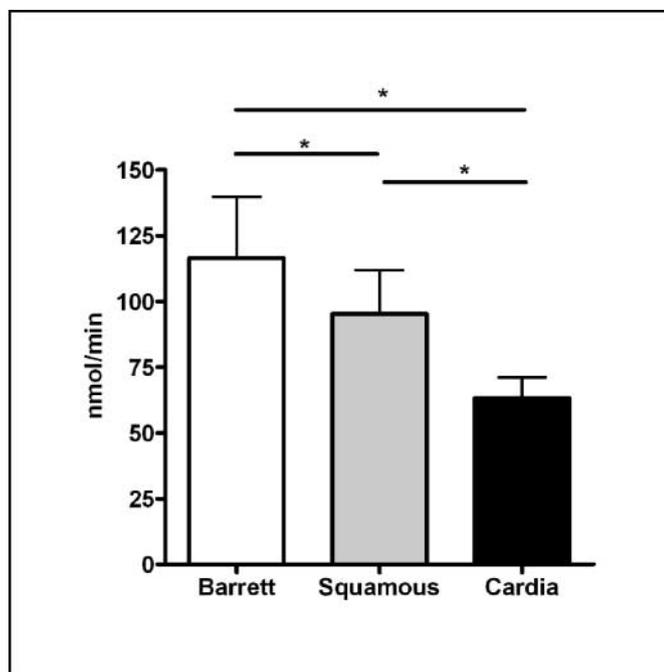
squamous esophagus. This is in contrast with a previously published article of Kumble et al. (30) where they describe that Src kinase activity is increased in the malignant and premalignant Barrett's esophagus. Although the phosphorylation site in their report, Src Tyr<sup>527</sup>, differs from the one that we studied, this is Src Tyr<sup>416</sup> that correlated to one of the phosphorylation sites on the PepChip.

Another highly interesting finding is the down-regulated activity of the MAPK signaling cassette that seems to be a major event in Barrett's esophagus compared with its surrounding tissue. To our knowledge, no research has been done on the MAPK activity in metaplastic Barrett's esophagus. Therefore, we validated the results with immunoblotting and confirmed that the level of ERK phosphorylation was indeed significantly down-regulated in Barrett's esophagus compared with normal squamous esophagus. Compared with gastric cardia, the down-regulated level of ERK phosphorylation in Barrett's esophagus was not significant, although a trend was seen ( $P = 0.066$ ; Fig. 5C). It is of interest that, in previous studies, it has been shown that acid and bile reflux can induce MAPK activity in Barrett's esophagus adenocarcinoma cell lines (31–33). It has also been described that malignant transformation of metaplastic Barrett's esophagus is related to ongoing bile and acid reflux (34). Therefore, we speculate that malignant transformation may be partly through activation of MAPK that is dormant in nonmalignant metaplastic Barrett's esophagus. Future kinase analysis comparing metaplastic, dysplastic, and malignant Barrett's esophagus will enlighten us on this matter.

Another important observation was the prominence of substrates in Barrett's esophagus that suggest enhanced glycolytic activity. These results closely correspond to an earlier study, analyzing the gene expression profiles of biopsies of Barrett's esophagus, normal squamous esophagus, and gastric cardia epithelium (16). In this study, clustering of genes in functional



**Figure 5.** Immunoblot analysis of expression levels of phosphorylated Src (*P-Src*), phosphorylated ERK (*P-ERK*), PKC- $\beta$ 1, and phosphorylated PDK (*P-PDK*; A) in Barrett's esophagus, normal squamous esophagus, and gastric cardia. Immunoblotting of phosphorylated Src shows high expression in normal squamous epithelium, whereas the expression in cardia and Barrett's esophagus is significantly lower (A and B). A and B, significantly lower expression levels of phosphorylated ERK and phosphorylated PDK in Barrett's esophagus and higher expression in both normal squamous and cardia epithelium. PKC- $\beta$ 1 is highly expressed in both Barrett's esophagus and gastric cardia but significantly lower in normal squamous esophagus.  $\beta$ -Actin was used as a control. B, quantification of immunoblot results of phosphorylated Src Tyr<sup>416</sup>, phosphorylated ERK Tyr<sup>204</sup>, PKC- $\beta$ 1, and phosphorylated PDK Ser<sup>241</sup> in Barrett's esophagus, squamous esophagus, and normal gastric cardia of 20 patients shows that these are significantly higher expressed in the normal squamous esophagus biopsies. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . The phosphorylation levels were determined by the ratio of signal intensity of the protein to that of the  $\beta$ -actin. Columns, mean; bars, SE.



**Figure 6.** Enzymatic assay for pyruvate kinase activity in Barrett's esophagus, normal squamous epithelium, and gastric cardia showing that pyruvate kinase is significantly more activated in Barrett's esophagus compared with its surrounding tissue. \*,  $P < 0.05$ . Columns, mean; bars, SE.

classes showed that metabolism was one of the most important biological processes in Barrett's esophagus compared with normal squamous and gastric cardia epithelium; in this biological process, the increase in expression of enzymes associated with the glycolysis

was a prominent change. In addition, Koss et al. (35) showed that pyruvate kinase type M2 was expressed in Barrett's esophagus and was even increased in dysplastic Barrett's esophagus. To further validate our kinase results, we did an enzyme activity assay for pyruvate kinase, which plays an important role in the final irreversible step in the glycolysis. Pyruvate kinase catalyzes the transphosphorylation of PEP and ADP to pyruvate and ATP (36). Results on biopsy material of seven patients showed that, indeed, pyruvate kinase activity was significantly up-regulated in Barrett's esophagus compared with normal squamous and gastric cardia epithelium (Fig. 6).

In summary, this study provides a comprehensive description of kinase activity specific to Barrett's esophagus and as such gives an important contribution to understanding the pathophysiology underlying this disorder. Here, the unique kinome profile of Barrett's esophagus is made available to the scientific community that as a comprehensive database can be used for future studies of cellular functions in Barrett's esophagus. We further speculate that manipulations of several of these cellular events will ultimately lead to more effective treatment of Barrett's esophagus and as such prevention of development of the esophageal adenocarcinoma.

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## References

- Haggitt RC, Tryzelaar J, Ellis FH, Colcher H. Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. *Am J Clin Pathol* 1978;70:1-5.
- Spechler SJ, Zeroogian JM, Antonioli DA, Wang HH, Goyal RK. Prevalence of metaplasia at the gastroesophageal junction. *Lancet* 1994;344:1533-6.
- Ransford RA, Jankowski JA. Genetic versus environmental interactions in the oesophagitis-metaplasia-dysplasia-adenocarcinoma sequence (MCS) of Barrett's oesophagus. *Acta Gastroenterol Belg* 2000;63:18-21.
- Winters C, Jr., Spurling TJ, Chobanian SJ, et al. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. *Gastroenterology* 1987;92:118-24.
- Mann NS, Tsai MF, Nair PK. Barrett's esophagus in patients with symptomatic reflux esophagitis. *Am J Gastroenterol* 1989;84:1494-6.
- Drewitz DJ, Sampliner RE, Garewal HS. The incidence of adenocarcinoma in Barrett's esophagus: a prospective study of 170 patients followed 4.8 years. *Am J Gastroenterol* 1997;92:212-5.
- Shaheen NJ, Crosby MA, Bozymski EM, Sandler RS. Is there publication bias in the reporting of cancer risk in Barrett's esophagus? *Gastroenterology* 2000;119:333-8.
- Hameeteman W, Tytgat GN, Houthoff HJ, van den Tweel JG. Barrett's esophagus: development of dysplasia and adenocarcinoma. *Gastroenterology* 1989;96:1249-56.
- Devesa SS, Blot WJ, Fraumeni JF, Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998;83:2049-53.
- Prach AT, MacDonald TA, Hopwood DA, Johnston DA. Increasing incidence of Barrett's oesophagus: education, enthusiasm, or epidemiology? *Lancet* 1997;350:933.
- Bytzer P, Christensen PB, Damkier P, Vinding K, Seersholm N. Adenocarcinoma of the esophagus and Barrett's esophagus: a population-based study. *Am J Gastroenterol* 1999;94:86-91.
- Moore JH, Lesser EJ, Erdody DH, Natale RB, Orringer MB, Beer DG. Intestinal differentiation and p53 gene alterations in Barrett's esophagus and esophageal adenocarcinoma. *Int J Cancer* 1994;56:487-93.
- Regalado SP, Nambu Y, Iannettoni MD, Orringer MB, Beer DG. Abundant expression of the intestinal protein villin in Barrett's metaplasia and esophageal adenocarcinomas. *Mol Carcinog* 1998;22:182-9.
- Wu GD, Beer DG, Moore JH, Orringer MB, Appelman HD, Traber PG. Sucrase-isomaltase gene expression in Barrett's esophagus and adenocarcinoma. *Gastroenterology* 1993;105:837-44.
- Levine DS, Rubin CE, Reid BJ, Haggitt RC. Specialized metaplastic columnar epithelium in Barrett's esophagus. A comparative transmission electron microscopic study. *Lab Invest* 1989;60:418-32.
- van Baal JW, Milano F, Rygiel AM, et al. A comparative analysis by SAGE of gene expression profiles of Barrett's esophagus, normal squamous esophagus, and gastric cardia. *Gastroenterology* 2005;129:1274-81.
- Barrett MT, Yeung KY, Ruzzo WL, et al. Transcriptional analyses of Barrett's metaplasia and normal upper GI mucosae. *Neoplasia* 2002;4:121-8.
- Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* 2004;118:217-28.
- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002;298:1912-34.
- Blume-Jensen P, Hunter T. Oncogenic kinase signaling. *Nature* 2001;411:355-65.
- Hunter T. Signaling—2000 and beyond. *Cell* 2000;100:113-27.
- Cohen P. Protein kinases—the major drug targets of the twenty-first century? *Nat Rev Drug Discov* 2002;1:309-15.
- Diks SH, Kok K, O'Toole T, et al. Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. *J Biol Chem* 2004;279:49206-13.
- Löwenberg M, Tuijnman J, Scheffer M, et al. Kinome analysis reveals nongenomic glucocorticoid receptor-dependent inhibition of insulin signaling. *Endocrinology* 2006;147:3555-62.
- Löwenberg M, Tuijnman J, Bilderbeek J, et al. Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn. *Blood* 2005;106:1703-10.
- Hardwick JC, Van Den Brink GR, Bleuming SA, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. *Gastroenterology* 2004;126:111-21.
- Vona-Davis L, Vincent T, Zulfiqar S, Jackson B, Riggs D, McFadden DW. Proteomic analysis of SEG-1 human

- Barrett's-associated esophageal adenocarcinoma cells treated with keyhole limpet hemocyanin. *J Gastrointest Surg* 2004;8:1018-23.
28. Streit JM, Jr., Madden MT, Marimanikkuppam SS, Krick TP, Salo WL, Aufderheide AC. Analysis of protein expression patterns in Barrett's esophagus using MALDI mass spectrometry, in search of malignancy biomarkers. *Dis Esophagus* 2005;18:170-6.
29. Jankowski J, Murphy S, Coghil G, et al. Epidermal growth factor receptors in the oesophagus. *Gut* 1992;33:439-43.
30. Kumble S, Omary MB, Cartwright CA, Triadafilopoulos G. Src activation in malignant and premalignant epithelia of Barrett's esophagus. *Gastroenterology* 1997;112:348-56.
31. Souza RF, Shewmake K, Pearson S, et al. Acid increases proliferation via ERK and p38 MAPK-mediated increases in cyclooxygenase-2 in Barrett's adenocarcinoma cells. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G743-8.
32. Souza RF, Shewmake K, Terada LS, Spechler SJ. Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. *Gastroenterology* 2002;122:299-307.
33. Jaiswal K, Tello V, Lopez-Guzman C, Nwariaku F, Anthony T, Sarosi GA. Bile salt exposure causes phosphatidylinositol-3-kinase-mediated proliferation in a Barrett's adenocarcinoma cell line. *Surgery* 2004;136:160-8.
34. Demeester SR, Peters JH, Demeester TR. Barrett's esophagus. *Curr Probl Surg* 2001;38:558-640.
35. Koss K, Harrison RF, Gregory J, Darnton SJ, Anderson MR, Jankowski JA. The metabolic marker tumour pyruvate kinase type M2 (tumour M2-PK) shows increased expression along the metaplasia-dysplasia-adenocarcinoma sequence in Barrett's oesophagus. *J Clin Pathol* 2004;57:1156-9.
36. Valentini G, Chiarelli L, Fortin R, Speranza ML, Galizzi A, Mattevi A. The allosteric regulation of pyruvate kinase. *J Biol Chem* 2000;275:18145-52.

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