Molecular mechanisms in the development of Barrett's esophagus

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Molecular mechanisms in the development of Barrett's esophagus

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Contents

Chapter 1

General introduction & outline of the thesis

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1. Introduction

Barrett's esophagus (BE) is the metaplastic change of the normal lined squamous epithelium of the distal esophagus to a columnar type of epithelium as a result of chronic longstanding gastro-esophageal reflux disease (GERD)[1, 2]. Patients with BE have a significantly increased risk of developing an esophageal adenocarcinoma (EA), with an estimated annual incidence varying from 0.4% to 1.8%[3-6]. Over the last 3 decades, the incidence of BE and its associated adenocarcinoma has increased in Western countries at a rate that exceeds that of any other malignancy[7-9]. Despite all the research performed on BE there is still an inadequate understanding of the biological basis of this mucosal transformation.

This introduction will provide a general introduction of BE and it's associated EA, describing the different types of intestinal metaplasia, GERD and biomarkers used for BE diagnosis. Furthermore, the malignant transformation of BE and the metaplastic transformation of BE is explained. Finally, the use of high through-put techniques like microarray, SAGE and pep-chips in BE is described.

2. Barrett's esophagus

2.1. Background

In 1950 Norman Barrett, a thoracic surgeon, published an article in the British Journal of Surgery entitled "Chronic peptic ulcer of the esophagus and 'esophagitis'"[10]. Well-known are his introduction of the expression 'reflux esophagitis' and his statement that it was a common condition. Barrett further noted that in patients with this condition, inflammation in the *muscularis propria* layer of the esophagus might subsequently end in a benign stricture. Furthermore, he proposed that a stricture of the esophagus produced by chronic inflammation had been mistaken as the esophagus because it was actually an area of the stomach partially enveloped by the peritoneum that had been drawn up by contraction of the scar tissue. Barrett concluded that it was this 'pulled up' stomach that was the location of the chronic gastric ulcer and that the ulcers that had been described in the lower esophagus before were actually 'gastric' and not esophageal in origin. He furthermore supported the idea that hiatal hernias were important causes of GERD and agreed that repair of this disorder was necessary for a successful therapy.

In retrospect, it now seems clear that although Barrett may have made a mistake in some of his hypotheses, his basic recognition of the relevance of the disease entity is still an important, novel clinical observation in an until now unexplored area of gastro-esophageal disease[11, 12].

Nowadays malignant degeneration of BE is thought to be a multi-step process in which metaplasia progresses through low grade and high grade dysplasia into eventually an invasive adenocarcinoma[13]. Once BE is diagnosed, patients enter an endoscopic surveillance program monitoring the development of BE associated dysplasia and adenocarcinoma, and determining when therapeutic intervention is required. Furthermore, management involving the control of reflux symptoms and any inflammatory sequel is necessary. Endoscopic surveillance can detect BE and its associated esophageal adenocarcinoma in an early and curable stage, still EA is mostly detected at an advanced stage[14-16].

2.2. Types of intestinal metaplasia

BE is characterized by an abnormal state of differentiation and proliferation. The development of BE arises when there is a switch from one differentiated epithelium to another cell lineage that

normally is not found in the esophagus. In more detail BE develops from normal stratified squamous mucosa into glandular epithelium. Three subtypes of BE have been illustrated. In 1976 Paull *et al*. described BE as esophageal columnar mucosa with a mosaic of intestinal goblet cells along with cardiac and fundic mucosa, suggesting a histological classification of BE into cardiac, fundic and specialized intestinal types[17]. However the specialized intestinal type is the only subtype clearly associated with malignant transformation[17, 18]. Three types of intestinal metaplasia are associated with BE. Type I metaplasia, or complete intestinal metaplasia, is a columnar epithelium with a complete brush border of mature goblet cells, additionally Paneth cells are commonly seen in the basal crypts[3, 19]. Types 2 and 3 are incompletely differentiated intestinal metaplasia because the columnar epithelium does not have the intestinal absorptive activity or ultra structural characteristics of characteristic intestinal cells[19]. Although, all types of intestinal metaplasia can become dysplastic and ultimately develop into EA, the type I metaplasia characterized by the presence of goblet cells has the highest risk for malignant transformation[3-6]. Previous studies demonstrated that BE show similarities with normal squamous and columnar epithelia. BE has common features with the small intestine, expressing villin and sucrase isomaltase[20-22]. Additionally BE has great similarities with gastric epithelium, because of the capacity to secrete mucus[23]. Currently BE is diagnosed on the histological finding of intestinal metaplasia, explicitly by the presence of goblet cells, in the biopsy[24]. According to the recent British classification of BE, the different types of intestinal metaplasia are included as BE and the specific type of differentiation should be mentioned in the classification, i.e. BE with gastric type of differentiation[25].

2.3 Gastro-esophageal Reflux Disease

A century ago, GERD was an almost unknown entity with less than 200 cases reported over the world[11]. In contrast, in the new millennium GERD has become a common condition that can be found in 20-30% of the general Western population[26-28]. GERD predisposes for BE, which replaces the normal squamous epithelium by an abnormal metaplastic columnar epithelium and as such this epithelium provides a better resistance to the effects of gastro-esophageal reflux. Up to 5-12% of patients who suffer from GERD will develop BE[29]. Literature suggests that there is a correlation of esophageal exposure to both acid and bile with an increasing severity of GERD, in more detail from benign erosive esophagitis to BE. Duodenal contents like pancreatic proteolytic

agents and bile salts are supposed to be essential in the development of metaplastic BE. In more detail it appears that acid is needed to activate proteolytic enzymes and enhance the capacity of bile salts to penetrate the mucosa of the esophagus. This is suggested by a study demonstrating that patients with a mixed acid-duodenal juice reflux have a higher prevalence of mucosal injury of the esophagus[30]. The majority of the current therapies are focused on the results of the acid-driven inflammation and its healing instead of the primary etiology. However it is clear that when a certain level of mucosal damage is caused in the lower esophagus, acid suppressive therapies may only be able to improve symptoms and can only maintain or stabilize the damage and scar that is already present. In this respect medical therapy, while successful in facilitating healing, can only do little more than maintain a steady state when at the same time the motor abnormalities of the lower esophageal sphincter, stomach and pyloric valve responsible for the reflux events, continue.

2.4 Biomarkers for Barrett's esophagus

A potential consensus requires the identification of the appropriate tools to detect BE early, identify the specific molecular markers associated with neoplastic transformation and establish a definitive therapeutic plan. Most of the factors studied in BE have been associated with cancer development in other organs. These include evaluation of cell proliferation, expression of cyclooxygenase 2, growth factors, oncogenes, secretory factors, cell cycle proteins, adhesion molecules, aneuploidy and other genetic abnormalities. Additionally to their role as potential biomarkers, these factors are gradually more reported as surrogate markers to examine the effectiveness of traditional treatments for BE.

Next to the above-mentioned largely immunohistochemical biomarkers, Cytokeratins (CKs) are promising candidates for use in the diagnosis of BE. CKs are the intermediate filaments as a part of the epithelial cytoskeleton. There are several CKs reported in the literature; CK1 to CK20. The CK expression profile is variable in epithelial cells; this expression pattern is depended on type, location and differentiation of the epithelium. Therefore CKs can be used for characterizing different tissues and also BE. Immunostaining for a subset of CKs have been reported to have potential use in the distinction of BE and intestinal metaplasia of the stomach. Ormsby *et al*. described a specific CK7-CK20 pattern for BE, characterized by a superficial CK20 staining of the surface epithelium and superficial glands and moderate and strong CK7 staining of superficial

and deep glands[31]. He reported that this CK7-CK20 pattern was not present in intestinal metaplasia of the stomach but this pattern was found in 100% of the biopsy specimens and in 94% of the resection specimens from patients with long-segment BE[31]. Nevertheless other groups did not obtain the same kind of results and currently there is debate regarding the contribution of CK immunohistochemistry in the diagnosis of BE[32-34].

The presence of goblet cells remains the defining feature in the histological diagnosis of BE. A new marker for BE, however, the homeobox gene CDX-2 has emerged. CDX-2 is a member of the caudal-type homeobox gene family. Members of this homeobox gene family are homologs of the caudal gene of *Drosophila melanogaster*. CDX-2 protein is an important transcription factor and plays a role in early differentiation and maintenance of the intestinal epithelium via regulating the transcription of intestine specific genes. In humans CDX-2 is normally expressed throughout the intestine, in general the gastro-duodenal junction is its proximal limit. Previously Phillips *et al*. showed that CDX-2 was expressed in the nuclei of goblet cells present in non-dysplastic BE, however occasionally goblet cells lacked CDX-2 nuclear staining[35]. All dysplastic BE tissue and adenocarcinoma showed nuclear CDX-2 stainings of the goblet cells, however the intensity of the staining varied according to the state of differentiation of the tissue.

3. Barrett's esophagus transformation

3.1 Transformation to esophageal adenocarcinoma

Several papers describe the pre-malignant BE and discuss the molecular events involved in the progression to EA and even the morphological cellular changes that characterize the dysplastic progression to EA are extensively reported. Chromosomal changes and accompanying genetic alterations occur, with resulting abnormalities in gene expression and cell cycle regulation. The incidence and timing of these changes are not reported in detail, however several papers describe a scheme of molecular events occurring in the progression to EA[13, 30, 36, 37]. Six major changes are necessary for a cell to become malignant, first a cell provides growth signals, ignores growth inhibitory signals, circumvents apoptosis, replicates with no boundary, maintains angiogenesis, invades and finally proliferates[38]. Recently, Morales *et al*. reported that these cancer hallmarks occur during the progression of BE to EA[13].

3.2 Transformation of squamous esophagus to Barrett's esophagus

Mechanisms underlying the progression to EA have been extensively studied, however the process by which squamous epithelium is replaced by specialized columnar epithelium is still poorly understood. When defense mechanisms like luminal secretion of mucus, growth factors and bicarbonate are overwhelmed by an continuing series of mucosal injury and repair, BE develops in the distal esophagus. The regions of damaged squamous epithelium are then gradually re-epithelized by a columnar epithelium. The origin of this epithelium is still debated. Initially several authors hypothesized upward cell migration from the junctional cardiac or gastric epithelium or glands[39]. This hypothesis however can be dismissed since several papers describe that columnar epithelium could develop in defective mucosa above a squamous barrier[40, 41]. In addition proximal migration of cardiac epithelium cannot explain the variety of epithelial cells in BE epithelium[42]. Therefore we can conclude that the cell that gives rise to the columnar epithelium is in the esophageal epithelium itself. Embryology taught us that the human embryonic esophagus is lined with columnar epithelium. At week 17 in embryology, the columnar epithelium of the mid-esophagus is progressively replaced by squamous epithelium. This process of squamous re-epitheliazation continues to the proximal and distal parts of the esophagus and is finished at birth[43]. These embryological developmental changes can explain the existence of an intrinsic cell within the esophagus that has the capacity to engage in columnar differentiation in specific circumstances. This specific cell could be the multi-potent stem cell that gives rise to the several different cell types that occur in BE[39]. However the columnar cells in BE may also originate from ductal cells of esophageal submucosal glands[44]. This hypothesis is based on the fact that the proximal two-thirds of the ducts of the esophageal glands are lined by columnar cells and the distal one-third by squamous cells. Superficial injuries would give rise to a mixed pattern of regeneration since both mucosal and glandular ductal cells survived, though the columnar cells would be more dominant since columnar cell turnover is more rapid[41, 45]. Nevertheless if due to harsh injuries the squamous cells are destroyed, the epithelium can only be rebuild by columnar cells[42]. The molecular events involved in this process are not well understood. Theoretically numerous factors can be involved in the process to induce metaplasia like the concentration of stem cells and expression of genes encoding for transcription factors involved in differentiation[46].

4. High trough-put technique for characterizing Barrett's esophagus

4.1 Gene Expression Profile Analysis

In order to understand the histological concept of a cell or tissue it is important to analyze the complete profile of gene expression present in a cell or tissue. In contrast to the genome, the transcriptome is variable and depends on gene function, developmental and disease state of the individual. Therefore analysis of the gene expression can help to more precisely classify and describe the different characteristics of a cell or tissue, furthermore it can help to improve the insight of the biological mechanisms and pathways involved in a cell or tissue. Especially diseased cells or tissues are important to analyze and compare to normal cells or tissues, since this information could help to develop novel screening and preventive strategies.

Several gene expression analysis techniques give the ability to compare sets of expressed genes. This set of expressed genes is the fingerprint or gene expression profile of this cell, tissue or organism. Previously several studies have pointed out differences in expression levels of one or a few genes, but until 2002 no comprehensive study on gene expression in BE had been reported[47, 48]. Therefore it was not known what differences there were in the BE gene expression profile in comparison to the gene expression profile of normal esophageal tissue, whether the majority of these differences are cell-autonomous or dependent on the Barrett micro-environment, and whether most differences are cell type specific or Barrett specific. Progression in technology made it possible to answer these questions using SAGE and microarray. Genes that are abundantly or exclusively expressed in BE may be important in the phenotypic changes that occur in the transition of squamous esophagus to BE and could help in defining and interpreting the differences that underlie in the cell specific phenotypes. Furthermore the identification of genes that are exclusively expressed in BE may be important for clinical implications, as these may be useful as tissue markers for BE. However, cellular changes within BE development may involve only a small subset of genes and expression of certain genes may be even at very low levels or not detectable at all. Several gene chip analysis studies have been performed, for instance through comparing BE and esophageal carcinoma, or through comparison of the gene expression profiles of BE and intestinal metaplasia of the cardia[47, 49]. Selaru *et al*. found that BE was separately clustered from adenocarcinoma, indicating that BE has its own profile on gene expression level, compared to its related cancer[47]. Recently, microarray analysis has been

performed in which BE was compared with duodenal, gastric and normal squamous epithelium[48]. In this study a closer correlation between BE and the surrounding normal epithelia was found, while the expression profiles between the fully differentiated normal epithelia showed less similarity[48].

One of the toughest parts in comparing different gene expression profiles is determining the biological significance of the significantly differentially expressed genes found in the comparisons. An option would be to analyze the genes in more detail by additional techniques and to predefine a field like biological phenomenon, assay, or marker.

4.2 Microarray analysis

In 1995, Adams *et al*. provided the first description of gene expression profiles from different human tissues. By sequencing cDNA clones he tried to identify new genes and analyze their expression patterns, but in the end this technique demonstrated to be useful for the identification of genes, however not for the determination of their quantitative expression profile[50]. Other methods, such as cDNA or oligonucleotide arrays were also used to compare the expression levels of thousands of genes in different tissues and organisms[51, 52]. However, these arrays were limited, because they had only the ability to analyze previously identified transcripts, currently whole human and mouse genome oligonucleotide arrays are commercially available. Nevertheless to analyze gene expression profiles for organisms with poorly characterized genomics and expressed sequences the microarray technique is still inadequate. Although the complete human genome is sequenced it is still a huge challenge to predict all encoded genes[53, 54].

Microarray technology uses relative measurements of mRNA transcription levels. On a glass slide DNA molecules are spotted at a fixed location (Figure 1). Each spot is related to a single gene, although multiple spots can represent the same gene to measure replicates. Microarrays use hybridization of the sample to the spots, in other words binding of complementary single-stranded nucleic acid sequences to the DNA molecules spotted on the array[55]. A strong fluorescent signal is the result of a good chemical binding and thus a good match between the mRNA and complementary DNA. A wide range of data analysis programs is available to extract the fluorescent intensity values for each spot in the array from the image. The initial step in microarray data analysis consists of grid finding, spot fitting, and spot measurements algorithms. Apart from the spot measurement a background measurement is performed. Before the actual

data can be investigated, analysis of the raw microarray data is necessary in more detail like background correction, data filtering and normalization and ratio calculation (transform Cy3 and Cy5 intensities to ratios). Finally the data can be investigated using several methods and data cluster analysis can be performed. For cluster analysis numerous different techniques can be applied, however whatever clustering technique is used, the results are not unique but dependent on the settings of the clustering technique and the steps that are applied on the raw data prior to clustering. Therefore it is extremely essential to have an expert in the field when doing microarray analysis.

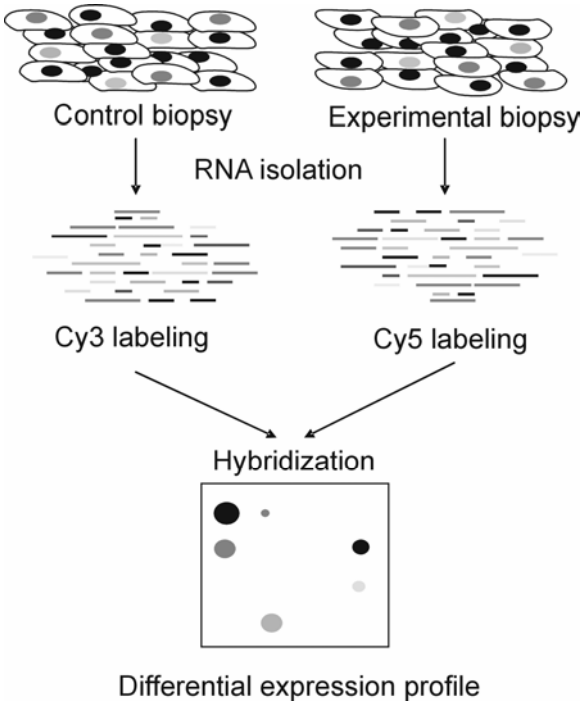

Figure 1: Microarray technique. RNA is isolated from the samples and labeled with Cy3 or Cy5. On a glass slide DNA sequences are spotted at a fixed location. These glass slides are hybridized with the Cy3/Cy5 labeled samples. After washing, hybridization of the samples to the spots can be relatively measured. Comparing the Cy3 with the Cy5 labeled samples provides differential gene expression profiles.

4.3 Serial Analysis of Gene Expression

Another technique to analyze gene expression profiles is Serial Analysis of Gene Expression (SAGE). The SAGE technique was first described by Velculescu *et al*. and allows a comprehensive quantitative and qualitative analysis of a large number of transcripts at once without prior knowledge of its abundance[56, 57]. SAGE is based on two principles: first, 10 bp tags are derived from the most 3' *Nla*III cleavage site of the transcripts (Figure 2). Since the location of the tags within the transcripts is exactly defined, these tags contain enough information to identify unique transcripts through public databases (SAGEgenie, http://cgap.nci.nih.gov). In

theory, a 10 bp tag can give 4^{10}, so over 1,000,000 different sequence combinations. This is adequate to distinguish between all transcripts from the human genome[58-60]. Secondly, by cloning these tags serially, along with a restriction enzyme recognition sequence that serves as an anchor, into a vector and transfect into *E. coli*, a large amount of transcripts can be identified efficiently by sequencing, revealing the identity of thousands of tags at the same time (Figure 2). The sequence data are matched to genome sequences to identify the gene corresponding to the tag. The amount of times a particular tag is observed in the library provides a quantitative measurement of the gene expression abundance in the sample. The 10 bp tags contain enough information to identify most of the genes from the human genome. Nevertheless it is possible that several tags correspond to the same gene if there are alternative 3' splice variants or poly-adenylation sites. Also a few examples of tags that are corresponding to several genes are reported in literature, frequently these tags have a low sequence complexity. Therefore it is strongly recommended to perform follow-up studies of interesting genes using additional techniques, like RT-PCR, Northern blotting and in situ hybridization.

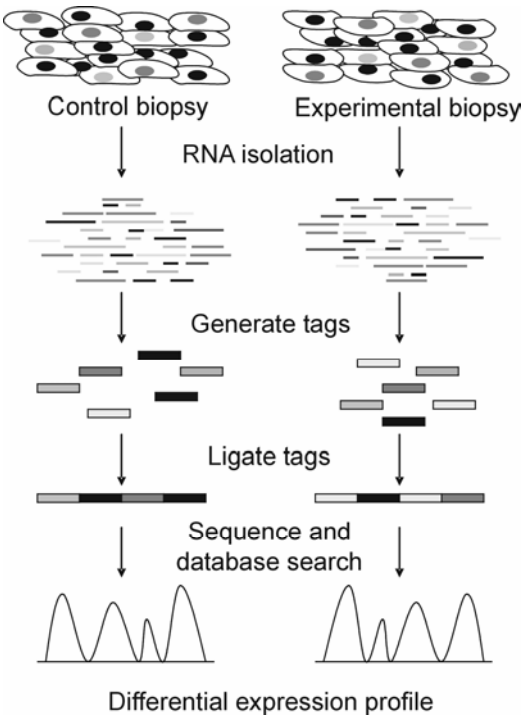

Figure 2: SAGE technology. RNA is isolated from the samples. From each mRNA transcript a 10 bp tag is isolated. These tags are ligated behind each other into concatemers. These concatemers are ligated into a vector, transfected into *E. coli* and sequenced. The sequence results provide quantitative and qualitative information of the samples. Comparison of the SAGE libraries provides differential gene expression profiles.

It is possible that tags, identified using SAGE, can have no match in public databases, and thus can represent potentially new genes. Yet, we should keep in mind that because of sequence artefacts, some tags can incorrectly be assigned to a certain gene cluster. Particularly, singleton tags should be carefully considered, although these generally correspond to mRNAs expressed at very low levels, some may be due to sequencing errors. Previously published SAGE libraries showed that singleton tags were found in approximately 25% of each library[61]. Furthermore it should be noted that the Cancer Genome Anatomy Project is developing continuously, so the assignment of tags to certain gene clusters can change over time. Constantly many Expressed Sequence Tags (EST's) have been better characterized and as more data is becoming available from the Human Genome Project our insight in the SAGE transcriptomes will further enlarge.

4.4 Microarray and SAGE

The choice of which technique to use, SAGE versus microarray, is dependent on diverse aspects, such as the amount of starting material, the number of samples to be analyzed, the genetic field of research interest and the availability of resources in the laboratory for example an automated DNA sequencer. With the current advances in technology, an automated sequencer should not be an issue anymore also good software to analyze microarray data should not be a problem. For small amounts of starting material, the technique SAGE is useful. The amount of RNA necessary for SAGE is reduced compared to microarray, because SAGE has a PCR amplification step. Recently SAGE libraries have been made using really small amounts of mRNA, 50,000 cells, 0.5 mg of tissue or 1 pg poly(A)+ mRNA were used to generate SAGE libraries[62-64]. This brings the SAGE technique reachable to a new dimension; analyzing gene expression profiles in a single cell. Schober *et al*. already reported a single cell SAGE library, but the quality of this library remains unclear[65].

Other aspects to choose between SAGE and microarray are the amount of samples. For analyzing the gene expression profiles of a large amount of samples, microarray analysis is more efficient and less time consuming. Regarding the genetic field of research interest, the technique SAGE is particularly appropriate, because it does not need a hybridization probe for each transcript and new genes can be discovered. Therefore using RNA of organisms whose genome is not completely sequenced, SAGE is preferably used since it allows the generation of a library of thousands of expressed genes without any previous knowledge of the cell's repertoire.

Several studies have compared SAGE with microarray. They report that there is a good correlation between the two techniques, though SAGE was found to be more quantitatively reproducible[61, 66, 67]. Ibrahim *et al*. reported a comparison of SAGE and Affymetrix arrays and concluded that a broader range of gene expression was obtained by SAGE, since almost 25% of the unique SAGE tags had no corresponding DNA probe on the microarray[61]. Ideal would be to combine both techniques and screen broadly using the SAGE technique, identify genes and interesting pathways and then develop a more focused microarray to analyze more samples[66]. Especially in cancer research the advantage would be that interesting genes and pathways not previously described in a certain cancer, can rapidly be identified and validated for clinical relevance in a larger number of samples, resulting in a possible tumor marker or prognostic factor.

The main advantage of the SAGE technique is that SAGE data often digital accessible is in databases of the NCBI-website and as such is accessible to the whole scientific community. Therefore it is possible that the whole scientific community can benefit from these data and can directly make a comparison between different SAGE libraries from different laboratories[68]. This powerful tool makes it possible to compare over a 100 different human SAGE libraries and perform virtual Northern blots of these SAGE libraries[68]. In contrast to this is the microarray technique. Comparing different microarray experiments from different investigators and different laboratories can be more of an issue. The main problem is that the statistical approaches for microarray research are not routine yet, there are numerous of potential sources of measuring random and systematic errors. Different investigators use their own source and in this way it is difficult to compare different microarray experiments from different investigators[69].

4.5 Kinome analysis

Recently gene expression profiling has been applied to verify differentially expressed genes that are involved in for example cancer development. These techniques are very potent to discover differentially expressed genes not known to be important in malignant transformation, however substantial gaps remain in understanding the development of cancer despite the use of these techniques. The main problem is that these techniques identify interesting genes but there is no information of the corresponding protein expression levels. Since RNA levels do not necessarily correlate with protein levels, it is as important to investigate protein expression[70, 71]. Therefore

proteomic techniques are in fashion. These methods can identify new biomarkers and therapeutic targets for the detection and treatment of cancer[72]. Using proteomics however, still leaves an important gap in understanding malignant transformation. Cellular events like growth and differentiation are regulated through signal transduction pathways, in which activated proteins are important. Therefore the expression levels of proteins are of less importance than the state of activation of the proteins. This kind of information is of significant value to elucidate the molecular mechanisms that govern esophageal cell physiology and differentiation. Furthermore, it is an important goal to define those proteins that contribute in signaling pathways that participate in the development of BE and provide critical information for understanding this pre-malignant condition.

Recently Irish *et al*. reported that only a small part of the transcriptome is important in characterizing the specific functions of a cell[73]. From genome and transcriptome analysis studies it is known that the greater part of the cell's transcripts is necessary for the cell to continue its basal level of functioning[73, 74]. The small part of the transcriptome that characterizes the cell specifically can result in huge differences in enzymatic activity which results in specific cell characteristics.

Recently a new technique, the pep-chip, has been developed[74]. This is an array containing 1176 different peptides, each peptide is a kinase specific consensus sequence. The method is first described by Diks *et al*. and can perfectly be used to analyze cellular metabolism of cell lysates or complete tissue lysates[74].

Pep-chip uses relative measurements of phosphorylation of the kinase consensus sequences. On a glass slide the kinase consensus sequences are twice spotted in a fixed location together with 12 control spots also in duplo, used as internal controls. Each sequence contains a tyrosine, serine or threonine site which can be phosphorylated by the kinases present in the sample applied to the pep-chip. A strong spot correlates with an optimal phosphorylation of the kinase consensus sequence. The same data analysis programs used for microarray analysis can be used for analyzing the pep-chip, like grid finding, spot fitting, background measurement and spot measurement.

Kinases that are up- or downregulated can lead to different cellular events and as such specifically direct a cell or tissue into its own characteristic. These analyzed cellular events gives information about the signal transduction pathways that are going on in this cell or tissue. Several

signaling cascades are dependent on enzymes that phosphorylate the tyrosine, serine and threonine sites on other proteins. These signaling cascades include cell growth, cell cycle, survival and differentiation fate of the cell or tissue.

The main advantage of the pep-chip is that analysis of multiple kinases is done at once, whereas the traditional genetic and biochemical approaches can only pursue one gene or pathway at a time. The kinome profiles of normal cells or tissues are substantially different from the kinome profile of cancer cells or tissues, because in cancer different signal transduction pathways are involved then in the maintenance of normal cells or tissues. These different signal transduction pathways can be identified using the pep-chip and as such can give a better insight in cancer development.

5. Aim and structure of this thesis

The aim of this thesis is to determine which genes and signal transduction pathways are involved in BE development. To achieve this purpose, mainly patient material was used.

Chapter 2 describes the transcriptome analysis of BE in a comparison to normal squamous esophagus and gastric cardia using the technique SAGE. Here we describe that certain factors are specifically expressed in the metaplastic BE, furthermore we report a specific CK expression pattern for the three epithelia. **Chapter 3** reports our findings when one of the factors found in the previous SAGE analysis, BMP4 was added to primary cell cultures. Furthermore it describes the role of the BMP pathway in inflamed squamous esophagus and BE. **Chapter 4** describes the kinome analysis of BE compared to the kinome profiles of normal squamous esophagus and gastric cardia, focusing on EGF receptor signaling, the glycolysis and MAPK signaling cascade. **Chapter 5** reports the SAGE analysis of primary cell cultures of BE and normal squamous esophagus in a comparison to the gene expression profiles of biopsies. This chapter describes which genes are specifically expressed by the epithelial cell layer in a comparison to the stromal epithelium, surrounding the epithelial cells. **Chapter 6** describes the gene expression profile analysis of EA compared to metaplastic BE in order to find genes involved in the malignant transformation. Furthermore this chapter reports our findings comparing the transcriptomes of esophageal squamous cell carcinoma compared to normal squamous esophagus. Finally **chapter**

7 reports our findings of expression levels of several CKs and CDX-2 in BE, normal squamous esophagus and gastric cardia in a Barrett population.

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Chapter 2

A comparative analysis by SAGE of gene expression profiles of Barrett's esophagus, normal squamous esophagus and gastric cardia

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Abstract

The metaplastic process in which the normal squamous epithelium of the distal esophagus is replaced by columnar lined epithelium, known as Barrett's esophagus (BE), is poorly understood. The aim of this study was to define, analyze and compare transcription profiles of BE, normal cardia and squamous epithelium, to gain more insight in the process of metaplasia and to identify uniquely expressed genes in these epithelia. Serial analysis of gene expression (SAGE) was applied for obtaining transcription libraries of biopsies taken from a BE patient with intestinal type of metaplasia, and from normal squamous and gastric cardia epithelia. Validation of results by RT-PCR and immunoblotting was performed using tissues of 20 BE patients. Over a 120,000 tags were sequenced. Between BE and squamous 776, and BE and gastric cardia 534 tags were significantly differentially expressed ($p<0.05$, Pair-wise comparison). In contrast squamous compared to cardia showed significant differential expression of 1316 tags. The most up-regulated genes in BE compared to squamous epithelium were Trefoil factors, Annexin A10 and Galectin 4. Each of the epithelia showed a unique cytokeratin expression profile. This study provides a comparison of the transcriptomes of BE, squamous and gastric cardia epithelia. BE proves to be an incompletely differentiated type of epithelium that shows similarities to both normal squamous and cardia epithelia. In addition several uniquely expressed genes are identified. These results are a major advancement in understanding the process of metaplasia that leads to BE.

Introduction

Barrett's esophagus (BE) is a pre-cancerous condition in which the normal squamous epithelium of the esophagus is replaced by metaplastic, columnar lined epithelium[1, 2]. In Western countries, the prevalence of BE and the incidence of esophageal adenocarcinoma has been increasing rapidly[3]. The increasing prevalence of BE is alarming and calls for screening programs of high risk populations and development of preventive therapies[4]. The transition of BE into cancer is a process known to go along with the accumulation of several genetic events such as aneuploidy, expression of oncogenes, and losses of cell surface receptors, and tumor suppressor genes[5-8]. To understand the histological concept of BE, several gene expression profile and gene chip analysis studies have been performed, for instance through comparing BE and esophageal carcinomas, or through comparison of the gene expression profiles of BE and intestinal metaplasia of the cardia[9, 10]. Recently, gene chip analysis has been performed in which BE was compared with duodenal, gastric and normal squamous epithelium[11]. Indeed, to develop novel screening and preventive strategies, it is of major importance to understand the biological pathways involved in the metaplastic transition of normal squamous epithelium into columnar epithelium. We hypothesized that objective, quantitative analyses of large molecular genetic data sets of BE and the surrounding normal epithelia such as normal squamous esophageal and normal gastric cardia mucosa, will accurately classify the different phenotypes of these epithelia and improve our insight in the biological mechanisms involved in the process of metaplasia.

In the present study, gene expression profiles were obtained by using serial analysis of gene expression (SAGE). The procedure as first described by Velculescu *et al.* allows rapid, quantitative and simultaneous analysis of thousands of genetic transcripts from tissue samples[12]. SAGE is based on two principles: first, a short nucleotide sequence, a tag, is generated. Since the location of the tags within the transcripts is exactly defined, these tags contain sufficient information to identify transcripts through public databases (SAGEgenie, http://cgap.nci.nih.gov). Secondly, by cloning these tags serially, along with a restriction enzyme recognition sequence that serves as an anchor, a large amount of transcripts can be identified efficiently by sequencing. This reveals the identity of thousands of tags and at the same time it quantifies their level of expression.

For the present study, SAGE profiles are made of RNA isolated from BE, normal squamous epithelium and normal gastric cardia mucosa of a BE patient. A panel of another 20 BE patients is used to validate the profiles by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and immunoblotting. SAGE is performed, resulting in three unique SAGE libraries with over a 120,000 identified tags. In particular, the whole spectrum of Cytokeratins (CK1 to CK20) as found in the three epithelia is described and expression of the most informative CKs are validated at protein level.

The results indicate that BE is not a fully differentiated phenotype, but rather an incompletely trans-differentiated lesion that has strong similarities to both, normal squamous esophageal epithelium and the columnar cardia mucosa. This study provides an important step toward a transcriptome of Barrett's metaplasia as a comparison to its surrounding epithelia in which Barrett's metaplasia develops. The unique profiles harbors a wealth of information and provides us the identity of several genes involved in several cell signaling pathways, which will contribute to understand and elucidate important biological processes involved in metaplasia. In addition, several unique genes that can be used as novel markers for distinguishing the different type of epithelia are identified.

Materials and Methods

Patients and Biopsy Specimens

Tissue samples were obtained during routine surveillance endoscopy of 21 patients with known BE but without dysplasia, 18 were male, mean age was 62 years (range 33-83 years). The average length of the BE segment measured endoscopically was 3.8 cm (range 2-9 cm). All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. BE was defined as histologically recognized incompletely differentiated intestinal type of metaplasia in the distal esophagus. 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 columnar type of metaplasia. Normal squamous epithelium was taken at least 2 cm above the Barrett's segment and gastric cardia was taken within 5 cm below the gastroesophageal junction. Endoscopically, none of the patients had reflux

esophagitis. All patients had proven incompletely differentiated intestinal type of columnar epithelium without dysplasia in the histological control biopsies with no signs of active or acute inflammation. Normal gastric cardia and normal esophageal squamous were also confirmed histologically, in all the pairwise taken control biopsies. All patients signed informed consent for the use of their biopsy material.

RNA isolation

Total RNA was isolated from biopsies using Trizol Reagent (Life Technologies Inc, Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. Spectrophotometry was performed with 1 µl of total RNA to quantitate on the Nanodrop® (type ND-1000, Wilmington, USA).

SAGE procedure

The SAGE libraries were obtained essentially following the SAGE protocol as described by Velculescu *et al*. using the Life Technologies I-SAGE kit and following manufacturer's instructions[12]. Electroporate transformation was done following manufacturer's protocol (Biorad, Hercules, CA). Colony PCR was performed with specific primers Sp6-F and T7A-R. DNA sequencing was done using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) and the T7A-R primers. Samples were run on an ABI3730 DNA Analyzer (Applied Biosystems) and analyzed with Sequence Analysis 5.1 software.

SAGE and Statistical Analysis

For analysis of the SAGE data the program USAGE V2 (Academic Medical Center, bioinformatics department) and the public databases of the NCBI-site and SAGE Genie (http://cgap.nci.nih.gov) were used[13, 14]. Statistical analyses and comparison of the SAGE libraries was done using a comparative *Z*-test (Pair-wise comparison, binominal approach) of the USAGE V2 program[15, 16].

RT-PCR

cDNAs from biopsies of 20 patients were synthesized from 1 µg of total RNA using an oligo dT primer and Superscript II MMLV-reverse transcriptase according to manufacturer's instructions

(Life Technologies). Primers for selected genes (Table 1) were derived from mRNA sequences as deposited in GenBank (NCBI-site). PCR analyses were carried out using Reddy Mix PCR Master Mix (Applied Biosystems). The mRNA expression level was determined by the ratio of signal intensity of the mRNA to that of the β-actin. Data are expressed as means ± Standard Error of Mean (SEM). Comparison between two groups was analyzed using two-tailed paired *t*-tests.

Immunoblotting

Immunoblotting was performed as described by Hardwick *et al*.[17]. Biopsies were lysed with 200 µl lysis buffer. Twenty mg of protein per lane was loaded onto SDS-PAGE. The blots were blocked with 2% BSA in Tris Buffered Saline supplemented with 0.1% Tween-20. The antibodies used and dilutions are summarized in Table 2.

Gene	Forward primer	Reverse primer	Annealing temperature	Fragment length
TFF1*	TTTGGAGCAGAGAGGAGG	TTGAGTAGTCAAAGTCAGAGCAG	60ºC	438 bp
TFF2	ATGGATGCTGTTTCGACTCC	GGCACTTCAAAGATGAAGTTG	55ºC	247 bp
TFF3	GTGCCAGCCAAGGACAG	CGTTAAGACATCAGGCTCCAG	58ºC	303 bp
CK7**	TGAATTAACCGCCGCACAG	TGCATTTGGCCATCTCCTCA	65ºC	277 bp
CK20	GGGACCTGTTTGTTGGCAATG	ATTTGCAGGACACACCGAGCAT	55ºC	247 bp
Annexin A10	TTGTTCTCTGTGTTCGAGACAAACC	GTAGGCAAATTCAGGATAGTAGGC	52ºC	609 bp
Galectin 4	GCTCAACGTGGGAATGTCTGT	GAGCCCACCTTGAAGTTGATA	60ºC	461 bp
FABP1***	TCATGAAGGCAATCGGTCTG	GTGATTATGTCGTCGCCGTTGAGT	55ºC	277 bp
β-actin	GTCAGAAGGATTCCTATGTGG	GCTCATTGCCAATGGTGATG	52ºC	628 bp
β-2-microglobulin	CTCGCGCTACTCTCTCTTTCT	TGCTCCACTTTTTCAATTCTCT	60ºC	185 bp

Table 1: Primer sequences. Primer sequences used for RT-PCR with corresponding used annealing temperatures and PCR fragment lengths. *) TFF= Trefoil Factor **) CK= Cytokeratin ***) FABP1= Fatty Acid Binding Protein 1

Antibody	Species	Company	Country	Dilution
Cytokeratin 5/6	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 7	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 8	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 10/13	Mouse monoclonal	Dako	Denmark	1:500
Cytokeratin 18	Mouse monoclonal	Sigma	USA	1:500
Cytokeratin 20	Mouse monoclonal	Progen	Germany	1:500
Actin (I-19)	Goat polyclonal	Santa Cruz	USA	1:2000

Table 2: Antibodies as used for immunoblot analysis.

Results

Three unique SAGE libraries were obtained, totally consisting of over 120.000 tags. The SAGE library characteristics are described in Table 3. The complete SAGE libraries can be found on the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/; Table 3). A minority of the identified tags correspond with different genes due to the presence of conserved sequences and common repeats in the 3' un-translated mRNA transcript. An example is the tag TTTTCTGAAA which matches with several genes, namely Thioredoxin and Surfeit 5. Various different tags can represent the same gene, for instance the Expressed Sequence Tags (ESTs) of GATACTGCCT, AAAGCACAAG and ATGTAATCAC correspond to the gene cluster of Keratin 6A. This variation may be the result of alternative splicing, alternative polyadenylation, or polymorphisms in the mRNA from which these tags are derived.

	Squamous	**Barrett**	**Gastric cardia**
Total tags	50.508	46.269	25.797
Unique tags	14.835	16.058	8.810
Singletons	4.168 (21%)	4.430 (25%)	6.485 (25%)
Tags 5-times present	1.201	1.202	612
Tags 10-times present	538	545	262
Accession code	GSM52501	GSM52502	GSM52500

Table 3: SAGE library characteristics. Number of total tags in the squamous, Barrett and gastric cardia libraries, together with the corresponding accession code in the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/), the number of unique tags, the number of singletons and the number of tags at least 5 times and 10 times present in each of the libraries. Calculation of the percentages of singleton tags was based on the total number of tags present in the libraries.

Comparison of the expression profiles of Barrett's esophagus with normal squamous epithelium and normal gastric cardia

Between the BE and squamous SAGE library 776 tags were significantly differentially expressed ($p<0.05$), 72 tags were more than 10 fold up-regulated and 26 more than 10 fold down-regulated (supplemental data, Table 1). The BE SAGE library as compared to the gastric cardia library showed 534 tags significantly differentially expressed ($p<0.05$). Thirty-one tags were more than 10 fold up-regulated and 76 tags were more than 10 fold down-regulated in BE compared to gastric cardia (supplemental data, Table 2). Between the squamous esophagus and gastric cardia SAGE libraries 1316 tags were significantly differently expressed ($p<0.05$). From these 108 tags were more than 10 fold up-regulated and 140 tags were more than 10 fold down-regulated in the squamous epithelium. Genes were also clustered in groups of biological processes (see supplemental data).

Validation of SAGE results

Expression levels of Trefoil factor (TFF) 1, TFF2, TFF3, Galectin 4, Annexin A10 and FABP1 were verified by RT-PCR. In all cases examined, the expression of genes represented by tags in either SAGE library was confirmed. TFF1, TFF2, TFF3, Annexin A10, Galectin 4 and FABP1 were significantly higher expressed in all BE samples compared to all control squamous esophageal samples (Figure 1).

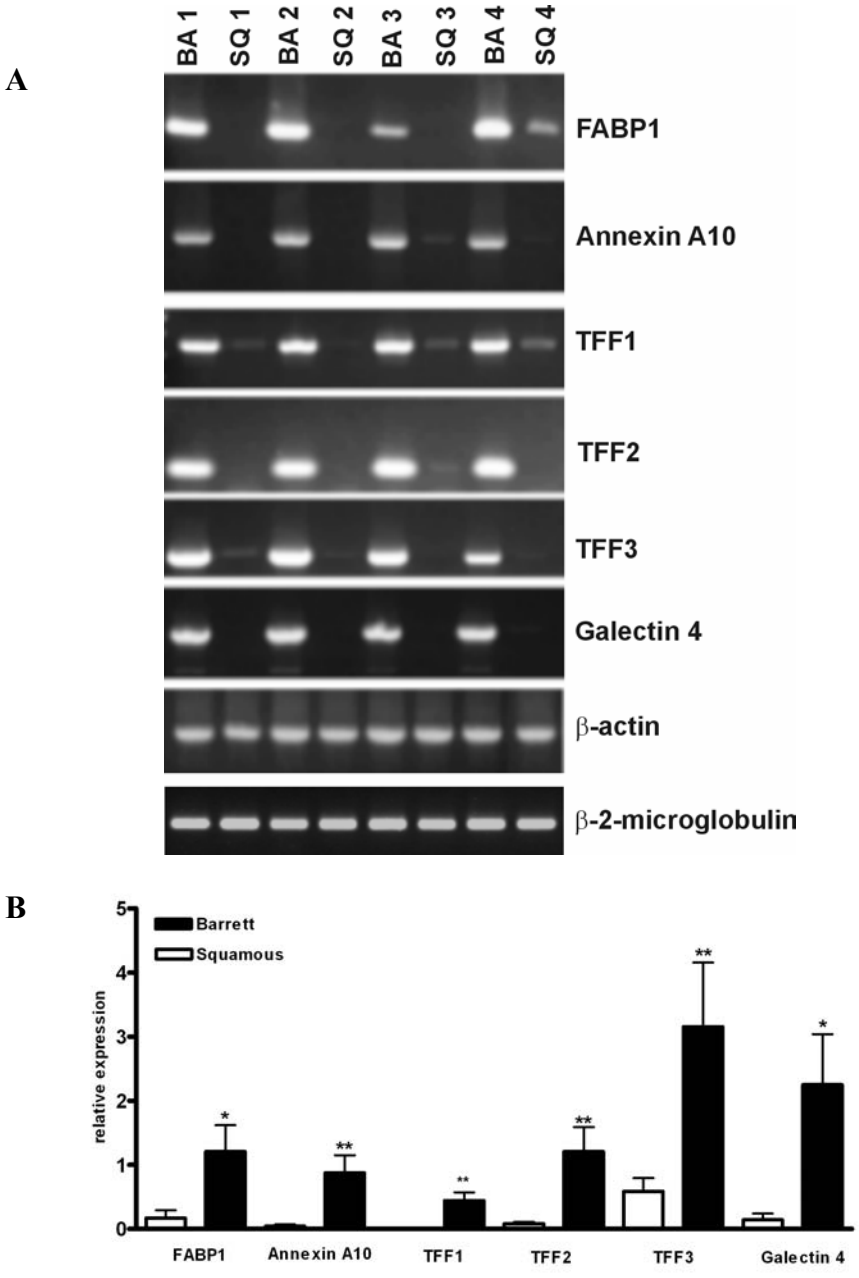

Figure 1: RT-PCR for validation of SAGE results of Barrett's and normal squamous epithelium. RT-PCR on RNA from Barrett's esophagus (BA) and Squamous esophagus (SQ) biopsies from different patients, demonstrates that Fatty Acid Binding Protein 1 (FABP1), Annexin A10, Trefoil Factor (TFF) 1, TFF2, TFF3 and Galectin 4 are highly expressed in the Barrett biopsies, but virtually absent in squamous epithelium (A). β-actin and β-2-microglobulin were used as a control. Quantitafication of RT-PCR results shows that FABP1, Annexin A10, TFF1, TFF2, TFF3 and Galectin 4 in Barrett and squamous esophagus of 20 patients are significantly higher expressed in the Barrett biopsies (B; two-tailed paired *t*-tests; * $p<0.05$, ** $p<0.01$). The gene expression levels were determined by the ratio of signal intensity of the mRNA to that of the β-actin. Data are expressed as means ± SEM.

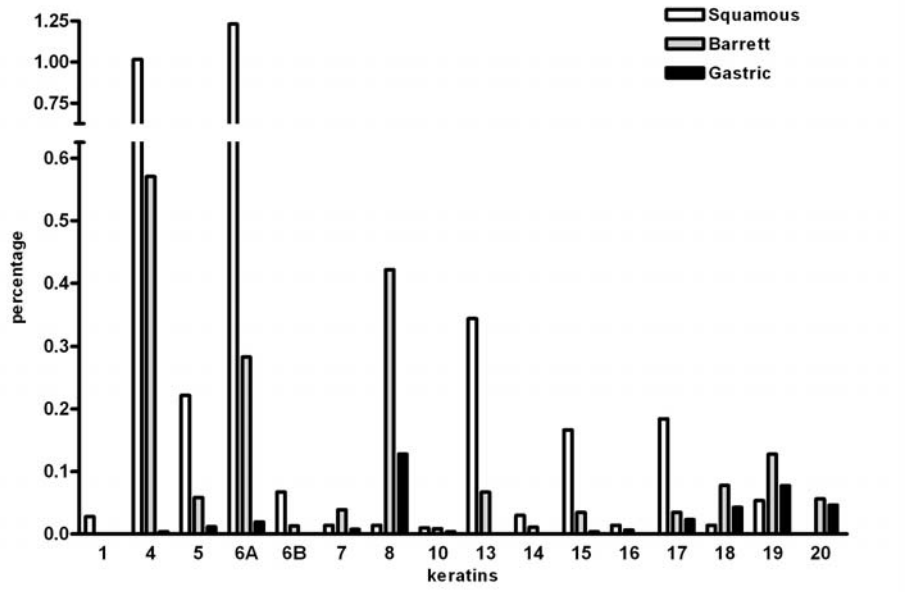

Figure 2: Cytokeratin gene expression patterns in Barrett, squamous esophagus and gastric cardia. Keratin expression patterns in the SAGE libraries of Barrett's esophagus, normal squamous epithelium and gastric cardia. Keratins 4, 6A, 6B and 13 were higher expressed in squamous epithelium. Keratins 7, 8, 18, 19 and 20 were higher expressed in Barrett's esophagus compared to squamous epithelium. Keratins 18 and 20 were highly expressed in both the Barrett's esophagus and the gastric cardia SAGE libraries.

Cytokeratin expression patterns

Specific CK expression patterns were found for the three epithelia by SAGE and verified by RT-PCR and immunoblotting. Tags corresponding to CKs 7, 8, 18, 19 and 20 were significantly higher expressed in BE compared to squamous ($p<0.05$), whereas tags corresponding to CKs 1, 4, 5, 6A, 6B, 13, 14, 15 and 17 were significantly higher expressed in squamous esophagus ($p<0.05$; Figure 2). Similar expression in BE and squamous was found for CKs 10 and 16. Compared to the gastric cardia SAGE library, CKs 4, 5, 6A, 7, 8, 13, 15 and 19 were significantly higher expressed in BE ($p<0.05$; Figure 2).

The CK expression profiles were validated by RT-PCR. CK7 and 20 were both significantly higher expressed in BE comparing to normal squamous epithelium (Figure 3). Validation on protein level confirmed the high expression of CKs 7, 8, 18 and 20 in BE epithelium as compared to normal squamous tissue (Figure 4). Immunoblot analysis also showed high expression of CKs 5/6 and 10/13 in normal squamous esophagus compared to BE (Figure 4). CKs 8, 18 and 20 were highly expressed and CK7 was less expressed in gastric cardia (Figure 4).

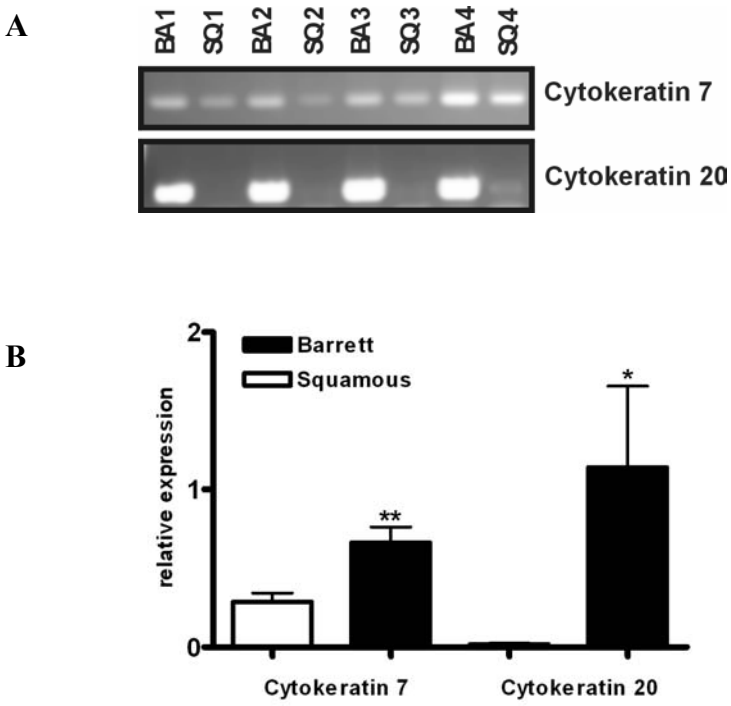

Figure 3: RT-PCR validation of Cytokeratin 7 and Cytokeratin 20 of Barrett and squamous epithelium. Validation by RT-PCR on RNA from Barrett's esophagus (BA) and squamous esophagus (SQ) biopsies from several patients. RT-PCR shows a higher expression of Cytokeratin 7 and Cytokeratin 20 in Barrett versus squamous epithelium (A). Quantitafication of RT-PCR results of gene expression of Cytokeratin 7 and Cytokeratin 20 in Barrett and squamous esophagus of 20 patients shows that these are significantly higher expressed in the Barrett biopsies (B; two-tailed paired *t*-tests; * $p<0.05$, ** $p<0.01$). The gene expression levels were determined by the ratio of signal intensity of the mRNA to that of the β-actin. Data are expressed as means ± SEM.

Discussion

In this study, SAGE technology was applied to identify the entire transcription profile of BE as a comparison to the profiles of normal squamous and gastric cardia epithelia. The specific information gained from this study helps us to identify factors involved in the metaplastic process and to identify uniquely expressed tissue specific genes.

The main advantage of SAGE compared to expression micro-arrays and other gene chip technologies is that it allows the generation of a library of thousands of expressed genes without any previous knowledge of the cell's repertoire. The obtained SAGE transcriptome conveys not only the identity of each expressed gene but also quantifies its level of expression.

In this study over a 120,000 tags were analyzed. Comparison of the SAGE-generated tag expression profiles of BE, normal esophageal squamous and gastric cardia epithelia identified hundreds of differentially expressed transcripts. Yet, it should be noted that because of sequence artefacts, some tags could incorrectly be assigned to a certain gene cluster. Particularly, singleton tags should be carefully considered, although these generally correspond to mRNAs expressed at very low levels, some may be due to sequencing errors. Singleton tags were found in

approximately 25% of each library, which is in accordance with previously published other SAGE libraries[18].

Mapping the SAGE tags to known genes and mRNAs in the SAGE Genie database revealed a large number of genes known to be expressed in BE, as well as many genes not previously recognized in BE. For instance, the Barrett SAGE library confirmed high expression of Mucin 5 (TGCACAATAT), TFF1 (CTGGCCCTCG) and TFF3 (CTCCACCCGA)[19]. In the gastric cardia SAGE library high number of tags were found for instance for Glutathione Peroxidase 2 (GGTGGTGTCT), known to be highly expressed in the stomach[20]. Also the tag CAGTGCTTCT

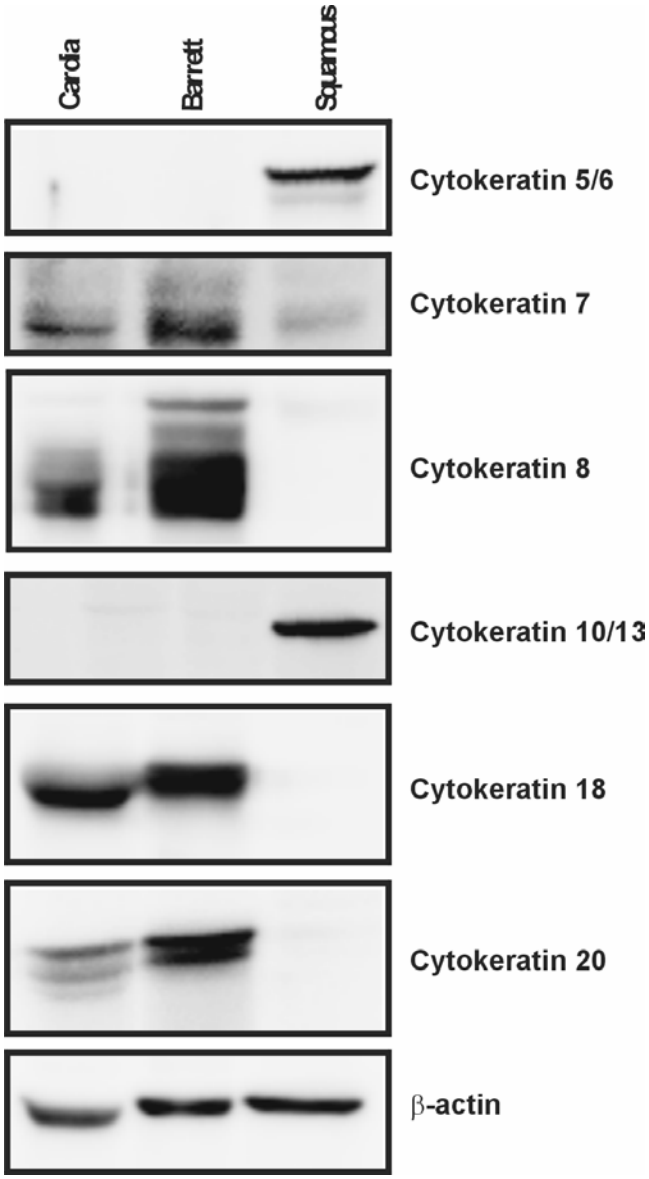

Figure 4: Cytokeratin expression in squamous esophagus, Barrett's esophagus and gastric cardia. Immunoblot analysis of Cytokeratins 5/6, 7, 8, 10/13, 18 and 20 expression in squamous esophagus, Barrett's esophagus and gastric cardia shows higher expression levels of Cytokeratin 5/6 and 10/13 in squamous epithelium, while Cytokeratin 7, 8, and 20 are highly expressed in Barrett's esophagus and less in the cardia biopsies. β-actin was used as a control.

(mapping to Deleted in Lymphocytic Leukemia 1 or Gastric Lipase) is known to be highly expressed in the stomach (http://cgap.nci.nih.gov/). The squamous SAGE library showed high expression of the tags GGCAGAGAAG and AAAGCGGGGC corresponding to Keratin 4 and 13, both keratins are known to be expressed on protein level in squamous esophagus and not in BE[21, 22].

Unlike the genome, the transcriptome is variable and depends on gene function, developmental and disease state of the individual. To prevent as far as possible confounding of the results by inflammatory factors, we only included patients with long term acid suppression and without active reflux esophagitis. We preferred to use tissue samples of one male individual known with non dysplastic BE, for making the SAGE libraries. For confirmation it is mandatory to verify RNA expression levels on a larger panel of samples. In this study tissue samples of 20 BE patients were used to validate expression of several genes by RT-PCR. For instance, the expression of FABP1, Galectin 4, Annexin A10, TFF1, TFF2, TFF3, CK7 and CK20 (Figures 1 and 3) were validated. The high expression of TFFs in BE and in gastric cardia as seen in our profiles are in concordance with several other reports[11, 23, 24]. TFFs are peptides often co-secreted with mucins by goblet cells and play an important role in protecting the epithelium[25-27]. Galectin 4 is a member of the Galectin family which are carbohydrate-binding proteins with high affinity for β-galactosides. They are involved in apoptosis and proliferation[28-31]. Several reports identified CKs 7 and 20 useful for identifying BE and distinguishing BE from gastric cardia[32-34].

Most interesting is the comparison between the three expression profiles. It is believed that the development of BE is a truly metaplastic process in which through chronic acid reflux, precursor cells of the esophageal squamous epithelium develops towards incomplete intestinal type of epithelium. Likewise, it has been hypothesized that BE may as well develop from gastric cardia cells or from glandular structures in the esophageal submucosa. To our knowledge there are no other SAGE expression profiles available on BE. Our data is in concordance with the results of Barrett *et al*., who used Affimatrix gene chips for comparison of gene expression of duodenum, gastric, squamous and BE tissues[11]. They found a closer correlation between BE and the surrounding normal epithelia, while the expression profiles between the fully differentiated normal epithelia showed less similarity. The present study supports their observation, moreover the differences between the normal epithelia in our study seems to be even more profound. Between the BE and normal squamous and cardia epithelia SAGE profiles we found a relative

low number of significantly differentially expressed genes: 776 genes (3.0% of all unique tags) and 534 tags (2.5%), respectively. In comparison, the highest number of significantly differential expressed tags, 1316 tags (6.4%), was found between normal gastric cardia and normal esophageal squamous epithelium. The relative large overlap of the BE profile with both squamous and columnar type of epithelium supports the hypothesis that BE is an incompletely differentiated type of epithelium that is related to the both anatomically neighbouring epithelia. To this fact, the remarkable expression pattern of CKs in BE, that partially overlaps with squamous and partially with gastric cardia, is illustrative (Figure 2). CK expression in epithelial cells vary depending on the location, type and differentiation of cells[35, 36]. However we should take in consideration that analyzing transcriptomes of duodenum and colon epithelium could further improve our insight in the process of metaplasia.

The identification of genes exclusively expressed in the three different epithelia may have important clinical implications, as these may be useful as tissue markers. To this end we compared the complete expression profiles of CKs 1 to 20 in the three epithelia (Figure 2) and verified expression of the most informative CKs on protein level (Figure 4). Several of these CKs are novel findings and may be applicable to clinical practice for instance as a screening tool for clinical diagnostics, e.g. for detection of BE for instance by brush cytology in population screening studies[37-39].

In summary, the comparison of the gene expression profiles of Barrett, normal squamous and gastric cardia shows that BE is related to both normal squamous and columnar type of epithelia. In addition, this analysis has provided us a wealth of information and identified novel genes that may be involved in the process of metaplasia or can be used as tissue identification markers.

Acknowledgements

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Supplemental Information

Clustering of genes within functional biological classes.

Genes, corresponding to tags, were clustered in groups of different biological processes according to Gene Ontology of the European Bioinformatics Institute (http://cgap.nci.nih.gov). Figure 1A of the supplemental data shows the clustering of significantly differentially expressed tags in groups of biological processes of the BE versus the squamous SAGE library. A higher expression of genes in the groups of cell death, cell adhesion, cell organization and biogenesis, cell proliferation and metabolism was found in BE compared to squamous esophagus. The biological clusters of BE compared with gastric cardia showed that cell motility, cell death, cell organization and biogenesis, development, biosynthesis and metabolism were more abundantly expressed in BE (Figure 1B of the supplemental data). The ratios of BE versus normal squamous and gastric cardia of these biological processes all showed an induction of at least 2 fold. Cell organization and biogenesis was the most important cluster comparing BE versus normal squamous esophagus (3 fold) and cell death comparing BE versus gastric cardia (3 fold).

Comparing the three profiles, we may assume that genes exclusively or highly expressed in BE may be involved in the profound phenotypic changes as takes place during the process of metaplasia. However, cellular changes within the transition of squamous esophagus to Barrett's metaplasia may involve only a small subset of genes and expression may be even at very low levels or not detectable at all at transcription level. In this study, clustering of genes in functional classes showed us the relative abundant genes involved in cell death, biosynthesis, cell adhesion, cell organization and biogenesis, cell proliferation and metabolism in BE (Figure 1 of the supplemental data). Uniquely expressed genes in BE for instance specifically involved in cell organization and biogenesis may help us to gain more insight in the process of metaplasia. Ongoing studies are being performed on functional analysis of several of these genes.

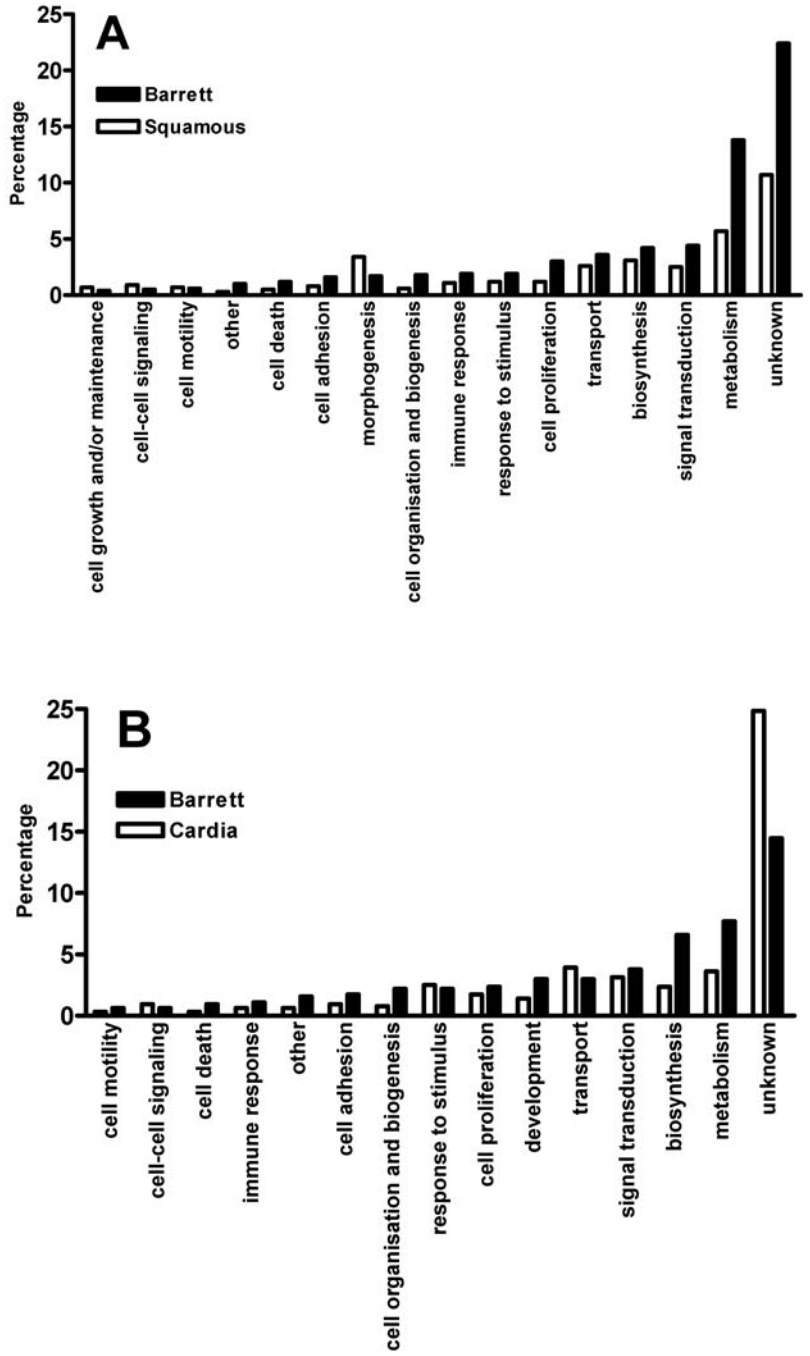

Figure 1: Clustering of genes in biological processes. Clustering of significantly differentially expressed tags, comparing Barrett's esophagus and squamous esophagus, in groups of biological processes (A). Cell death, cell adhesion, cell organization and biogenesis, cell proliferation and metabolism were more important in Barrett's esophagus (A). Clustering of significantly differentially expressed tags, comparing Barrett's esophagus with gastric cardia, in groups of biological processes showed that cell motility, cell death, cell organization and biogenesis, development, biosynthesis and metabolism were more important in Barrett's esophagus (B).

Tag	p BB	p SB	up	gene ID
CTGGCCCTCG	0.746	0.000	345	Trefoil factor 1
GGAAGGTTTA	0.568	0.000	263	Regenerating islet-derived family, member 4
AAGAAAGCTC	0.419	0.000	194	Chromosome 1 open reading frame 22
AAATCCTGGG	0.387	0.000	179	Trefoil factor 2
ATTTTCTAAA	0.707	0.004	178	Anterior gradient 2 homolog
GGAAAACAGA	0.305	0.000	141	Galectin 4
GCCAGTCTGT	0.242	0.000	112	Mucin 5
CCTCCAGCTA	0.391	0.004	99	Keratin 8
CTCCACCCGA	0.212	0.000	98	Trefoil factor 3
GTGATCAGCT	0.169	0.000	78	Mucin 5AC
GAATGATTTC	0.149	0.002	75	Putative nuclear protein ORF1-FL49
GCCCAGGTCA	0.151	0.000	70	CDNA clone IMAGE:5759948, partial cds
ATGGAATAAT	0.117	0.000	54	Annexin A10
TGCTCCTACC	0.106	0.002	53	Fc fragment of IgG binding protein
CAGTGCTTCT	0.102	0.002	51	Deleted in lymphocytic leukemia, 1
ATGTAAAAAA	0.333	0.008	42	Lysozyme
CCAAAGCTAT	0.084	0.000	39	Transmembrane 4 superfamily member 3
TCATTCTGAA	0.076	0.000	35	Gastrokine 1
TAGACTAGCA	0.069	0.002	35	Transmembrane 4 superfamily member 8
GTATGAGTAG	0.063	0.002	32	Calmodulin-like 4
TGACTAATTG	0.067	0.000	31	Anterior gradient 2 homolog
GTACGTATTC	0.061	0.002	31	HSPC082
GTTCTCTGAA	0.065	0.000	30	Cathepsin E
AAATAAAAGA	0.063	0.000	29	Hypothetical protein FLJ11767
AGATCCCAAG	0.061	0.000	28	Intelectin 1
TTTATAAAGG	0.058	0.000	27	Diffuse panbronchiolitis critical region 1
TAAATTGCAA	0.056	0.000	26	Keratin 20
CTGTACCTGG	0.041	0.000	19	Bone morphogenetic protein 4
GAAAGGCAAA	0.037	0.002	19	Sialyltransferase 7
AAGAAAACCT	0.039	0.000	18	Breast cancer membrane protein 11
TTACGAGGAA	0.035	0.002	17	SEC13-like 1
GGTGGGAACA	0.037	0.000	17	Regenerating islet-derived family, member 4
TAAAAGTTTA	0.037	0.000	17	Transcribed locus
TTGGCCCTCG	0.037	0.000	17	Unknown
AACTAATCTG	0.032	0.002	16	Unknown
GACCCAAGAT	0.035	0.000	16	Polymeric immunoglobulin receptor
TTTTTAGAAT	0.030	0.002	15	Thiosulfate sulfurtransferase
GAGAGCTCCC	0.030	0.002	15	Unknown
TTTAGGATGA	0.032	0.000	15	Down-regulated in gastric cancer GDDR
TAAGTAAAGT	0.056	0.004	14	Hypothetical protein MGC11242
AGAACCTTCC	0.026	0.002	13	Major histocompatibility complex, class I, A
ACATTGGGTG	0.028	0.000	13	Fatty acid binding protein 1, liver
AATGGAATGG	0.028	0.000	13	Melanophilin
CCATAATGTT	0.028	0.000	13	Pleckstrin 2
TGAAGGTTTA	0.028	0.000	13	Unknown
GAGTTTGTTA	0.024	0.002	12	Claudin 18

GGCACCGTGC	0.024	0.002	12	Hypothetical gene supported by BC022385; BC035868; BC048326
ATGATGCGGT	0.024	0.002	12	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin)
TACACTTGAA	0.026	0.000	12	Chaperonin containing TCP1
TGCACAATAT	0.026	0.000	12	Mucin 3A
AGGTGGACAG	0.026	0.000	12	Potassium channel, subfamily K, member 5
GTGCACTGAG	0.093	0.008	12	Major histocompatibility complex, class I, A
CAAACCATCC	0.069	0.006	12	Keratin 18
TTTCCTTCCT	0.024	0.000	11	Clathrin, light polypeptide
TTTAATTTGT	0.024	0.000	11	Golgi phosphoprotein 2
CTCCCCCAAG	0.024	0.000	11	Hypothetical protein MGC27165
GAAATAAAGC	0.024	0.000	11	Immunoglobulin heavy constant gamma 1
GCTAGGGTTC	0.024	0.000	11	Transmembrane 4 superfamily member 4
CTCATACACC	0.024	0.000	11	Unknown
TACTGAGGAG	0.024	0.000	11	Unknown
TGGAAAGTGA	0.024	0.000	11	V-fos FBJ murine osteosarcoma viral oncogene homolog
AATATTTATA	0.022	0.002	11	Carcinoembryonic antigen-related cell adhesion molecule 5
GTGGCGGGCA	0.022	0.002	11	Hypothetical protein FLJ38482
CTTTTCTAAA	0.022	0.002	11	Septin 8
GACATCAAGT	0.119	0.012	10	Keratin 19
GCCAAAGGAA	0.022	0.000	10	Hemopoietic cell kinase
GCCCAGCATT	0.022	0.000	10	Prostate stem cell antigen
TAGAAAGCTC	0.022	0.000	10	Rab6-interacting protein 2
AGCTCTTGGA	0.022	0.000	10	Selenium binding protein 1
AGAAGGTTTA	0.022	0.000	10	Unknown
AGCAGGCTCA	0.022	0.000	10	Unknown
TAATCCTGGG	0.022	0.000	10	Unknown

Table 1A: Tags more than 10 fold up-regulated in Barrett versus squamous epithelium. Tags more than 10 fold up-regulated in Barrett's esophagus compared with squamous epithelium along with their counts in percentage (p) in Barrett's esophagus (BB) or squamous esophagus (SB). The fold up induction of each tag and their corresponding gene ID's are provided.

Tag	p BB	p SB	down	gene ID
GCAGAGAGGA	0.000	0.044	22	Keratin 13
ATCCTGCAGA	0.000	0.042	21	Fizzy/cell division cycle 20 related 1
TACCCTGCAG	0.000	0.042	21	Phosphatidylinositol glycan, class F
CTGATGGCGA	0.006	0.125	19	S100 calcium binding protein A9
GAAATGAGTG	0.002	0.038	17	Arachidonate 12-lipoxygenase
AGCAGGAGCA	0.002	0.038	17	S100 calcium binding protein A16
AATCTTGTTT	0.000	0.034	17	Dermokine
GAAATTCCCC	0.002	0.036	16	Eukaryotic translation elongation factor 1 alpha 1
TTATTATTTG	0.000	0.032	16	Unknown

AATCTCTTGG	0.002	0.032	15	Unknown
ACATTTCAAA	0.000	0.028	14	Keratin 1
ACTCAGTAGC	0.002	0.030	14	G protein-coupled receptor 126
TGTAAAGCAA	0.002	0.030	14	SH3-domain binding protein 1
TTGAATCCCC	0.009	0.113	13	Protease inhibitor 3, skin-derived
GATATGTAAA	0.004	0.053	12	Chloride channel, calcium activated, family member 4
TGTATGTAAA	0.002	0.026	12	Kallikrein 10
TTGTGATGTA	0.002	0.026	12	Metastasis associated lung adenocarcinoma transcript 1
TCTGAATAGC	0.002	0.026	12	Thioredoxin
TGGAACTGTG	0.000	0.022	11	Sialic acid binding Ig-like lectin 8
TAGATAAATG	0.000	0.022	11	Unknown
AGCGCTGATT	0.004	0.048	11	Unknown
ACTGTGGTAG	0.002	0.022	10	Gap junction protein, beta 2, 26kDa
CAAATATATC	0.002	0.022	10	Hypothetical protein DKFZp686G0786
CAGCTGTCCC	0.000	0.020	10	Keratin 16
GAGACAGTGG	0.000	0.020	10	Similar to Intersectin 1
AATGGATGAA	0.000	0.020	10	Unknown

Table 1B: Tags more than 10 fold down-regulated in Barrett versus squamous epithelium. Tags more than 10 fold down-regulated in Barrett's esophagus compared with squamous epithelium along with their counts in percentage (p) in Barrett's esophagus (BB) or squamous esophagus (SB). The fold down induction of each tag and their corresponding gene ID's are provided.

Tag	p BB	p GB	up	gene ID
GGAAGGTTTA	0.568	0.000	147	Regenerating islet-derived family, member 4
GGCAGAGAAG	0.508	0.000	131	Keratin 4
CTCCACCCGA	0.212	0.004	55	Trefoil factor 3
TAATTTGCAT	0.104	0.004	27	Epithelial membrane protein 1
TGTGCCAGTG	0.097	0.000	25	Unknown
GAGATAAATG	0.082	0.004	21	Lymphocyte antigen 6 complex, locus D
GGGTCTGAGG	0.073	0.000	19	BAI1-associated protein 2
GAAGCACAAG	0.067	0.000	17	Unknown
AAGGATAAAA	0.065	0.000	17	Carcinoembryonic antigen-related cell adhesion molecule 6
AAATAAAAGA	0.063	0.000	16	Hypothetical protein FLJ11767
CTGTCACCCT	0.063	0.000	16	Small proline-rich protein 1A
GTATGAGTAG	0.063	0.004	16	Calmodulin-like 4
AGATCCCAAG	0.061	0.000	16	Intelectin 1
CTTCCTGCTC	0.061	0.000	16	Aconitase 2, mitochondrial
GCCCCTGCTG	0.056	0.004	14	Keratin 5
AAAGCGGGGC	0.052	0.000	13	Keratin 13
GGCTTCTAAC	0.050	0.000	13	CDNA clone IMAGE:6653118
CTGTACAGAC	0.050	0.004	13	Tubulin, beta, 2

CACCTGCAGA	0.048	0.004	12	G protein-coupled receptor kinase 1
TATGACTTAA	0.043	0.004	11	Ras-related C3 botulinum toxin substrate 1
ACGTGTGTAA	0.041	0.000	11	Unknown
TGTGAAGCCT	0.041	0.000	11	Lactate dehydrogenase A-like 6A
TTTCCTCTCA	0.039	0.004	10	Stratifin

Table 2A: Tags more than 10 fold up-regulated in Barrett comparing with gastric cardia. Tags more than 10 fold up-regulated in Barrett's esophagus compared with gastric cardia along with their counts in percentage (p) in Barrett's esophagus (BB) and gastric cardia (GB). The fold up induction of each tag and their corresponding gene ID's are presented.

Tag	p BB	p GB	down	gene ID
AACCTCCCCA	0.000	2.326	1076	Pepsinogen 5, group I
TCAAACATTA	0.002	0.543	251	Unknown
CAGTGCTTCT	0.102	11.901	117	Deleted in lymphocytic leukemia, 1/ gastric lipase
TAGTGCTTCT	0.004	0.434	100	Unknown
AGTGCTCTTC	0.002	0.167	77	Pepsinogen C
TCATTCTGAA	0.076	5.787	77	Gastrokine 1
TTGCCCCTAC	0.000	0.155	72	Eosinophil chemotactic cytokine
CTGACTGTGC	0.000	0.151	70	ATPase, H+/K+ exchanging, alpha polypeptide
GGAACGCAAG	0.000	0.143	66	ATPase, H+/K+ exchanging, beta polypeptide
GAGTGCTTCT	0.000	0.128	59	DUTP pyrophosphatase
GTAAAAACCA	0.006	0.333	51	Unknown
CAGTGCTTCC	0.000	0.109	50	Chromodomain helicase DNA binding protein 2
AATGTACCAA	0.000	0.093	43	Lipase, gastric
CACCTCCCCA	0.000	0.093	43	Chromosome 1 open reading frame 6
TTTTGAACAG	0.000	0.085	39	Lipase, gastric
CCATTCTGAA	0.004	0.171	39	Tripartite motif-containing 32
AACGTCCCCA	0.000	0.081	38	Pepsinogen 5, group I
CAGTGCCTCT	0.000	0.081	38	KIAA0303 protein
CAGTGCTCCT	0.000	0.074	34	Unknown
GACCTCCCCA	0.000	0.074	34	Unknown
CAGCGCTTCT	0.000	0.070	32	Unknown
CTGTGCTTCT	0.000	0.070	32	Unknown
GCTGAGGAGA	0.004	0.136	31	Gastrokine 1
CACCCCTGAT	0.000	0.062	29	Creatine kinase, brain
CGGTGCTTCT	0.000	0.062	29	Unknown
TTATTCTGAA	0.002	0.062	29	KIAA0143 protein
AAGGGAGCAC	0.002	0.054	25	Immunoglobulin lambda constant 2
GGTCAGTCGG	0.002	0.054	25	Unknown
AAAATAAAAT	0.004	0.105	24	Gastric intrinsic factor
CCGTGCTTCT	0.000	0.050	23	Unknown

GAGAACCACT	0.000	0.050	23	Gastric intrinsic factor
GTTTGCTTGC	0.000	0.050	23	ATP-binding cassette
TACCTCCCCA	0.000	0.050	23	Unknown
TCATTCCGAA	0.000	0.050	23	Unknown
TATTTGTTGA	0.004	0.089	21	ADMP
TCATCCTGAA	0.000	0.043	20	Unknown
TCATTCTAAA	0.000	0.043	20	Transcribed locus
TTTAGGATGA	0.032	0.585	18	Down-regulated in gastric cancer GDDR
TACAAACCTG	0.000	0.039	18	Metallothionein 1F
TCATTCTGGA	0.000	0.039	18	Laminin, alpha 1
GATCCCAACT	0.002	0.039	18	Metallothionein 2A
GGCCCAGGCC	0.004	0.078	18	Aldehyde dehydrogenase 3 family, memberA1
GTCGGGCCTC	0.002	0.035	16	Folate receptor 1
TCACTCTGAA	0.002	0.035	16	Leucine rich repeat containing 27
AAGGTAACAG	0.009	0.136	16	Serine protease inhibitor, Kazal type 1
CAGTGCTTTT	0.002	0.031	14	CDNA clone IMAGE:4603971
CAGTGCTTTC	0.002	0.027	13	Unknown
GCATTTGACA	0.002	0.027	13	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2
GAATCCTGGG	0.004	0.050	12	Unknown
AAGTCCTGGG	0.002	0.023	11	Unknown
GGGCAAGCCA	0.002	0.023	11	Estrogen-related receptor alpha
TATCCCAGAA	0.002	0.023	11	KIAA1229 protein
TCATTCTGAG	0.002	0.023	11	Forkhead box A1

Table 2B: Tags more than 10 fold down-regulated in Barrett comparing to gastric cardia. Tags more than 10 fold down-regulated in Barrett's esophagus compared to gastric cardia along with their counts in percentage (p) in Barrett's esophagus (BB) and gastric cardia (GB). The fold down induction of each tag and their corresponding gene ID's are presented.

Chapter 3

Bone Morphogenetic Protein (BMP) 4 mediates transformation of inflamed squamous esophageal mucosa into Barrett's esophagus

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Abstract

Although the premalignant Barrett's esophagus (BE) is considered a metaplastic condition in which normal squamous esophageal epithelium dedifferentiates into columnar epithelium, the pathophysiology through which this metaplasia occurs is unknown. A recent study by SAGE analysis showed that Bone Morphogenetic Protein 4 (BMP4) is uniquely expressed in BE. In this study the role of the BMP pathway in the metaplastic transformation of normal squamous epithelium into BE was examined. Tissues from an esophagitis-BE rat model and patients were examined for activation of the BMP-pathway. Short term cultures of primary normal squamous esophageal cells were treated with BMP4 and cell biological changes were examined by Western blot analysis, immunohistochemistry and microarrays. In both rat and human tissues the BMP pathway proved to be up-regulated in esophagitis and BE. Upon incubation of squamous cultures with BMP4, the Cytokeratin (CK) expression pattern showed a shift towards a Barrett specific CK pattern, for instance with up-regulation of CK20. Involvement of the BMP pathway was marked by up-regulation of P-Smad 1/5/8 that was effectively blocked by Noggin, a BMP antagonist. Comparison of the gene expression profiles of squamous cells, BMP4 treated squamous cells and BE cells, showed a shift in the profile of the BMP4 treated squamous cells towards that of the BE cells ($p<0.01$). These results indicate that the transformation of normal esophageal squamous cells into columnar cells is a BMP pathway dependent event. Future manipulation of this pathway may prevent BE and the associated esophageal adenocarcinoma.

Introduction

BE is a pre-malignant condition of the distal esophagus that is associated with an increased risk of developing esophageal adenocarcinoma. In recent years, the incidence of BE and associated adenocarcinoma has been increasing dramatically and death from esophageal adenocarcinoma has become a major health concern[1-3]. BE is believed to be a metaplastic change in which the normal stratified squamous epithelium in the distal esophagus is replaced by columnar epithelium[4]. This process is assumed to be the result of longstanding gastroesophageal reflux disease (GERD). GERD is a common condition that can be found in 20-30% of the general Western population[5]. It has been estimated that BE can be found in 6-12% of patients who suffer from GERD and it is assumed that individuals with GERD and esophagitis subsequently may develop BE[6, 7]. Although the concept of metaplasia is largely accepted, the actual pathophysiology through which these changes take place is unknown. In order to identify genes specifically involved in the transition of normal squamous esophageal epithelium into metaplastic BE, we previously used serial analysis of gene expression (SAGE) as a technique to compare the expression profile of BE with that of its surrounding epithelia, *i.e.*, normal squamous esophageal and gastric cardia mucosa. BMP4 was found to be abundantly and uniquely expressed in the SAGE library of BE, but not in normal squamous or stomach cardia epithelium. BMP4 is a protein belonging to the transforming growth factor beta family (TGF-β). Members of the TGF-β family are involved in controlling cellular differentiation, migration and proliferation[7, 8]. BMPs are 30-35 kD hetero- or homodimeric proteins and are originally identified to play a role in bone formation, but they are also essential during embryonic development[9, 10]. BMPs induce the formation of a heterodimeric complex of the BMP receptor type I and type II. This receptor complex signals downstream by phosphorylating specific BMP receptor regulated Smads (Smad 1, 5 and 8). The phosphorylated Smad (P-Smad 1/5/8) forms a heterocomplex with Smad 4, this complex translocates into the nucleus, where certain target genes, such as ID2, can be transcribed[11]. Previous studies demonstrated that BMPs are induced during inflammation and injury[12-15]. In present study, immunohistochemistry and Western blot analysis showed that BMP4 is highly expressed in patient biopsies with esophagitis, suggesting

that increased levels of BMP4 in the inflamed esophagus may trigger the process of the epithelial transformation. Increased BMP4 was as well observed in esophagitis and the metaplastic columnar BE epithelium in a BE rat model. To further reveal the role and pathway through which BMP4 is involved in the development of BE, primary cell cultures of biopsy specimens of normal squamous esophageal epithelium and of BE obtained from patients were established. The squamous cells were treated with recombinant (rec) human (h) BMP4. Upon treatment the phosphorylation level of Smad 1/5/8 was increased, this was effectively blocked through addition of the BMP antagonist Noggin. The activation of the BMP pathway was also demonstrated on a rat esophagitis-BE animal model, as shown by the increase in the expression of P-Smad and ID2 in the Barrett tissues compared to the normal not inflamed tissue. Phenotypically, a shift of the CK expression pattern of the treated squamous cells towards that of columnar type of cells was found. To further prove that BMP4 stimulation of the squamous cells induced a transformation into a columnar cell type, analysis by whole human genome chip arrays was applied. The micro-array analysis demonstrated a shift of the expression profile of the BMP4 treated squamous cells towards that of BE cells.

In this study evidence is provided that the process of metaplastic transformation, which is initiated in inflamed squamous esophageal mucosa and results in BE, is mediated by BMP4. We demonstrate that this transformation is a BMP pathway dependent phenomenon. As these results indicate that BMP4 plays a key role in the development of BE, manipulation of this pathway may be used for future prevention of BE and thus reduce the occurrence of the related esophageal adenocarcinoma.

Materials and Methods

Patient and Biopsy Specimens

Biopsy specimens were obtained during routine surveillance endoscopies from patients with a known BE but without dysplasia. All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. Paired biopsies, taken next to each other, were obtained of the Barrett's segment and of normal squamous esophagus. The Barrett biopsies were taken 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 taken at least 2 cm above the Barrett's segment. Special care was taken to avoid sampling Barrett's areas containing isles of squamous mucosa. All patients had proven incompletely differentiated intestinal type of columnar epithelium without dysplasia in the histological control biopsies with no signs of active or acute inflammation. Normal squamous esophageal epithelium was as well confirmed histologically in all the pairwise taken control biopsies. For Western blot analysis biopsy material of 13 BE patients was used. Ten patients were male; mean age was 66 years (range 46-86 years). The average length of the BE segment measured endoscopically was 5.4 cm (range 2-11 cm).

In addition biopsies of another 6 patients were obtained that at endoscopy at least had grade B (Los Angeles classification) esophagitis. Paired biopsies were taken next to each other from the inflamed squamous mucosa and normal squamous segment. None of the esophagitis patients had a concomitant BE. The inflammation was histologically confirmed on the pairwise taken control biopsies as well as the pairwise taken normal control biopsies from these patients. Four patients were male and the mean age was 54 years (range 27-70 years).

Primary Cell Culture

Specimens for tissue culture were placed immediately in essential medium (Sigma Chemical Co., St Louis, MO, USA) on ice before processing. Biopsy specimens were processed within 4 hours after endoscopy. The explant method described by K. Washington *et al*. was used[16]. With this method, biopsy specimens were minced into fragments of 1-2 mm^3 in size. The pieces of tissue were placed in a 24 wells culture plate and anchored by a sterile glass microscope slide before addition of growth medium. Primary cell cultures were initiated by maintaining the cells in Barrett's plus media, as previously described, containing 5% fetal bovine serum (FBS), 0.4 μg/mL hydrocortisone (Sigma Chemical Co.), 20 ng/mL epidermal growth factor (GIBCO BRL, Grand Island, NY, USA), 10^{-10} mol/L cholera toxin (Sigma Chemical Co.), 140 μg/mL bovine pituitary extract (Sigma Chemical Co.), 20 μg/mL adenine (Sigma Chemical Co.), 100 U/mL penicillin (GIBCO BRL, Life Technologies), 0.25 μg/mL streptomycin (GIBCO BRL),

0.25 μg/mL amphotericin B (GIBCO BRL), 4 mmol/L glutamine (GIBCO BRL), 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium (Sigma Chemical Co.)[17, 18]. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and fed two times a week. After two to three weeks culturing, squamous cells were incubated with 100 ng/ml rec h BMP4 (R&D Systems, Minneapolis, MN, USA) for several time points and/or 50 μg/ml recombinant Mouse noggin/Fc chimera (R&D).

For the preparation of cells on glass slides**,** after harvesting the cells, cells were re-suspended in medium and dropped on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides and air-dried overnight.

RNA isolation

Total RNA was isolated from primary cell cultures using TRIzol Reagent (Life Technologies Inc, Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. Briefly, $1x10^6$-$5x10^6$ cells were lysed by adding 1 ml TRIzol. After phenol/chloroform extraction, RNA was precipitated with isopropanol, washed with 70% ethanol and air-dried, dissolved in RNase-free H_2O and stored at -80 °C until required. Spectrophotometry was performed with 1 μl of total RNA to quantitate on the Nanodrop® (type ND-1000). Assessment of the quality was performed with the RNA 2100 Pico Labchip kit (Agilent Technologies, Amstelveen, The Netherlands) using 1 μl of total RNA.

Micro-Array analysis: RNA Amplification, Labeling and Hybridization

The mRNA was double amplified using the Amino Allyl MessageAmp kit (Ambion, Austin, USA). The labeling, hybridization and data extraction were performed at ServiceXS (Leiden, The Netherlands). Briefly, 20 ng total RNA was mixed with 1 μl of T7 Oligo(dT) primer in a total volume of 12 μl. Primer and template were denatured by incubating at 70°C for 10 minutes and annealed by putting the reaction tubes on ice. The First Strand Reaction was performed by adding 8 μl Reverse Transcription Master Mix (containing 10x First Strand buffer, Ribonuclease Inhibitor, dNTP Mix and Reverse Transcriptase) and incubating at 42°C for 2 hours. Second Strand cDNA Synthesis was

done by adding 63 µl Nuclease-Free Water, 10 µl 10x Second Strand Buffer, 4µl dNTP Mix, 2µl DNA Polymerase and 1 µl RNase H and incubating at 16°C for 2 hours. cDNA purification was done according to the manufacturer's protocol (Ambion). *In vitro* transcription was initiated by addition of 3 µl UTP solution (50mM), 12 µl ATP, CTP, GTP Mix (25mM), 4 µl T7 10x Reaction Buffer and 4 µl T7 Enzyme Mix and incubated at 37°C for 5 hours. Purification of aRNA was done according to manufacturers protocol (Ambion). The second round First Strand Synthesis was done using 2 µg purified aRNA, adding 2 µl 10x First Strand Buffer, 1 µl Ribonuclease Inhibitor, 4 µl dNTP Mix and 1 µl Reverse Transcriptase and incubating at 42°C for 2 hours. After adding 1 µl of RNase H, samples were incubated at 37°C for 30 minutes. Five µl of T7 Oligo (dT) primer was added and the sample was denatured by incubating at 70°C for 10 minutes and annealed by putting the reaction tubes on ice. The second round Second Strand cDNA Synthesis was done by adding 58 µl Nuclease-Free Water, 10 µl 10x Second Strand Buffer, 4 µl dNTP Mix and 2 µl DNA Polymerase and incubating at 16°C for 2 hours. cDNA purification was done according manufacturers protocol (Ambion). *In vitro* transcription was initiated by addition of 2 µl aaUTP Solution (50mM), 12 µl ATP, CTP, GTP mix (25mM), 3 µl UTP Solution (50mM), 4 µl T7 10x Reaction Buffer and 4 µl T7 Enzyme Mix and incubation at 37°C for 9 hours. Qiagen's RNeasy mini spin columns were used for purification of the cRNA as described in Agilent's user manual. Dye Coupling Reaction was performed using 5 µg amino allyl aRNA, 9 µl Coupling Buffer and 11 µl NHS ester dye, prepared according manufacturers protocol (Amersham, Buckinghamshire, United Kingdom). After an incubation at room temperature for 30 minutes, 4.5 µl 4M Hydroxylamine was added and incubated at room temperature for 15 minutes. Dye labeled aRNA was purified according manufacturers protocol (Ambion) and the samples were checked on concentration and dye incorporation on the Nanodrop ND-1000. The cRNA yield was between 91 µg and 123 µg. Hybridization was performed with 600 ng or 1 µg of each labeled target together with control targets, fragmentation and hybridization buffer at 60°C for 17 hours onto Agilent Human Whole Genome Oligo arrays (Amsterdam, The Netherlands) following manufacturer's protocol.

Microarray Imaging, Data and Statistical Analysis

The microarray slides were washed following the user manual instructions and scanned on the Agilent dual laser DNA microarray scanner. The microarray data was normalized using the Agilent feature extraction software (version 7.5) with regards to local background correction and analyzed using the Rosetta Resolver v5.0 Expression Data Analysis System (Rosetta Biosoftware, Seattle, USA). To compare the normal squamous cells with the BMP4 incubated squamous cells we hybridized every sample to the BE sample. To make the statistical comparison between the Barrett samples and the non treated squamous cells and BMP4 treated squamous cells we had to build new ratios using Ratio-split software of the Rosetta Resolver Package. Using this Ratio-split, statistical analysis using ANOVA (on intensity data) is immediately performed between the 3 different experiments ($p<0.01$). Further statistical analysis was done using the genes that were more than 2 fold up- or down-regulated compared to the BE samples, chi square test was performed to compare differences in the amount of genes of 3 experiments that were more than 2-fold up- or down-regulated ($p<0.001$).

Western blot analysis

Treated cells were washed with ice cold PBS and scraped into 25 µl lysis buffer (Cell Signaling) with the addition of 1 mM Pefablock (Sigma). Biopsies were lysed with 200 µl lysis buffer, 1 mM Pefablock. The lysates were sonicated and then centrifuged at 20g for 10 minutes at 4ºC. The pellet was discarded and the protein concentration was measured with the BCA protein assay kit (Pierce chemical co. Rockford, IL, USA). Lysates were diluted 1:2 in protein sample buffer (125 mM Tris/HCl, pH 6,8; 4%SDS; 2% β-mercapto ethanol ; 20% glycerol; 1 mg bromphenol blue) and incubated at 95ºC for 5 minutes. Twenty mg of protein per lane was loaded onto SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore, Amsterdam, The Netherlands). The blots were blocked with 2% BSA in Tris Buffered Saline supplemented with 0.1% Tween-20 (TBST) for one hour at room temperature and washed in TBST before overnight incubation at 4ºC with primary antibody in 2% BSA in TBST. Blots were then washed with TBST and incubated for 1 hour at room temperature in 1:1000 Horse Radish Peroxidase (HRP) conjugated secondary antibody in 2% BSA in TBST. After a final

wash with TBST, blots were incubated for 5 minutes in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Fuji LAS3000 illuminator (Fuji Film Medical Systems, Stamford, USA). The antibodies used and dilutions are summarized in Table 1.

Rat model of Barrett's esophagus

Healthy, six-week-old male Sprangue-Dawley rats were purchased from Harlan (Indianapolis, IN, USA). A detailed description of the procedure and conditions of the preparation of the BE rat model has been described by Buttar *et al*.[19]. Briefly, a midline laparotomy was carried out and Levrat's esophagojejunostomy technique was performed to induce enteroesophageal reflux[20]. Rats were sacrificed between 20 to 22 weeks post-operatively using CO_2 narcosis, intramuscular injection of 12 mg/kg xylazine hydrochloride and removal of 5 ml intra-cardiac blood. The whole body was cooled to 4ºC and a midline incision was made from the laryngopharynx to the lower abdomen. The site of anastomosis was identified by finding the polypropylene sutures. It was freed of any adhesions and then dissected free of surrounding tissue up to the laryngopharynx. The esophagus was then cut at the level of the larynx and 2 mm above the site of anastomosis and opened longitudinally. Tissues were snap frozen and stored at -80ºC. The animal care committee (IACUC) at the Mayo Clinic (Rochester, MN, USA) approved the animal study.

Fluorescent immuno-histochemistry

Slides were fixed for 20 minutes in Phosphate buffered Saline (PBS) with 4% Paraformaldehyde (PFA) and 0.1% Triton and washed in PBS. Blocking of aspecific antigens was performed by incubating slides for 45 minutes with PBS with 1% Bovine Serum Albumin (BSA) with 10% Fetal Calf Serum (FCS). Slides were washed with PBS and incubated overnight at 4ºC with the appropriately diluted primary antibody in PBS with 1% BSA with 0.1% Triton. After incubation the slides were washed with PBS and incubated with the secondary antibody FITC conjugated (Dako, Denmark) 1:500 diluted in PBS. Slides were washed and mounted with DAPI (Roche, Mannheim,

Germany)/vectashield (Vector laboratories Inc, Burlingame, CA, USA) 1:1000. The antibodies used and dilutions are summarized in Table 1.

Antibody	Species	Company	Country	Dilution
Cytokeratin 7	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 10/13	Mouse monoclonal	Dako	Denmark	1:500
Cytokeratin 20	Mouse monoclonal	Progen	Germany	1:500
BMP4	Mouse monoclonal	R&D	USA	1:1000
P-Smad 1/5/8	Rabbit monoclonal	Cell Signaling	USA	1:1000
BMP RIA	Goat polyclonal	R&D	USA	1:1000
BMP RII	Goat polyclonal	Abcam	USA	1:500
ID2	Rabbit polyclonal	Santa Cruz	USA	1:1000
Smad 4	Mouse monoclonal	Santa Cruz	USA	1:1000
β-Actin	Goat polyclonal	Santa Cruz	USA	1:2000

Table 1: Antibodies as used for Western blotting and immunohistochemistry.

Confocal microscopy on rat esophagitis-Barrett's esophagus animal model

Rat tissues from the control rats and the esophagitis-BE rat model were sampled from the normal esophagus from the upper part of the esophagus, from inflamed epithelium taken above the esophagojejunostomy and of the metaplastic columnar epithelium taken at the anastomosis were collected in liquid nitrogen and stored at -80˚C until processing for immunohistochemistry and H&E staining.

Tissues were embedded in Tissue-Tek® Optimum Cutting Temperature compound (Sakura Finetek, USA, Torrance, Calif.) at -20°C and sectioned using a cryo-microtome (Microm HM 550) in serial cryostat sections of 6 μm, placed on Superfrost + glass slides and air-dried overnight. For routine histological examination, of each block one slide was routinely stained by hematoxylin and eosin. Other slides were used to perform fluorescent immunohistochemistry for BMP4, P-Smad 1/5/8 and ID2, as described above and examined by confocal microscopy using a Leica TCS-SP2 filter-free Spectral Confocal Microscope (Heidelberg GmbH, Molecular Imaging Center, Bergen, Norway). A 40X magnifying objective was used with the numerical aperture (NA) at 1.25, type HCX PL fluotar.

Results

Expression of BMP4 and downstream targets in Barrett's esophagus, squamous and inflamed squamous epithelium of patients

In a recently published SAGE analysis we found that BMP4 is 19 fold up-regulated in BE compared to normal squamous epithelium[8]. Validation by Western blot analysis of BE and normal squamous esophageal biopsies confirmed the SAGE data and showed a high expression of BMP4 on protein level in BE and a low expression in normal squamous esophagus. Importantly, we now also demonstrate a high expression of BMP4 in the inflamed squamous epithelium (Figure 1). Western blot analysis shows that Smad 4 and both BMP receptors: BMP RIA and BMP RII, are expressed in BE, in normal squamous epithelia and in esophagitis specimens (Figure 1), yet ID2, a downstream BMP4 target, and P-Smad 1/5/8 are only detectable in BE and in esophagitis but not in normal non-inflamed squamous epithelium (Figure 1). These results indicate that in the non-inflamed squamous/ esophagitis/ BE -sequence, the BMP pathway is already activated in esophagitis and is still active in BE.

BMP4 and downstream targets expression in Barrett's esophagus, squamous and inflamed squamous epithelium in a BE rat model

In the control animals, normal rat esophageal mucosa showing keratinizing squamous epithelium is found (Figure 2A). Reflux esophagitis and/or intestinal type of metaplasia of the epithelium resembling BE are found in the operated rats just above and/or at the esophago-jejunostomy (Figure 2B, C). Confocal analysis of immunohistochemical stainings of the fresh frozen material of the normal rat esophagus shows low expression of BMP4, P-Smad 1/5/8 and ID2. BMP4 and the downstream BMP4 targets, P-Smad 1/5/8 and ID2, are only seen in the inflamed esophagus and BE in the rat BE model (Figure 3). BMP-4 expression in the inflamed esophagus and BE is typically localized in the mesenchymal tissue (Figure 3) while P-Smad 1/5/8 is localized in nuclei indicating transcriptional activity, and ID2 is seen in the nuclei and cytoplasm of the epithelial cells[21]. This esophagitis-BE rat model confirms that activation of the BMP pathway is

already in the inflamed esophagus and that the pathway is still actived in the metaplastic BE.

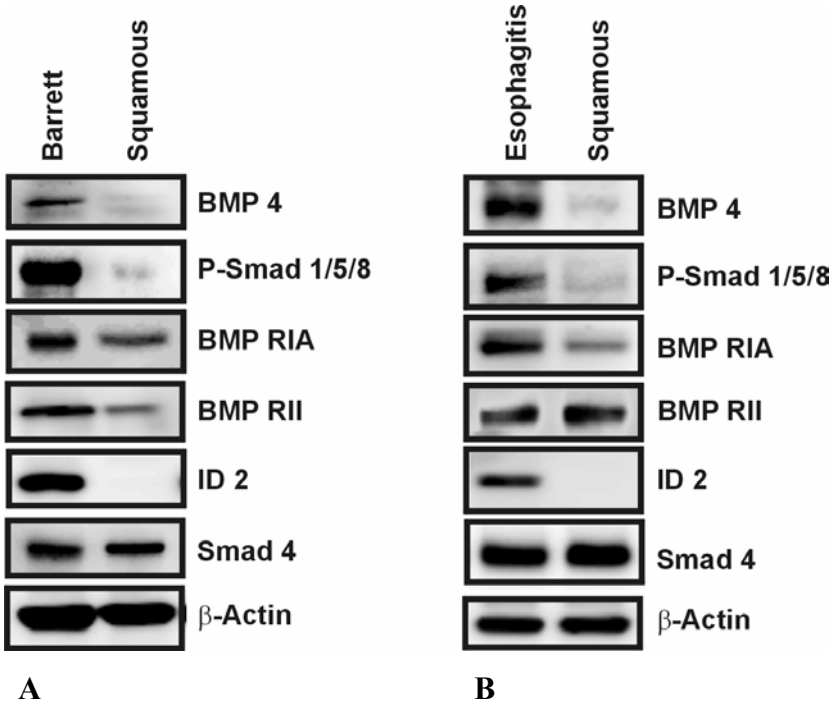

Figure 1: Expression of proteins of the BMP pathway in Barrett's esophagus, esophagitis and normal squamous epithelium. Western blot analysis of BMP4, P-Smad 1/5/8, ID2, BMP RIA, BMP RII and Smad 4 expression in Barrett's esophagus and normal squamous esophagus biopsies (A) and in inflamed squamous (esophagitis) and normal squamous epithelium (B). Results show that BMP4 and its downstream targets P-Smad 1/5/8 and ID2 are expressed in Barrett's esophagus and inflamed squamous epithelium, while they are not expressed in normal squamous epithelium. Smad 4 and both receptors, BMP RIA and BMP RII are expressed in Barrett's esophagus, inflamed squamous epithelium and normal squamous epithelium. β-Actin was used as a control.

Figure 2: H&E staining of normal squamous esophagus, inflamed squamous esophagus and Barrett's esophagus rat model. Hematoxilin and Eosin staining of the rat esophagitis-Barrett's esophagus animal model showing: Normal keratinizing squamous esophageal mucosa of a rat (A); Inflamed esophageal mucosa (B); Metaplastic intestinal type of epithelium resembling Barrett's esophagus, illustrated by the presence of goblet cells (C).

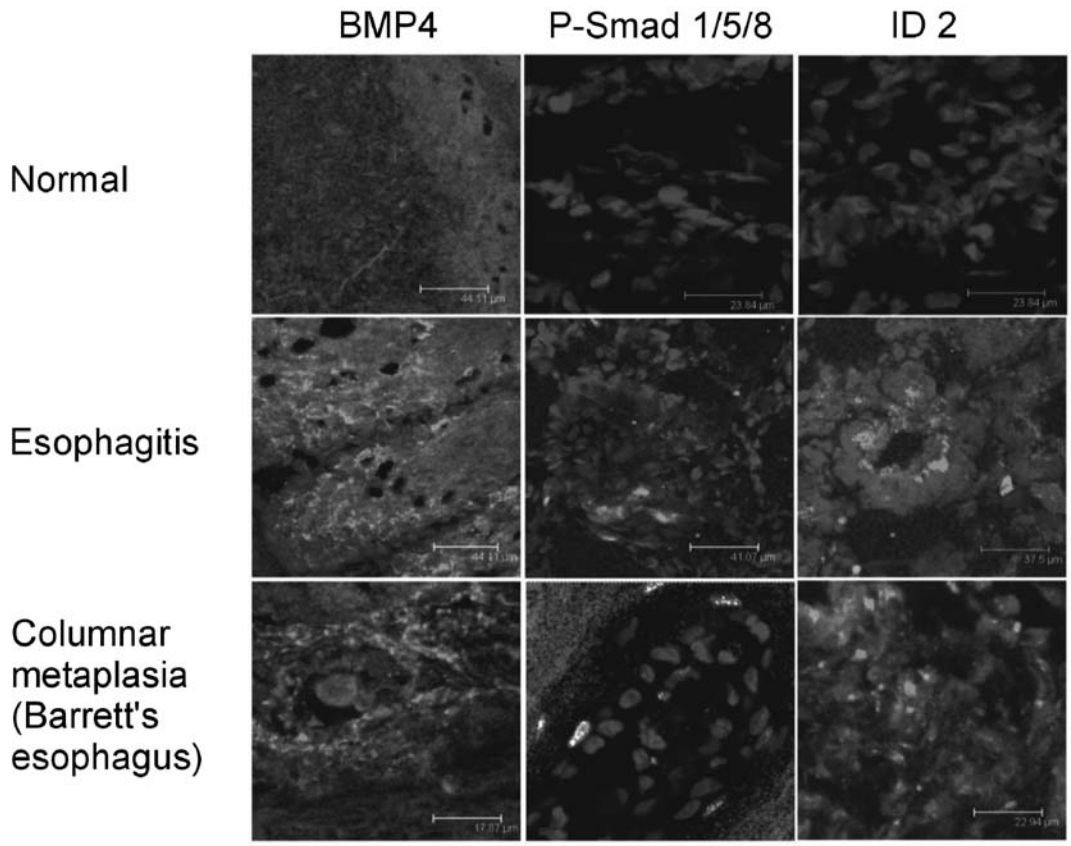

Figure 3: BMP4 pathway expression in normal squamous esophagus, inflamed squamous epithelium and Barrett's esophagus rat tissue. Confocal analysis of immuno-histochemical staining of the esophagitis-Barrett's esophagus rat model shows that in normal rat keratinizing esophageal mucosa staining for BMP4 and the downstream targets is negative. The inflamed mucosa and Barrett mucosa show an increased expression of the BMP downstream targets P-Smad 1/5/8 and ID2, as well as up-regulation of the BMP4 expression, confirming the BMP pathway activation in the rat model.

BMP4 activation in primary cultures of normal squamous esophageal cells

Short term primary cell cultures were established of biopsies from BE and normal squamous esophagus. Time-course incubation of primary cultured normal squamous cells with 100 ng/ml rec h BMP4 was performed. Western blot analysis show that after 5 minutes of BMP4 incubation there are increased levels of P-Smad 1/5/8 (Figure 4A). This phosphorylation level is increased even more at 10 minutes and 20 minutes of incubation, whereas the untreated squamous cell cultures do not show any phosphorylation of Smad 1/5/8 (Figure 4A). Pretreatment of squamous cells with the BMP4 antagonist Noggin for 10 minutes decreases the phosphorylation level of Smad 1/5/8 to a basal level, indicating that the BMP pathway is blocked (Figure 4B).

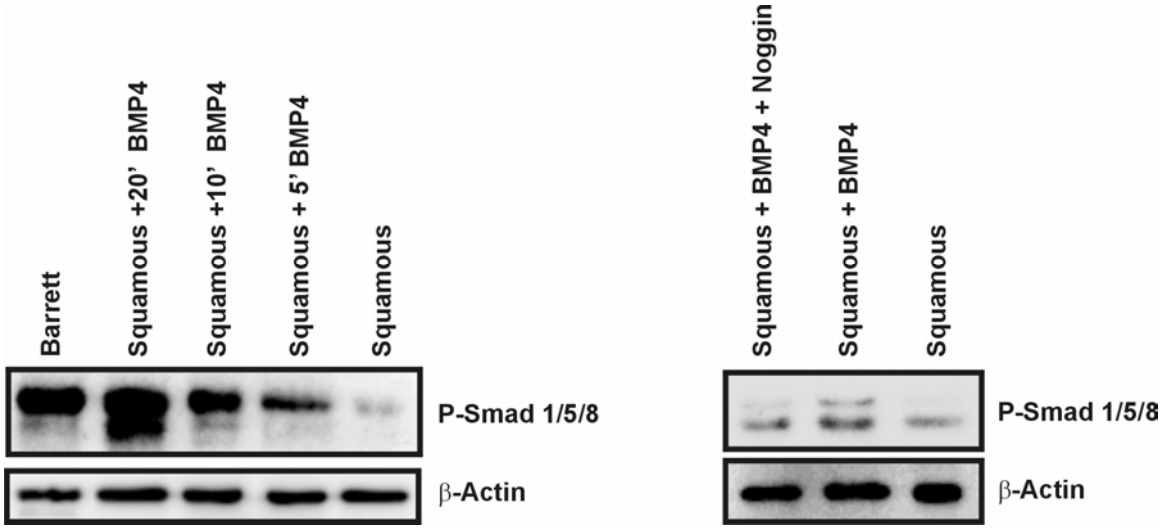

Figure 4: P-Smad 1/5/8 expression in cell cultures stimulated with BMP4. Western blot analysis of P-Smad 1/5/8 in primary cultured Barrett and squamous esophageal cells. In cultured squamous cells there is no phosphorylation of Smad 1/5/8, while cultured Barrett cells show high levels of P-Smad 1/5/8. Upon treatment of the squamous cell cultures with BMP4 for 5, 10 or 20 minutes P-Smad 1/5/8 levels are up-regulated (A). This up-regulation in phosphorylation level of Smad 1/5/8 is efficiently inhibited when the squamous cells are incubated with Noggin, a BMP antagonist (B). β-Actin was used as a control.

Cytokeratin expression in cultured squamous cells before and after BMP4 treatment

CK10 and CK13 are normally only expressed by squamous cells, while CK7 and CK20 are more specific for columnar types of cells such as BE[8]. The effects of BMP4 on expression levels of CK7, CK10/13 and CK20 were investigated in squamous cell cultures that were incubated for 5 days with rec h BMP4, and compared with non treated squamous and BE cell cultures. Immunohistochemistry shows that the normal squamous cells do express CK10/13 but do not express CK7 or CK20 (Figure 5), while Barrett cells do not express CK10/13 but do express CK7 and CK20. Upon treatment of squamous cells with BMP4, expression of CK7 and CK20 is up-regulated whereas down-regulated expression of CK10/13 is seen (Figure 5). This is confirmed by Western blot analysis, showing increased CK7 expression after 5 hours of treatment (Figure 6). These results indicate that BMP4 expression causes a shift in the CK expression pattern of normal squamous mucosa to a CK pattern resembling BE.

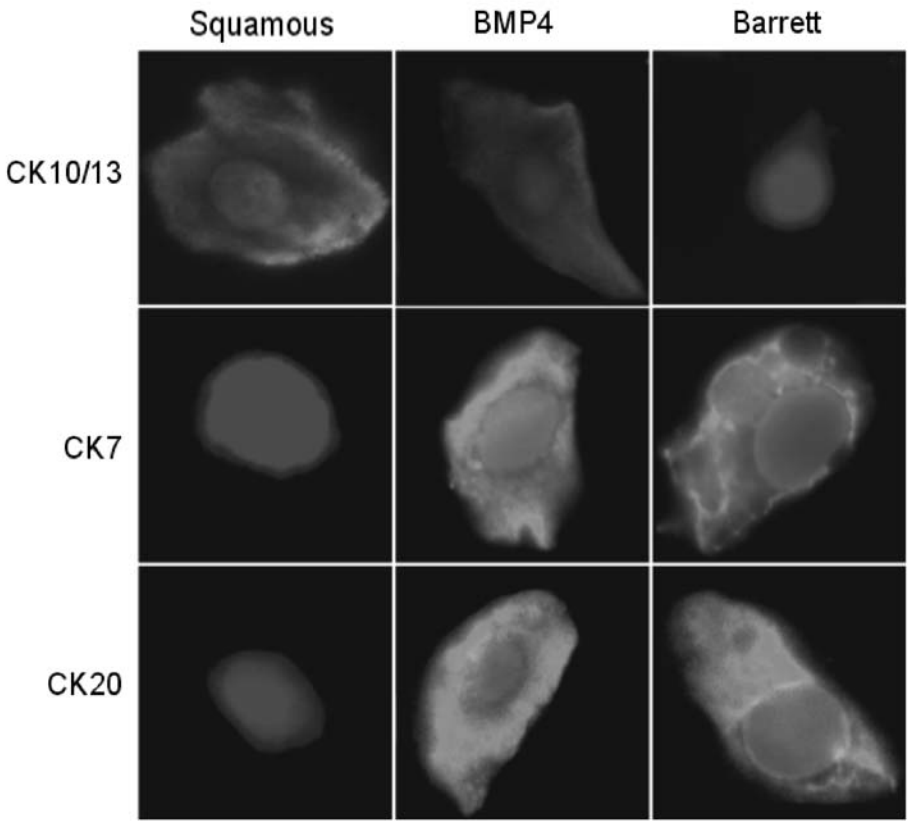

Figure 5: Cytokeratin 10/13, 7 and 20 expression in cultured Barrett, non treated squamous and BMP4 treated squamous cells. Immunofluorescent pictures showing the expression of CK7, CK10/13 and CK20 in primary cultured Barrett and squamous esophageal cells. Normal squamous cells show no expression of CK7 and CK20 but strongly express CK10/13 while Barrett cells are positive for CK7 and CK20 but not for CK10/13. After 5 days of treatment of the cultured squamous cells with BMP4 there is up-regulation of CK7 and CK20, while CK10/13 expression is decreased. DAPI was used to stain nuclei.

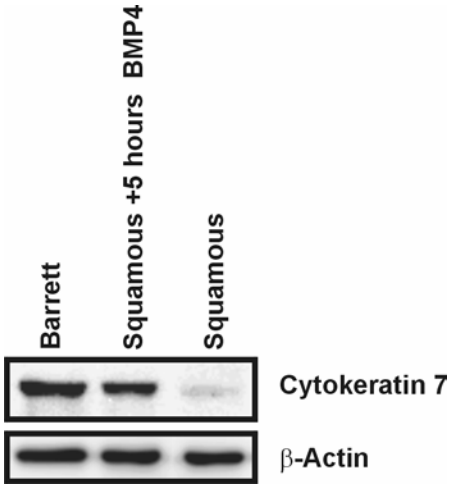

Figure 6: Expression of Cytokeratin 7 in cultured squamous cells incubated with BMP4. Western blot analysis of CK7 expression in cultured squamous cells and Barrett cells. In cultured squamous cells there is no CK7 expression, while in Barrett's esophagus cells there is CK7 expression. Upon BMP4 treatment of squamous cells for 5 hours, CK7 expression is up-regulated. β-Actin was used as control.

Whole human genome microarray analysis

The gene expression profiles of BMP4 treated, not treated squamous cells as well as Barrett cell cultures were obtained and compared. The overall gene expression profile of the BMP4 treated squamous cells in comparison to the Barrett cells and untreated squamous cells, indicate that there is a shift of the gene expression profile of squamous treated cells towards that of Barrett cells (Figure 7). Analysis of a subset of genes that are at least 2-fold up- or down-regulated show that on average 11099 genes are differentially expressed when comparing not treated squamous with Barrett cells, while on average 8226 differentially expressed genes are found when comparing BMP4 treated squamous with Barrett cells. The decreased number of the at least 2-fold differentially expressed genes as seen upon treatment of the squamous cells with BMP4 is statistically significant (chi square test; $p<0.001$). Further statistical analysis of the 3 different microarray experiments show that comparing Barrett with non treated squamous cells, 446 genes are significantly differentially expressed, while the comparison of the Barrett with the BMP4 treated squamous cells show that 392 genes are significantly different (ANOVA; $p<0.01$). This is a decrease of 12% on gene expression level. These results indicate that on gene expression level, 5 days of incubation of normal squamous cells with BMP4 tends to trans-differentiate these cells towards Barrett cells.

Discussion

In previous studies BMPs have been found to directly change the pathophysiology of certain inflammatory conditions[13, 14]. Due to their chemotactic activity on inflammatory cells and fibroblasts, BMPs have been considered to influence inflammatory processes in adults[12]. BMP4 is a protein belonging to the TGF-β protein family. So far, the involvement of members of the TGF-β family in BE has not been investigated in detail. With microarray analysis Barrett *et al*. found that a TGF-β superfamily protein was one of the genes that was up-regulated in BE[22]. Through SAGE, we recently found that BMP4 is exclusively expressed in BE compared to normal squamous epithelium[8]. Since BE is caused by chronic inflammation as a result of reflux of gastric contents damaging the esophageal mucosa, we hypothesized that these inflammatory changes could induce

the production of BMP4, which subsequently triggers trans-differentiation into a columnar cell type that replaces the normal squamous esophageal cells.

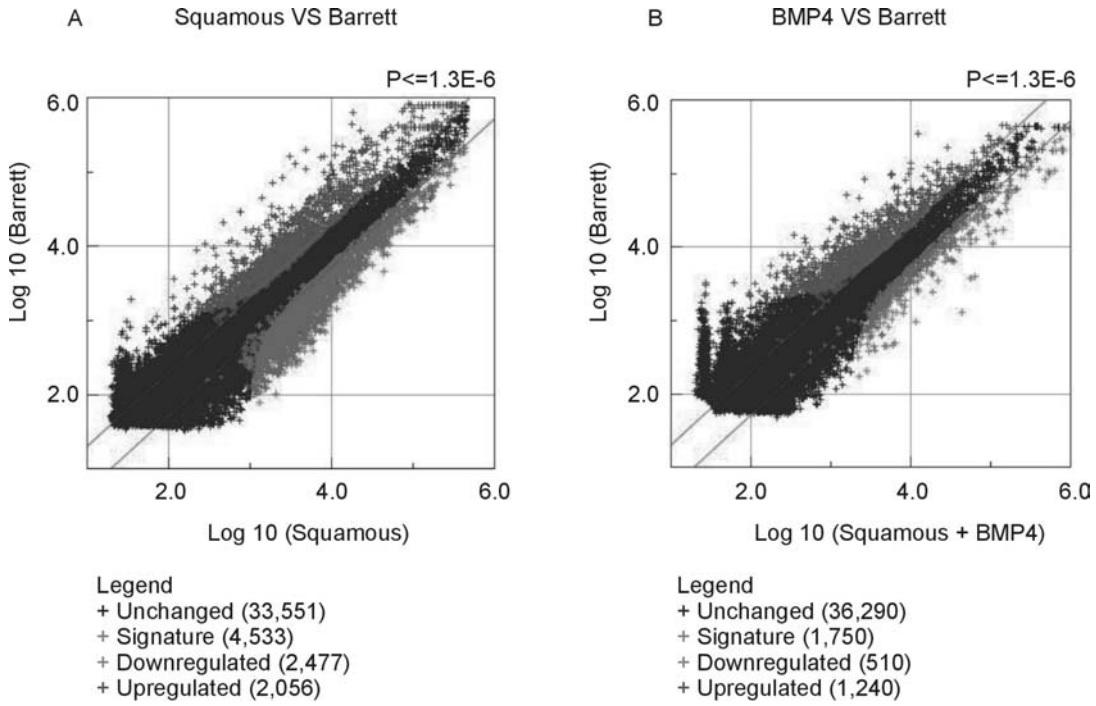

Figure 7: Microarray analysis comparing differentially expressed genes in squamous and BMP4 treated squamous cells versus Barrett cells. Plots showing the comparison of microarray analysis of Barrett versus non treated squamous cells (A) and Barrett versus BMP4 treated squamous cells (B). The plots are representative for 3 different experiments. Depicted in red are genes up-regulated in Barrett; depicted in green are genes down-regulated in Barrett. A shift in gene expression profile is visible, based on the decreased number of genes that are up- or down-regulated in Barrett versus BMP4 treated squamous cells.

In this study, we provide evidence that BMP4 is a key factor that induces phenotypic changes that are reminiscent of metaplastic BE, which is a result of esophagitis caused by GERD. By Western blot analysis we found that BMP4 and its downstream targets, P-Smad 1/5/8 and ID2 are present in patient biopsies with BE and esophagitis, but not in normal non inflamed squamous esophageal mucosa (Figure 1). This indicates that indeed in inflamed esophageal mucosa, BMP4 is up-regulated and activates its pathway. The finding of BMP receptor (RIA and RII) expression in normal squamous esophageal tissue supports the possibility that under certain conditions the BMP pathway can be activated in squamous epithelium. Analysis of a esophagitis-BE rat model in which reflux

esophagitis and subsequently BE is induced through a gastro-jejunostomy, confirmed that the BMP pathway is activated in both the inflamed esophageal mucosa and BE illustrated by increased expression of BMP4 and the downstream targets P-Smad and ID2 (Figure 3). We further explored in an *ex-vivo* set up whether the BMP pathway was active in BE cell cultures and whether BMP4 could induce dedifferentiation of cultured squamous cells into a columnar cell type that resembles BE. To this end we established short term primary cultures of normal squamous epithelial cells and Barrett cells from patient biopsies. Treatment of squamous epithelial cells with recombinant human BMP4 for 5, 10 or 20 minutes showed expression of P-Smad 1/5/8, that increased in time, while the control squamous cell cultures did not show any expression level of activated Smad proteins (Figure 4A). Blocking the pathway by pre-incubating squamous cells, treated with BMP4, with its antagonist Noggin, showed a decreased phosphorylation level of Smad 1/5/8 (Figure 4B). From these *ex-vivo* experiments we conclude that the BMP pathway can be activated in normal squamous cell cultures and that this effect can be inhibited by blocking with Noggin.

To further study the phenotypical trans-differentiation process we examined the CK expression profiles in BMP stimulated and non stimulated squamous cells. CKs are the building blocks for the intermediate filaments as a part of the cytoskeleton. The CK expression profile is variable in epithelial cells depending on type, location and differentiation of the epithelium and is therefore important for characterizing certain epithelia, for instance characterizing the difference between BE and normal squamous cells[8, 23-26]. CK7 and CK20 are known to be expressed in BE and other columnar types of epithelium and can be used to distinguish BE from normal squamous epithelium. In contrast CK10/13 is expressed in normal squamous epithelium but not in BE or other columnar types of mucosa[8, 27, 28]. After treatment of squamous cell cultures with BMP4 for 5 days, expression of the Barrett's specific markers CK7 and CK20 were induced, whereas non-treated cells did not express these CKs (Figures 5 and 6). Furthermore we saw decreased CK10/13 expression in BMP4 treated squamous cells. Western blot analysis revealed that there was up-regulation of CK7 within 5 hours of BMP4 incubation (Figure 6). From these results we can conclude that upon treatment with BMP4, the CK expression pattern was shifted towards a BE type of CK expression, indicating that the

induction of these specific CKs is a direct BMP4 target. To further investigate to what extent a columnar type of phenotype is induced by BMP4, whole human genome arrays were used to compare the total gene expression profiles of primary Barrett's cell cultures with the BMP4 treated and non treated squamous cell cultures. A remarkable change towards a BE gene expression profile was seen of the BMP4 treated squamous cell cultures (Figure 7). We found a diminished number of significantly differentially expressed genes (ANOVA; $p<0.01$) between BMP4 treated cells and Barrett cells as compared to non treated cultured squamous and Barrett cells. This reduction was 12%. The results were more impressive when looking at the genes that were more abundantly (> 2-fold) up- or down-regulated. In this category of genes the overall number of differentially expressed genes diminished from 11099 to 8226 between non treated normal squamous cells and BMP4 treated squamous cells compared to Barrett cells, which is a reduction of 26%.

In summary, in the present study BMP4 is noted to be a key player in the process of BE as occurs in the distal esophagus in which normal squamous epithelial cells are dedifferentiated into columnar type of cells, as a result of GERD. We showed that the BMP pathway is activated in inflamed esophageal epithelium and in BE of either human and rat tissues and that the BMP4 pathway can be blocked effectively with Noggin, a specific BMP antagonist. In our *ex-vivo* experiments the phenotypical dedifferentiation of normal squamous cells into a columnar cell type as induced by BMP4 is illustrated by changes of the CK expression patterns and gene expression profiles. Here we demonstrated that BMP4 is a key player in transforming normal squamous esophageal cells into columnar cells. Future manipulation of the BMP pathway may help us to prevent BE and subsequently the highly malignant BE associated esophageal adenocarcinoma.

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Chapter 4

Comparison of kinome profiles of Barrett's esophagus, normal squamous esophagus and gastric cardia

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Abstract

The precursor metaplastic mucosal lesion that predisposes for esophageal adenocarcinoma is Barrett's esophagus (BE). In Western countries the incidence of the highly malignant esophageal adenocarcinoma and the prevalence of BE are rapidly rising. Since, the signal transduction events that occur in BE are poorly understood, this study aimed on generating a comprehensive description of cellular kinase activity in BE, normal squamous esophagus and gastric cardia for gaining more insight into the pathogenesis of BE. Peptide arrays, exhibiting 1176 specific consensus sequences for protein kinases, were used to produce a global analysis of cellular kinase activity in biopsies of BE, 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 BE patients. Three unique kinome profiles are described and compared. We identified cascades of activated kinases showing that MAPK and EGF receptor activity are both significantly altered in BE compared to squamous and gastric cardia epithelia. Another novel finding is that the glycolysis pathway is significantly up-regulated in BE, which is illustrated by an up-regulated pyruvate kinase activity. Here, the unique kinome profile of BE is made available as a comprehensive database. Several signaling pathways are revealed as specifically expressed in BE when compared to the adjacent normal epithelia. These unique findings provide novel insight in the pathogenesis of BE that will ultimately help to resolve the increasing problem of BE and prevention of esophageal adenocarcinoma.

Introduction

BE is widely recognized as a pre-malignant 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]. BE 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]. BE is further associated with the highly malignant esophageal adenocarcinoma with an estimated annual incidence of approximately 0.5%[6-8]. Over the last 3 decades, the prevalence of BE and BE adenocarcinoma has been rising rapidly in Western countries[9-11]. The phenotypic changes during the development of BE have been described in several studies[12-15]. Recently microarray and other gene expression profile studies have been performed, showing that also at the gene expression level, BE has strong similarities with the anatomical surrounding epithelia[16, 17]. Nevertheless at the level of cellular functions and processes, the pathophysiology of BE 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]. Dys-regulation and mutations of these enzymes play central roles in several human diseases like BE, 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 BE with the surrounding normal squamous and gastric cardia epithelia would generate profound insight in the biological processes that are specifically activated in BE. In the present study, kinome profiles were obtained using a peptide array containing 1176 different kinase specific consensus sequences. The method as first described by Diks *et al*., allows a comprehensive detection of the cellular metabolism in lysates[23]. Up- or down-regulation of a particular kinase activity may lead to a cascade of cellular events. These can be fit into specific cell signalling 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 pathological processes, like a metaplastic lesion such as BE. In this study we have attempted to obtain further insight into the molecular processes by applying peptide arrays to clinical samples of BE and employing this technology we delineate active cell signalling pathways in BE and the anatomically adjacent squamous and gastric cardia epithelium from 3 individual BE patients. The three unique kinome patterns as described in the present study provide important information on kinase activity of BE as compared to its surrounding epithelia. Important differences in kinase activity were confirmed by conventional technology in tissue samples of 27 BE patients. It was found that at kinome level, BE does have similarities with both adjacent epithelia, yet several unique kinase patterns were found as well. For instance, the important Epidermal Growth Factor (EGF) cell signalling pathway was found to be down-regulated in BE when compared to normal squamous but up-regulated when compared to normal cardia. An important up-regulation of glycolysis was seen in BE compared to the both anatomical surrounding epithelia.

In summary, the present study contributes to a better understanding of BE by providing a comprehensive description of kinase activity specific to this disease. The unique kinome profile of BE is made available to the scientific community as a comprehensive database that can be used for future studies of cellular functions in BE. Intervening with these cellular events will ultimately lead to more effective treatment of BE and as such prevention of development of the esophageal adenocarcinoma.

Materials and Methods

Patients and tissue specimens

Tissue samples of 30 BE patients (21 males, 9 females; mean age 61 years, age range 44-86; average length of BE segment measured endoscopically 2.9 cm, range 2-11 cm) taken during routine surveillance endoscopy were used. All patients had known BE without dysplasia and were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. 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 above 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 histopathological 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 histological 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. The first three consecutive patients with histopathological confirmed intestinal metaplasia were selected for kinome analysis, whereas the remainder was employed for confirmation of results using immunoblot analysis and enzyme activity assays.

Kinome array analysis

Kinome array analysis was performed 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 website: http://www.pepscan.nl/pdf/Manual%20PepChip%20Kinase%200203.pdf. Full biopsies were weighted and lysed with lysis buffer (Cell Signaling, Beverly, MA, USA) in a final concentration of 0.4 mg biopt/µl lysis buffer (average biopsy weight was 20 mg) with the addition of 20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodiumvanadate, 1 mM sodiumfluoride, 1 µg/ml leupeptin and 1 µg/ml aprotinin. The lysates were centrifuged at 20000 g for 10 minutes at 4ºC and the pellet was discarded. In order to study kinase activity 50 µl lysate was added to 12 µl activation mix, containing 50% glycerol, 250 µM ATP, 60 mM $MgCl_2$, 0.05% v/v Brij-35, 0.25 mg/ml BSA and 2000 µ Ci/ml ^{33}P-γ-ATP. The peptide arrays (Pepscan, Lelystad, The Netherlands), containing 1164 different kinase pseudo-substrates 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 Phosphate Buffered Saline (PBS) containing 0.1% Triton X-100, twice with 2 M NaCl containing 0.1% Tween-20 and twice in dH_2O. Slides were air dried and exposed to a phospho-imaging 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 ^{33}P-γ-ATP to peptide motifs, ^{33}P-α-ATP was employed: no radioactivity was detected. Furthermore as a control, we have analyzed frozen versus fresh material using the pep-chip since 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 performed as described by Diks *et al*. and Löwenberg *et al*.[23-25]. Briefly, ScanAlyze software (http://www.bio.davidson.edu/Biology/GCAT/protocols/scanalyze.html) was used. Using grid tools, spot density and individual background was corrected and spot intensities and background intensities were analyzed. Data, from 3 individual experiments, was 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 in order to extract kinases of which activity was either significantly induced or reduced. Different kinase activities in lysates from BE, 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 performed as described by Löwenberg *et al*., briefly; a minimal modification for the algorithm originally developed for microarray analysis (http://www.stat.stanford.edu/~tibs/SAM/) was used[24].

Immunoblotting

Immunoblotting was performed as described by van Baal *et al*. and Hardwick *et al*.[16, 26]. Blots were incubated with the primary antibody over night at 4˚C. Antibodies used were p-EGF Receptor Tyr 845 (Cell Signaling; 1:1000), p-EGF Receptor Tyr 992 (Cell Signaling), p-EGF receptor Tyr 1068 (Cell Signaling), p-HER2 Tyr 1248 (Cell Signaling), p-Src Tyr 416 (Cell Signaling), p-ERK Tyr 204 (Santa Cruz), PKC β1 (Santa Cruz), p-PDK Ser 241 (Cell Signaling) and β-Actin I-19 (Santa Cruz); (the used dilutions are summarized in Table 1). After a final wash, blots were incubated for 5 minutes in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemi-luminescence detected using a Fuji LAS3000 illuminator. The

phosphorylation level was determined by the ratio of signal intensity of the protein to that of the β-actin. Statistical analysis of phosphorylation levels were conducted using GraphPad Prism version 4.00, GraphPad software (San Diego, CA, USA). Data are expressed as means ± SEM. Comparison between two groups was analyzed using paired *t*-tests (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Antibody	Species	Company	Country	Dilution
P-EGF Receptor (Tyr 845)	Rabbit polyclonal	Cell Signaling	USA	1:1000
P-EGF Receptor (Tyr 992)	Rabbit polyclonal	Cell Signaling	USA	1:1000
P-EGF Receptor (Tyr 1068)	Rabbit polyclonal	Cell Signaling	USA	1:1000
P-HER2 (Tyr 1248)	Rabbit polyclonal	Cell Signaling	USA	1:1000
P-Src (Tyr 416)	Rabbit polyclonal	Cell Signaling	USA	1:1000
P-ERK (Tyr 204)	Mouse monoclonal	Santa Cruz	USA	1:1000
PKC β1	Rabbit polyclonal	Santa Cruz	USA	1:1000
P-PDK (Ser 241)	Rabbit polyclonal	Cell Signaling	USA	1:1000
β-Actin (I-19)	Goat polyclonal	Santa Cruz	USA	1:2000

Table 1: Antibodies as used for immunoblot analysis

Pyruvate kinase activity assay

Biopsies were homogenized using 200 µl homogenization mix, containing 10 mM MOPS buffer (3-N-Morpholino-propanesulfonic acid) pH 7.4, 150 mM 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 to convert the pyruvate generated in the pyruvate kinase reaction into lactate with the concomitant oxidation of NADH to NAD. To this end, 15 µl homogenate was added to a medium containing 86 mM TRA buffer pH 7.6, 10 mM KCl, 2.5 mM $MgSO_4$, 4.7mM ADP, 0.2 mM NADH, 9 U/ml LDH and 0.1% Triton X-100. Reactions were initiated by adding phosphoenolpyruvate (PEP) at the final concentration of 0.53 mM. The absorbance at 350 nm was measured using a COBAS FARA centrifugal analyzer (Roche, Switzerland). Data are expressed as means ± SEM. 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 BE, normal squamous esophagus and gastric cardia contain substantial kinase activity; almost all substrate peptides incorporate ^{33}P-γ-ATP (Figure 1). Subsequent analysis of the kinome profiles of 3 individuals revealed 130 kinase substrates showing a significantly differential expression profile when comparing BE versus normal squamous epithelium ($p<0.05$; supplemental data). Comparison of the kinase activity patterns of 3 individuals of BE with gastric cardia identified 274 kinase substrates that were significantly differentially phosphorylated ($p<0.05$; supplemental data). Figure 2 shows scatter plots comparing substrate phosphorylation in BE to normal squamous esophagus and normal gastric cardia, revealing the kinase signature of BE to be an intermediate between the surrounding epithelia ($R^2=0.80$ and $R^2=0.80$ respectively; $p<0.0001$; Figures 2A and B). Accordingly, a scatter plot comparing substrate phosphorylation of normal esophagus to normal cardia shows poor correlation ($R^2=0.66$; $p<0.0001$; Figure 2C). Thus, overall kinome profiling suggests that BE has similarities with both squamous and cardia epithelium.

Barrett's Esophagus associated changes in kinase activity

One of the most prominent effects in BE compared to normal squamous epithelium was significantly decreased phosphorylation of Src consensus substrates. Comparing BE with gastric cardia, phosphorylation of Glycogen Synthase Kinase-3 (GSK3) α consensus substrates were one of the major effects that was significantly decreased in BE. A complete list of the peptide substrates with significantly altered phosphorylation in BE versus normal squamous and in BE versus gastric cardia can be found as supplemental information with corresponding fold induction.

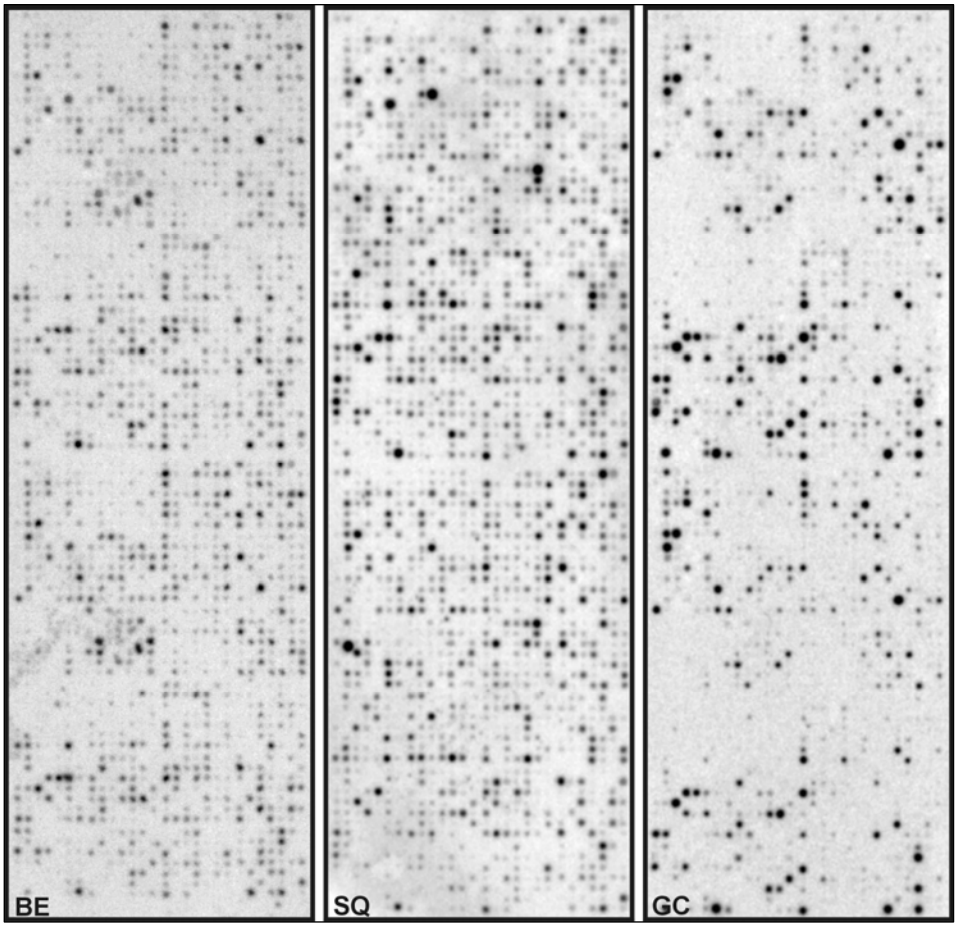


Figure 1: Kinase peptide arrays showing phosphorylation of the substrates by biopsy lysates of Barrett's esophagus, normal squamous esophagus and normal gastric cardia. Scans of the peptide arrays after incubation with lysates of Barrett's esophagus (BE), normal squamous esophagus (SQ) or normal gastric cardia (GC) in the presence of ^{33}P-γ-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.

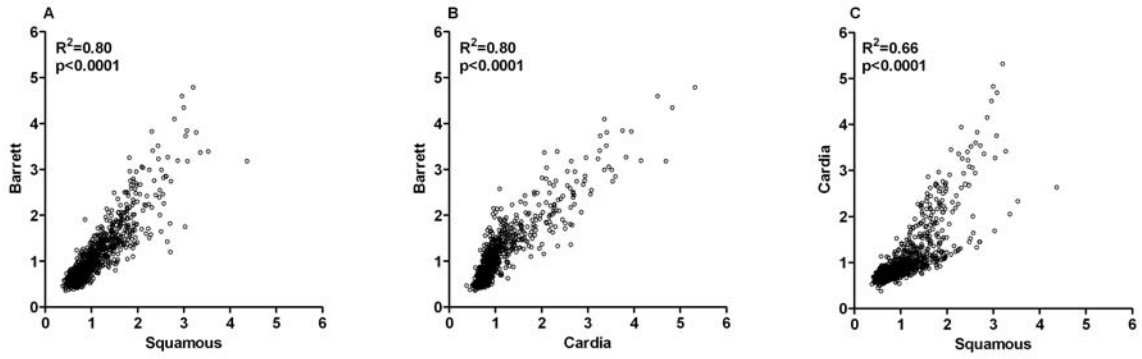
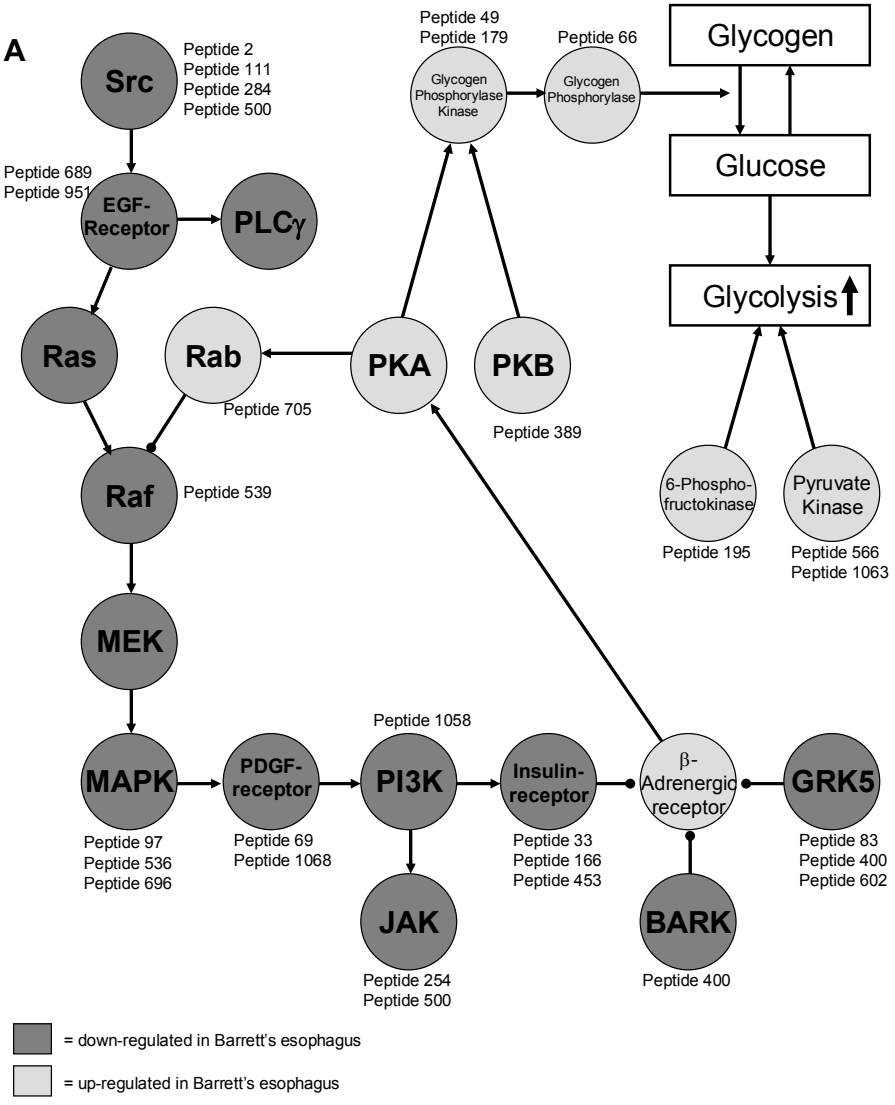


Figure 2: Scatter plots of kinase activities showing the correlations between Barrett's esophagus, normal squamous esophagus and gastric cardia. Scatter plots showing the overall distribution of all different kinase consensus substrates from 3 individual experiments in Barrett's esophagus versus normal squamous esophagus (A), Barrett's esophagus versus normal gastric cardia (B) and normal gastric cardia versus normal squamous esophagus (C) with the corresponding correlation in R^2 and p-value.

Delineation of cell signaling pathways and functions in BE

The results obtained were employed to construct provisional signal transduction schemes showing the differences in cellular signaling between BE and the surrounding epithelia (Figure 3). The results show a strong increase in glycolytic metabolism in BE compared to cardia epithelium, which was associated with down-regulated signaling through the insulin receptor and its downstream mediators Phosphatidylinositol-3 kinase (PI3K), Protein Kinase B (PKB) and GSK3 (Figure 3B).



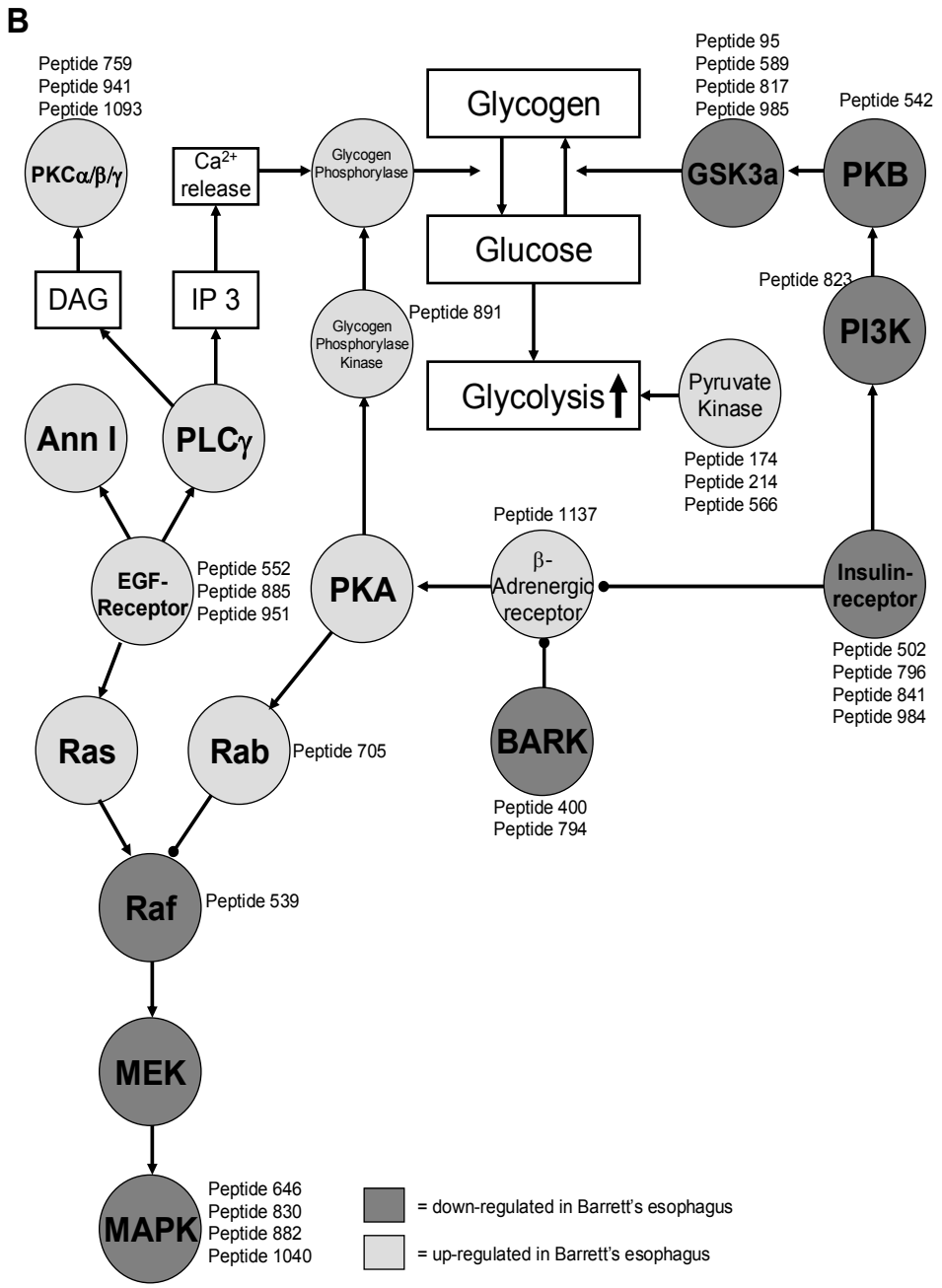


Figure 3: Provisional signal transduction schemes demonstrating differences in cell signaling. Provisional signal transduction schemes of several cellular signaling pathways comparing Barrett's esophagus and normal squamous epithelium (A) and Barrett's esophagus versus gastric cardia (B). Depicted in light grey are the significantly higher activated proteins in Barrett's esophagus and depicted in dark grey are the significantly less activated proteins in Barrett's esophagus. Important in Barrett's esophagus are decreased activity of the MAP kinase signaling cassette and the increased glycolytic metabolism. The EGF receptor signaling is decreased in Barrett's esophagus compared to normal squamous tissue, while EGF receptor activity in BE is increased compared to gastric cardia. Peptide numbers provided, correspond to the substrates on the peptide arrays.

The EGF receptor signaling was increased when BE versus cardia were compared (Figure 3B), and decreased in BE when compared to normal squamous (Figure 3A). The EGF signal in BE resulted in enhanced activation of Phospholipase-C (PLC) γ and PKC (Figure 3).

At the same time, signaling through the Mitogen-Activated Protein Kinase (MAPK) signaling cassette is decreased in BE, probably partly due to increased inhibition of c-Raf through Rab (Figure 3) and in case of the comparison between BE and normal squamous epithelium also because of diminished activation of c-Raf through the down-regulation of EGF receptor signaling (Figure 3A).

Surprisingly strong deactivation of the β-adrenergic receptor kinase, of which activation is associated with inhibition of the β-adrenergic receptor, was seen in BE when compared to normal squamous and cardia epithelium suggesting activation of β-adrenergic receptors in BE (Figure 3).

Validation of kinome profile results

Biopsies of a panel of 20 BE 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 EGF receptor (normal squamous esophagus > BE > normal gastric cardia) is suitable for such analysis as various different phospho-specific antibodies are available of which the immunoreactivity correlates well with EGF receptor activation.

Figure 4A shows that in all patients investigated the differential activation of the EGF receptor was confirmed when three different tissues were compared for EGF receptor activation. EGF receptor is significantly more activated in normal squamous esophagus compared to both BE and gastric cardia tissue (Paired *t*-test, $p<0.05$). For all different tyrosine sites for the EGF receptor investigated in this study, the EGF receptor was significantly more activated in BE compared to gastric cardia (Paired *t*-test, $p<0.05$). Also other key signaling elements were according to expectation, figure 5 shows significant reduced Src activation in BE, significant diminished activation of the MAPK signaling cassette (P-ERK Tyr 204) as expected from Rab activation in BE, and to a certain extent the expected enhanced activation of PKC-β1 in BE versus squamous epithelium. The kinome results suggest diminished activation of insulin receptor signaling in BE and we confirmed this employing a phospho-Phosphoinositide-Dependent Kinase (PDK) 1 antibody immunoreactivity as a read out. Figure 5 shows that BE is indeed associated with reduced activity of this pathway. Significantly decreased activity of PDK1 was seen in BE

compared to normal squamous esophagus and gastric cardia (Paired *t*-test; $p<0.01$ for BE versus squamous esophagus; $p<0.05$ for BE versus gastric cardia; Figure 5E).

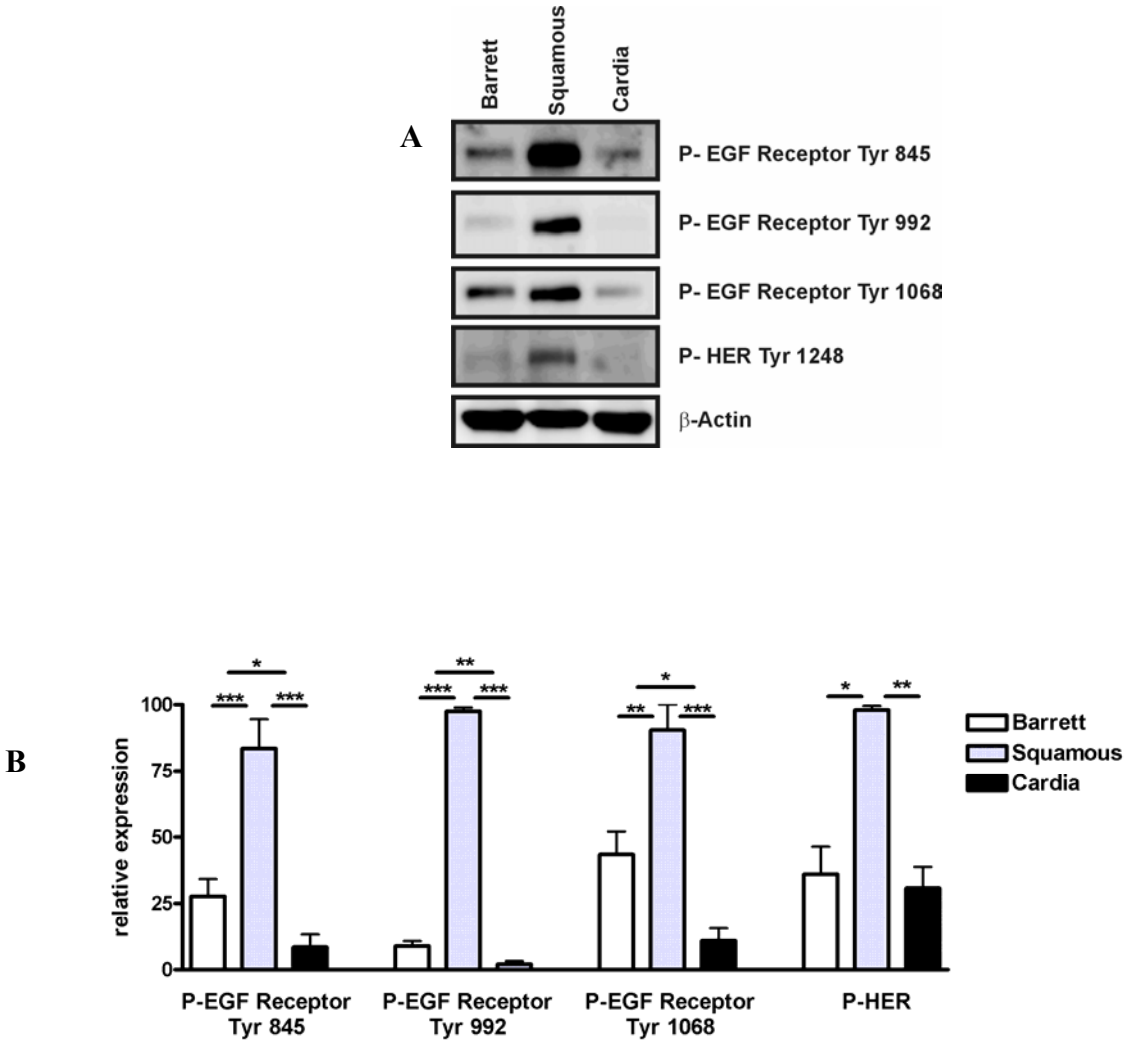


Figure 4: Immunoblot analysis for EGF Receptor activation in Barrett's esophagus, normal squamous esophagus and normal gastric cardia. Immunoblot analysis of expression levels of several phosphorylation sites of the EGF receptor (A) in Barrett's esophagus, normal squamous esophagus and gastric cardia. The pictures show high expression levels of P-EGF receptor in squamous epithelium, lower expression in Barrett's esophagus and lowest in gastric cardia (A). β-Actin was used as a control. Quantification of immunoblot results of P-EGF Receptor Tyr 845, Tyr 992, Tyr 1068 and P-HER2 Tyr 1245 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 (B; * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Phosphorylation levels of EGF receptor were also significantly higher in Barrett's esophagus compared to gastric cardia. The phosphorylation levels were determined by the ratio of signal intensity of the protein to that of the β-actin. Data are expressed as means ± SEM.

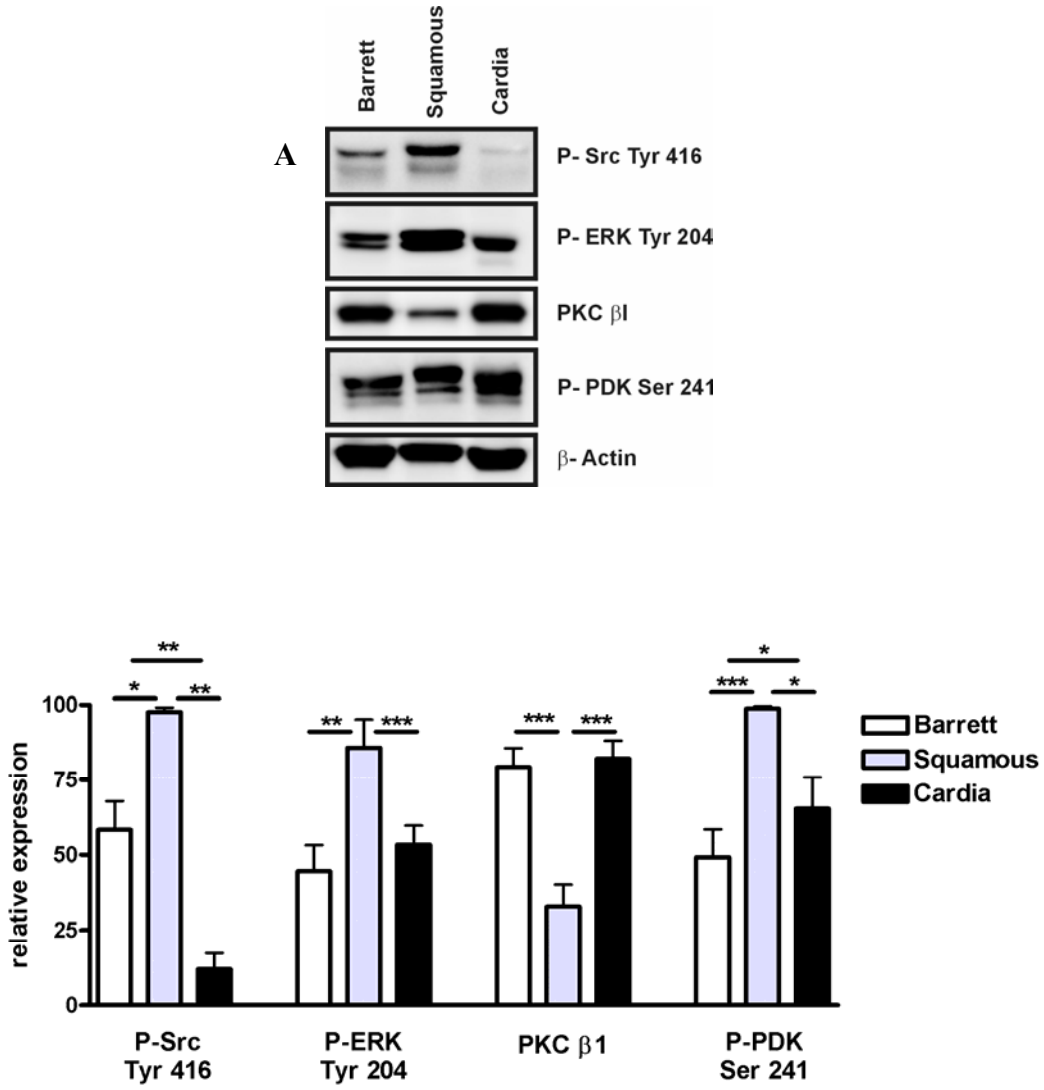


Figure 5: Immunoblot showing the activity of Src, ERK and PDK in Barrett's esophagus, normal squamous esophagus and gastric cardia. Immunoblot analysis of expression levels of P-Src, P-ERK, PKC βI and P-PDK (A) in Barrett's esophagus, normal squamous esophagus and gastric cardia. Immunoblotting of P-Src shows high expression in normal squamous epithelium, whereas the expression in cardia and Barrett's esophagus is significantly lower (A and B). Furthermore the pictures show significantly lower expression levels of P-ERK and P-PDK in Barrett's esophagus and higher expression in both normal squamous and cardia epithelium (A and B). PKC βI is highly expressed in both Barrett's esophagus and gastric cardia, but significantly lower in normal squamous esophagus (A and B). β-Actin was used as a control. Quantification of immunoblot results of P-Src Tyr 416, P-ERK Tyr 204, PKC βI and P-PDK Ser 214 in Barrett's esophagus, squamous esophagus and normal gastric cardia of 20 patients show that these are significantly higher expressed in the normal squamous esophagus biopsies (B; * $p<0.05$, ** $p<0.01$, *** $p<0.001$). The phosphorylation levels were determined by the ratio of signal intensity of the protein to that of the β-actin. Data are expressed as means ± SEM.

For validation of the glycolytic metabolism we performed an enzyme activity assay for pyruvate kinase. Results show that pyruvate kinase was significantly up-regulated in BE compared to normal squamous esophagus and gastric cardia (Paired *t*-test; $p<0.05$; Figure 6). Thus, assaying signal transduction in BE and its surrounding epithelia by conventional technology corresponds to the results obtained with the peptide arrays.

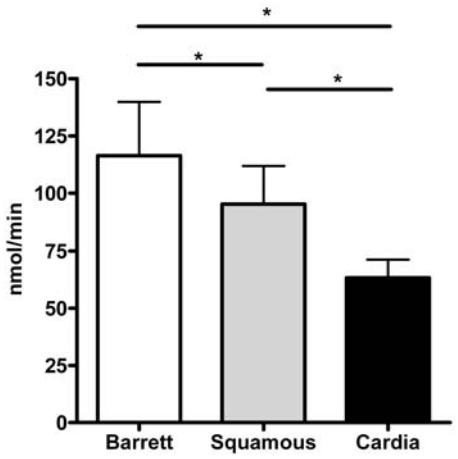


Figure 6: Pyruvate kinase activity assay of Barrett's esophagus, normal squamous and gastric cardia epithelium. Enzymatic assay for pyruvate kinase activity in Barrett's esophagus, normal squamous epithelium and gastric cardia showing that pyruvate kinase is significantly more activated in Barrett's esophagus compared to its surrounded tissue (* $p<0.05$). Data are expressed as means ± SEM.

Discussion

Over the last years array and mass spectrometry technologies have enabled analysis of the transcriptome and proteome of BE in comparison to 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 BE 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 certainly 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 BE 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 premalignant condition. In this study BE, normal squamous esophagus and gastric cardia were used for the analysis, since recently microarray and SAGE studies reported that at transcriptome level, BE has strong similarities with the anatomical 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 depending 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, since differences in metabolic conditions could influence the results analysed with the pep-chip.

Here, we choose to use biopsies of 3 male individuals known with non dysplastic BE for analyzing the cellular 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. Here upon conventional technologies were used to validate these pathways in biopsy specimens of another 27 BE patients. The results unambiguously show that the kinome signature of BE has strong similarities with both the kinome profiles of normal squamous and normal cardia epithelium. Confirming that BE does not represent a true trans-differentiation 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 BE. Of particular interest is the kinase activity of the phospho-EGF receptor, which is a transmembrane protein receptor that may trigger numerous signalling pathways[20]. Previously Jankowski *et al*. reported expression of EGF receptor in BE, however to our knowledge no research has been performed on EGF receptor activation in BE[29]. The immunoblot results show that EGF receptor is significantly more activated in normal squamous esophagus compared to BE. Yet the EGF receptor activity is

significantly higher in BE compared to 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 EGF receptor activation in BE as compared to cardia epithelium, suggests that although BE has a columnar phenotype, there may be aberrant up-regulation of cell growth and differentiation that at least partly is induced through the EGF receptor activation.

We found a decreased activity of Src kinase consensus sequences in BE compared to normal squamous esophagus. This is in contrast with a previously published paper of Kumble *et al* where they describe that Src kinase activity is increased in the malignant and pre-malignant BE[30]. Although the phosphorylation site in their report, Src Tyr 527, differs from the one that we studied, this is Src Tyr 416 that correlated to one of the phosphorylation sites on the pep-chip.

Another highly interesting finding is the down-regulated activity of the MAPK signalling cassette that seems to be a major event in BE compared to its surrounding tissue. To our knowledge no research has been performed on the MAPK activity in metaplastic BE. Therefore we validated the results with immunoblotting and confirmed that the level of ERK phosphorylation was indeed significantly down-regulated in BE compared to normal squamous esophagus. Compared to gastric cardia, the down-regulated level of ERK phosphorylation in BE was not significant, although a trend was seen ($p=0.066$; Figure 5C). It is of interest that in previous studies it has been demonstrated that acid and bile reflux can induce MAPK activity in BE adenocarcinoma cell lines[31-33]. It has also been described that malignant transformation of metaplastic BE 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 non-malignant metaplastic BE. Future kinase analysis comparing metaplastic, dysplastic and malignant BE will enlighten us on this matter.

Another important observation was the prominence of substrates in BE that suggest enhanced glycolytic activity. These results closely correspond to an earlier study, analysing the gene expression profiles of biopsies of BE, normal squamous esophagus and gastric cardia epithelium[16]. In this study, clustering of genes in functional classes showed that metabolism was one of the most important biological process in BE comparing to normal squamous and gastric cardia epithelium, in this biological process the increase in expression of enzymes associated

with the glycolysis was a prominent change. To further validate our kinase results we performed 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 phosphoenolpyruvate and ADP to pyruvate and ATP[35]. Results on biopsy material of 7 patients showed that indeed pyruvate kinase activity was significantly up-regulated in BE compared to normal squamous and gastric cardia epithelium (Figure 6).

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

Acknowledgements

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Supplemental Information

Peptide number	Sequence	Kinase	Barrett / Squamous
183	SSLKSRKRA		2.46
152	KRPSKRAKA	PKC	2.39
164	SSKRAK	PKC	2.25
995	RQRKSRRTI	PKC	2.11
926	KYRKSSLKS		2.07
195	LRGRSFMNN	PKA	1.85
389	SRTASFSES	PKB	1.77
650	EDTLSDSDD	CKII	1.75
23	SPRKSPKKS	sperm-specific histone kinase	1.73
179	RKQISVRGL	PK	1.73
239	KASASPRRK	sperm-specific	1.67
369	SLRASTSKS	S6K	1.66
368	RSGYSSPGS		1.66
34	RKRSAKE	PKA,PKG	1.65
1062	SPRKSPRKS	sperm-specific	1.65
900	AAASFKAKR	PKC	1.61
201	HMRSSMSGL	PKA	1.55
316	RGKSSSYSK	PKC	1.52
566	RRATPA		1.51
1096	AAASFKAKK	PKC	1.51
45	KRAKAKTAKKR	PKC	1.50
941	DPTMSKKKK	PKC	1.49
352	DAGASPVEK	PKC	1.49
380	PLTPSGEAP	Src	1.49
356	EGTHSTKRG	PKC	1.48
351	GSRGSGSSV	PKA	1.47
30	TLASSFKRR	PKC	1.46
793	ALGISYGRK	PKC	1.46
833	LTRRASFSAQ	PKA	1.45
807	SPKKSPRKA	sperm-specific	1.45
705	ARKKSSAQL	PKA	1.44
364	GEINTEDDD	CKII	1.44
231	VIKRSPRKR	CDK	1.43
180	AGTTYAL	MHCK	1.43
348	KRPSIRAKA	PKC	1.43
169	RLSPSPTSQ	CDK	1.42

969	LGSALRRR		1.40
166	AVDRYIAIT	IR	1.39
1044	RRLSSLRAS	S6K	1.38
244	KRSGSVYEP	PKA	1.37
1110	REILSRRPS	GSK3	1.36
574	YKNDYYRKR		1.36
167	DPLLTYRFP	PKC	1.36
1033	EQEEYEDPD	Lyn,Syk	1.35
189	VKRGISGL	H4-PK-I,PKA	1.35
154	PYKFPSSPLRIPGZ	na	1.33
349	NPGFYVEAN	EGFR	1.32
1149	RKRKSSQAL	PKA	1.31
827	KAKTTKKRP		1.31
151	ESSNYMAPY	PDGFR	1.30
551	GTVPSDNID	GRK2,GRK5	1.30
337	KLRRSSSVG	PKA,PKC	1.29
571	RKFSSARPE	PKA	1.28
362	AVDGYVKPQ	Lyn,Src,JAK2	1.28
188	PQPEYVNQP	erbB2	1.27
786	KRPSNRAKA	PKC	1.25
158	GGRDSRSGS	PKA,PKC	1.25
1063	RRRASVA	PKA,PKC	1.25
156	DAHKSKRQH	CKII	1.24
915	GRGLSLSR	PKA	1.20
49	KRKQGSVRGL	PhK,PKA	1.16
127	TAYGTRRHL	PKC	1.12
747	GTRLSLARM	PKA,PKC	1.12
1076	KKDVTPVKA		0.84
602	EKESSNDST	GRK	0.82
951	DNLYYWDQD	erbB2	0.82
260	RRRASQLKI	PKC	0.81
453	GRILTLPRS	PKC	0.80
3	EDNEYTARQ	Src	0.79
689	EEQEYVQTV	EGFR	0.77
307	EENVSVDDT	CKII	0.77
696	DLPLSPSAF	MAPK	0.77
259	EPAVSPLLP	CDK	0.76
427	VHNRSKINL	AMP-PK	0.76
539	QLIDSMANS	Raf1	0.75
254	AQDTYLVLD	Lyn,JAK2	0.74
517	LGEGTP		0.74
232	SPVKSPEAK		0.73
1040	FLTEYVATR		0.73
834	HRQETVEAL	CaM-II	0.73
716	TRQTSVSGQ	CaM-II	0.73
531	PINGSPRTP	CDK	0.73
208	GMGTSVERA	PDK	0.73
33	SPGEYVNIE	IR	0.72
1080	MPGETPPLS	GSK3	0.72
284	STGIYEALE	Src	0.71
512	RGAISAEVY	PKG	0.71
500	DLPMSPRTL	JAK1,Src	0.71
734	LIEDAEYTA	Lck,Fyn,Fes,Yes	0.70
1067	PSPKYPGPQ		0.69
102	ERTNSLPPV	PKA	0.69
516	SGYSSPGSP		0.68
533	KLINSIADT		0.67

311	NDITSL	tropomyosin kinase	0.67
805	ESVDYVPML	PDGFR	0.66
69	DGHEYIYVD	PDGFR	0.66
400	GHQGTVPSD	GRK5	0.66
529	SVFSSPSAS	CDK	0.65
591	EDAESEDEE		0.65
393	HDALSGSGN	CKI	0.65
880	KKIDSFASN	PKC	0.65
709	RKISASEA		0.64
673	SRKGSGFGH	PKA,PKC	0.64
76	PRRASATSS	? CKI	0.64
501	GDLQSAEFH		0.64
536	SRQLSSGVS	MAPKAPK2	0.64
1068	SSVLYTAVQ	PDGFR	0.64
94	LSVSSLPGL	CKI	0.64
409	NVFSSPGGT	CDK	0.63
246	HSSQSQGGG	PKA,PKC	0.63
444	EEELYLEPL		0.63
1051	RDDTYTAHA	Abl	0.63
111	EEPQYEEIP	Src	0.62
97	KVPQTPLHT	MAPK	0.61
261	SFTTTAERE	AFK	0.61
519	TAESSQAEE	CKII	0.60
921	STSVSAVAS	GRK	0.60
819	VGPGYLGSG		0.59
255	DIPESQMEE	CK,CKII	0.59
62	EEDLSDENI	CKII	0.59
1059	TPPLSPIDM	ERT,PK	0.58
1058	STNEYMDMK	PI3K,CHK	0.57
66	KQISVR	PK	0.57
919	PASPSPQRQ	CDK	0.55
1001	ESSYSYEEI		0.54
41	LSDDSFIED	CKII	0.52
143	QASSTPLSP	CDK	0.50
610	YTRFSLARQ	PKC	0.50
510	DLFGSDDEE	CKII	0.49
83	STNDSLL	GRK5	0.47

Table 1: Peptide substrates with significantly altered phosphorylation in Barrett's esophagus versus normal squamous esophagus. The fold induction of each substrate, their corresponding protein kinases and chip peptide number are provided.

Peptide number	Sequence	Kinase	Barrett / Cardia
955	LRRGSLG	PKA,H4-PK	2.34
214	RRASI	PKA,PKC	2.23
573	LRRASLG	PKA,H4-PK	2.07
625	LRRASLGAA	PKA,H4-PK	2.06
174	LRRASLAG	PKA	2.04
650	EDTLSDSDD	CKII	2.02
941	DPTMSKKKK	PKC	1.99
804	DDAYSDTET	CKI	1.88
1093	RRRRAASVA	PKC	1.87
534	LDDQYTSSS		1.87
958	RRASLG		1.83
1151	LRRATLG	PKA,H4-PK	1.79
694	GRRESLTSF	PKA,PKG	1.79
588	QRRTSVSGE	PKA	1.78
152	KRPSKRAKA	PKC	1.73
134	SRRSSLGSL	PKA	1.72
891	ARTKRSGSV	PKA	1.71
890	GRPITPPRN	CDK	1.69
784	QRRTSLTGS	PKA	1.69
747	GTRLSLARM	PKA,PKC	1.67
566	RRATPA		1.65
917	VTRSSAVRL		1.62
977	SRSRSRSPG	RS	1.61
1110	REILSRRPS	GSK3	1.60
552	EGSAYEEVP	EGFR	1.59
1145	ADSFSLHDA	CKI	1.59
1078	LYSGSEGDS		1.59
972	PPRRSSIRN		1.56
946	RVLESFRAA	PKC	1.56
898	SIYSSDDDE	CKII	1.56
769	LRRASGG	PKA,H4-PK	1.56
177	TVTRSYRSV	PKC	1.55
915	GRGLSLSR	PKA	1.55
959	AAVDTSSEI	CKII	1.55
951	DNLYYWDQD	erbB2	1.54
949	ADSFSLNDA	CKI	1.54
498	GRRQSLIED	PKA,PKC,Cam-II	1.52
688	MRRNSFTPL	PKA	1.51
187	TTPLSPTRL	CDK	1.51
294	PRRRTRRAS	PKA	1.51
1137	DPPGTESFV	beta-ARK	1.50
759	LRRPSLG	PKC	1.50
146	LLQDSVDFS	CaM-II	1.50
911	SYPLSPLSD	MAPK	1.49
20	DDINSYEAW		1.49
103	IRRASTIEM	PKA,CaM-II	1.49
167	DPLLTYRFP	PKC	1.49
835	LRRFSLATM	PKA	1.49
705	ARKKSSAQL	PKA	1.48
973	RTKGSGSV	PKA	1.48
813	SSSNTIRRP	PKC	1.48
549	DRLVSARSV	PKC	1.48
276	VPRTPGGRR	MAPK	1.48
901	ARKFSSARP	PKA	1.47
692	NDSVYANWM		1.46
967	SSEESIISQ	CKI	1.45

768	LRRASLDG	PKA,H4-PK	1.43
928	SRLHSVRER		1.43
23	SPRKSPKKS	sperm-specific histone kinase	1.43
865	RAAASRARQ	PKC	1.42
256	FPRASFGSR	PKA	1.42
18	RRASL	PKA	1.41
244	KRSGSVYEP	PKA	1.40
128	TRRISQTSQ	CaM-II	1.40
275	TRAPSRTAS	MFPK	1.39
752	RRPTPA	PKA	1.38
556	RRPTVA	PKA	1.37
935	ESMESYEVS		1.37
807	SPKKSPRKA	sperm-specific	1.37
453	GRILTLPRS	PKC	1.36
719	GTKRSGSV	PKA	1.36
1169	RTGRSGSV	PKA	1.36
37	LRRFSLATM	PKA	1.36
135	PETVYEVAG	Src	1.35
481	TPAISPSKR		1.35
929	KNDKSKTWQ	IR	1.32
905	RAKRSGSV	PKA	1.32
846	EDSTYYKAS	FAK	1.32
444	EEELYLEPL		1.31
282	LSGFSFKKS	PKC	1.31
1154	RGYSLG	PKA	1.31
731	PLRRTLSVA	AMP-PK,CKI,PKA,PK	1.28
1149	RKRKSSQAL	PKA	1.25
1106	SSKRA	PKC	1.25
885	EEQEYIKTV	EGFR	1.24
141	KLSPSPSSR	CDK	1.24
956	RRSVSEAAL	AMP-PK,CaM-II	1.23
836	EEEAYGWMD	Src	1.21
1160	RKRTLRRL	PKC	1.21
1146	ATRRSYVSS	PKA,PKC	1.20
728	KQGSGRGL	PK	1.20
857	TKRSGSV	PKA	1.19
220	RRAASVA	PKA,PKC	1.18
348	KRPSIRAKA	PKC	1.18
260	RRRASQLKI	PKC	1.18
1044	RRLSSLRAS	S6K	1.17
374	YDKEYYSVH	Met	1.17
1045	SFKKSFKLS	PKC	0.94
24	RRKASGP	PKA	0.91
575	SSEITTKDL	CKII	0.89
542	EILNSPEKA	AKT	0.88
652	RRPTSPVSR		0.85
169	RLSPSPTSQ	CDK, cdc2 kinase	0.85
274	STSKSESSQ	S6K	0.84
829	PLSKTLSVSSL	AMP-PK,CKI,PKA,PK	0.84
28	LRRAS	PKA	0.84
702	SKAGSLGNI	PKA	0.83
1012	YRKGSLKSR		0.82
691	IREESPPHS		0.82
644	PYKFPSSPLRIPGZ		0.81
491	IGSVSEDNS	CaM-II	0.81
661	VKRGSGL	H4-PK-I	0.80
380	PLTPSGEAP	Src	0.80

501	GDLQSAEFH		0.80
539	QLIDSMANS	Raf1	0.79
176	THERSPSPS	proline-rich kinase	0.79
124	SIDEYFSEQ	Tyk2	0.78
640	EEEEYMPME	Src	0.78
641	EQFSTVKGV	GRK5	0.78
699	EEKGSPLNA		0.77
156	DAHKSKRQH	CKII	0.77
197	HDLSSEMFN		0.77
667	TPQVSDTMR	PKA	0.77
529	SVFSSPSAS	CDK	0.76
38	PRPASVPPS	DYRK	0.76
540	IVYKSPVVS		0.76
1122	KYLASASTM	CaM-II	0.75
796	GKTDYMGEA	IR	0.75
286	LYSSSPGGA	CDK	0.74
218	YVTTSTRTY	PKC	0.74
297	EETQTQDQP	ds-DNA	0.74
119	GAFSTVKGV	RK	0.74
616	KGYSLG		0.74
651	ENQASEEED	CKII	0.74
414	YTTNSPSKI	CDK	0.74
620	YRLPSNVDQ	CRYS-K	0.73
482	LVVASAGPT		0.73
344	KGGSYSQAA		0.73
812	KEAKSD	CKII	0.73
985	HHHATPSPP	GSK3	0.72
882	PAAPSPGSS	MAPK	0.72
653	SFMDSSGLG		0.72
531	PINGSPRTP	CDK	0.72
841	GNGDYMPMS	IR	0.71
800	SAYGSVKAY	PKC	0.71
895	EEKESSNDS	GRK	0.70
74	RRKMSRGLP	PKA	0.70
990	DDPSYVNVQ	Fyn,Lck,IR,PDGFR	0.70
798	EKEISDDEA	CKII	0.70
75	SEVPYREVQ	JNK2	0.69
823	SSNEYMDMK	PI3-KINASE	0.69
880	KKIDSFASN	PKC	0.69
609	ETAESSQAE	CKII	0.68
299	IRQASQAGP	CaM-II	0.68
502	GSEEYMNMD	IR	0.68
723	PASQTPNKT	CDK	0.68
602	EKESSNDST	GRK	0.68
232	SPVKSPEAK		0.68
991	EVEKSPVKS		0.68
727	PGPQSPGSP		0.67
791	PYKFPSSPLRIPGZ		0.67
52	EEGISQESS	CKII	0.67
432	TKAASEKKT	PKC	0.67
84	VRTFTHEVV	CDK	0.67
974	KRAASPRKS	sperm-specific	0.67
95	SRHSSPHQS	GSK3, CKII	0.67
646	APVASPAAP	MAPK	0.67
830	QSGMTEYVA	MAPK	0.67
730	LASSSKEEN		0.67
888	NDSTSVSAV	GRK	0.67

1030	HRQETVDAL	CaM-II	0.66
613	RPPGFSPFR		0.66
519	TAESSQAEE	CKII	0.66
1121	KKRFSFKKS	PKC	0.66
271	SETKTEEEE	CKII	0.65
878	KRKQISVAGL	PK	0.65
679	PTRHSRVAE	PKA	0.65
822	PRHLSNVSS	PKA	0.65
61	GRLSSMAMI	CaM-II	0.65
1164	PKKGSKKAV	PKA	0.65
67	SLKDH	PKC	0.65
795	EYVQTVKSS	PKC	0.64
1040	FLTEYVATR		0.64
817	SNVSSTGSI	GSK3	0.63
840	PYKFPSSPLRIPGZ		0.63
984	ELSNYIAMG	IR	0.63
986	KTETSQVAP	RK	0.63
60	FPVSYSSSG	Ret	0.63
425	SPALTGDEA	CKII	0.62
82	SPSSSPTHE	proline-rich kinase	0.62
91	PVSPSLVQG	GRK	0.61
834	HRQETVEAL	CaM-II	0.61
630	PSAPSPQPK	CDK	0.60
794	DDSGSAMSG	GRK	0.60
400	GHQGTVPSD	GRK5	0.60
819	VGPGYLGSG		0.55
1170	KQSPSSSPT	PKC	0.55
598	DEAATKTQT	MHCK	0.54
66	KQISVR	PK	0.53
393	HDALSGSGN	CKI	0.51
618	TKKTSFVNF	PKA	0.44
589	HATPSPPVD	GSK3	0.43

Table 2: Peptide substrates with significantly altered phosphorylation in Barrett's esophagus versus gastric cardia. The fold induction of each substrate, their corresponding protein kinases and chip peptide number are provided.

Chapter 5

Gene expression profile comparison of Barrett's esophagus epithelial cell cultures and biopsies

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Abstract

Barrett's esophagus (BE) is the metaplastic process in which the normal squamous epithelium of the distal esophagus is replaced by columnar lined epithelium. To gain more insight in the process of metaplasia and to identify which genes are specifically expressed by the epithelial cells and the surrounding tissue, the aim was to define and analyze gene expression profiles of squamous and BE primary cell cultures and compare with BE and normal squamous esophageal biopsies. To specifically obtain the epithelial cell layer, epithelial cells out of biopsies of BE and normal squamous epithelium were cultured using a Barrett specific culturing medium. Serial analysis of gene expression (SAGE) was applied to obtain transcription libraries of these primary epithelial cell cultures. The transcriptomes were analyzed and compared with previously described transcriptomes of BE and normal squamous esophageal biopsies. Validation of results by RT-PCR was performed using tissues of 16 BE patients and 16 primary cell cultures. Over 85,000 tags were sequenced. The gene expression profiles of the cultured epithelial BE and squamous cells showed remarkable similarities that most likely is due to culturing artifacts causing de-differentiation towards a primordial epithelial cell type. Genes specifically expressed by the Barrett epithelial cells were for instance Lipocalin 2 and Cyclin D1, whereas Annexin A10, TFF1 and TFF2 were specifically expressed in the BE biopsies.

Introduction

Gastroesophageal reflux disease (GERD) is a common condition found in 20% of the population[1]. The key feature of GERD is reflux of gastric contents into the esophagus. One of the most important complications of chronic GERD is BE, a pre-cancerous condition in which the normal squamous epithelium of the esophagus is replaced by a metaplastic, columnar lined epithelium[2,3]. BE is associated with a 30 to 125 fold increased risk for developing esophageal adenocarcinoma[4-6]. For understanding the biological processes underlying the process of metaplasia and malignant transformation, numerous gene expression profile and microarray studies have been performed. For instance several studies compared BE and esophageal carcinomas, or BE, normal squamous esophagus and gastric cardia[7-10]. One limitation of these studies is that most of the time full biopsies were used to describe and compare gene expression profiles. Full biopsies consist of a heterogeneous cell population; that do not only contain epithelial cells but also stromal tissue and inflammatory factors. These factors may be expressed at higher levels and obscure the analysis of several specific epithelial genes of which the expression may be more subtle. Therefore, we hypothesized that analyzing and comparing gene expression profiles of non transformed primary cultures of epithelial cells of BE and normal squamous esophagus may more precisely illustrate which genes are involved in the transformation of squamous epithelium into BE. By studying the gene expression of these primary cultures in comparison to that of biopsies, genes solely expressed by epithelial cells can be identified and distinguished from stromal factors. The culturing method for obtaining these epithelial cultures has been described before[11-13]. The cultures are considered as models for Barrett and normal squamous epithelial cells and as such have been previously used in several studies[13-15]. In the present study, gene expression analysis is performed by using Serial analysis of gene expression (SAGE) which has been first described by Velculescu *et al*.[16]. In the SAGE methodology short nucleotide sequences of each expressed gene are generated. These 10 base-pair tags contain sufficient information to uniquely identify the transcripts through public websites (SAGEgenie, http://cgap.nci.nih.gov). By ligating these tags into concatemers, serial analysis of thousands of gene specific tags can be performed at once. This technique quantitatively evaluates the expression pattern of transcripts by determining the abundance of individual tags and identifying the gene corresponding to each tag.

For SAGE analysis, RNA was isolated from non transformed primary squamous and BE cell cultures containing epithelial cells. A panel of another 16 squamous and 16 BE primary cell cultures, 16 BE biopsies and 16 normal squamous esophageal biopsies was used to validate the obtained expression profiles by Reverse-Transcription Polymerase Chain Reaction (RT-PCR). SAGE of the cultured cells resulted in two unique gene expression profiles with over 85,000 identified tags. The SAGE expression profiles of the squamous and BE cell cultures were first compared to identify differences between these two epithelial cell types. In order to identify genes expressed by the epithelial cell layer from stromal tissue in BE and squamous esophagus, the SAGE libraries of the BE and squamous cell cultures were compared with previously published SAGE libraries of BE and squamous esophageal biopsies[10].

In summary, this study gives an overview of the expression profiles of non transformed squamous and BE epithelial cell cultures, and several factors that are expressed by either epithelial or stromal tissues in BE and squamous esophagus are identified. The results of this study greatly improves our insight in the molecular and biological mechanisms that may be involved in the development of BE and open avenues for potential diagnostic or therapeutic opportunities.

Materials and Methods

Biopsy Specimens

Tissue samples were obtained during routine surveillance endoscopy. Thirty-four patients with known BE but without dysplasia were included in this study. To prevent reflux esophagitis, all patients were on long term proton pump inhibitors of 40 to 80 mg daily. BE was defined as histologically recognized incompletely differentiated intestinal type of metaplasia in the distal esophagus. Endoscopically, none of the patients had reflux esophagitis. Paired biopsies, taken next to each other, were obtained of the Barrett's segment and normal squamous esophagus. The Barrett's segment was biopsied at least 2 cm above the gastroesophageal junction yet within the Barrett's segment. The Barrett's segment was recognized endoscopically as typically pink colored columnar type of metaplasia. The normal squamous epithelium was biopsied at least 2 cm above the Barrett's segment. Of each set of biopsies, one biopsy was used for

histopathological confirmation. All patients signed informed consent for the use of their biopsy material.

Cell Culture Medium and Maintenance of Cells

Specimens for tissue culturing were placed immediately in essential medium (Sigma Chemical Co., St Louis, MO) on ice before processing. Biopsy specimens were processed within 4 hours from the time of the biopsy. The explant method described by K. Washington *et al*. was used[11]. With this method, biopsy specimens were minced into fragments of 1 to 2 mm in size. The pieces of tissue were placed in a 24 wells culture plate and anchored under glass coverslips before addition of growth medium. Primary cell cultures were initiated by maintaining the cells in Barrett's plus media, as previously described, containing 5% fetal bovine serum (FBS), 0.4 μg/mL hydrocortisone (Sigma Chemical Co.), 20 ng/mL epidermal growth factor (GIBCO BRL, Grand Island, NY), 10^{-10} mol/L cholera toxin (Sigma Chemical Co.), 140 μg/mL bovine pituitary extract (Sigma Chemical Co.), 20 μg/mL adenine (Sigma Chemical Co.), 100 U/mL penicillin (GIBCO BRL), 0.25 μg/mL streptomycin (GIBCO BRL), 0.25 μg/mL amphotericin B (GIBCO BRL), 4 mmol/L glutamine (GIBCO BRL), 5 μg/mL insulin, 5 μg/mL transferring, and 5 ng/mL selenium (Sigma Chemical Co.)[12, 13]. Cells were maintained in a humidified atmosphere containing 5% CO_2 in air at 37°C and fed two times a week. After five to six weeks of culture, RNA was isolated.

RNA isolation

Total RNA was isolated using Trizol Reagent (Life Technologies Inc, Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. In brief: tissues and epithelial primary cell cultures were lysed by adding 200 μl Trizol. After phenol/chloroform extraction, RNA was precipitated with isopropanol, washed with 70% ethanol and air-dried. The RNA was then dissolved in RNase-free H_2O and stored at -80°C until required. The RNA concentration was determined spectrophotometrically with 1 μl of total RNA on the Nanodrop® (type ND-1000, Wilmington, USA).

SAGE procedure

The SAGE libraries were obtained essentially following the SAGE protocol as described by Velculescu *et al*. using the Life Technologies I-SAGE kit and following manufacturer's instructions[10, 16]. Five μg of total RNA was used per SAGE analysis. The isolated concatemers, consisting of serially ligated tags, were ligated into the pZErO-1 vector (Life Technologies) and transformed in TOP10 Electrocompetent *Escherichia coli* cells (Life Technologies) by means of electroporate transformation, following manufacturer's protocol (Biorad, Hercules, CA). PCR was performed on obtained colonies with specific primers Sp6-F (5'-GATTTAGGTGACACTATAG-3') and T7A-R (5'- TAATACGACTCACTATAGGG-3') and PCR products were analyzed by agarose gel electrophoresis. A total of 1920 PCR fragments were selected for DNA sequencing using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) and the T7A-R primers. Samples were run on an ABI3730 DNA Analyzer (Applied Biosystems) and analyzed with Sequence Analysis 5.1 software.

SAGE and Statistical Analysis

For analysis of the SAGE data the program USAGE V2 (Academic Medical Center, bioinformatics department) and the public databases of the NCBI-site and SAGE Genie (http://cgap.nci.nih.gov) were used[17, 18]. Statistical analyses and comparison of the SAGE libraries was done using a comparative *Z*-test (Pair-wise comparison, binominal approach) of the USAGE V2 program[19, 20].

RT-PCR

cDNAs from squamous and BE primary cell cultures as well as squamous and BE biopsies were synthesized from 1 μg of total RNA using an oligo dT primer and Superscript II MMLV-reverse transcriptase according to manufacturer's instructions (Life Technologies). Primers were derived from mRNA sequences as deposited in GenBank (NCBI-site). Amplicons using specific primers were generated for TFF1, TFF2, Annexin A1, Annexin A10, Lipocalin 2, Prosaposin, Cyclin D1, Plakophilin 3, Galectin 7, Keratin 17, Keratin 19, Cytokeratin 7, Smurf 1, Diacylglycerol kinase α, SOX 4, β-2-microglobulin and β-actin (Table 1). Subsequent PCR analyses were carried out in 25 μl reactions containing 1 μl cDNA, 23 μl Reddy Mix PCR Master Mix (Applied Biosystems), 200 ng Forward primer and 200 ng Reverse primer. The products were electrophoresed on 1.5%

agarose gel. The mRNA expression level was determined by the ratio of signal intensity of the specific mRNA to that of the β-2-microglobulin and β-actin. Statistical analysis of mRNA expression levels was conducted using GraphPad Prism version 4.00, GraphPad software (San Diego, CA). Data are expressed as means ± Standard Error of Mean (SEM). Comparison between two groups was analyzed using two-tailed *t*-tests.

Gene	Forward primer	Reverse primer	Annealing temperature	Fragment length
TFF1*	TTTGGAGCAGAGAGGAGG	TTGAGTAGTCAAAGTCAGAGCAG	60ºC	438 bp
TFF2	ATGGATGCTGTTTCGACTCC	GGCACTTCAAAGATGAAGTTG	55ºC	247 bp
CK7**	TGAATTAACCGCCGCACAG	TGCATTTGGCCATCTCCTCA	65ºC	277 bp
SOX 4***	CTTGACATGATTAGCTGGCATGATT	CCTGTGCAATATGCCGTGTAGA	64ºC	100 bp
Cyclin D1	AGAGGCGGAGGAGAACAAAC	GGCACAAGAGGCAACGAAG	60˚C	207 bp
Annexin A1	TGGTATCTCCCTTTGCCAAGC	TTTCCTCCACAAAGAGCCACC	60˚C	80 bp
Annexin A10	TTGTTCTCTGTGTTCGAGACAAACC	GTAGGCAAATTCAGGATAGTAGGC	52ºC	609 bp
Lipocalin 2	GGAGCTGACTTCGGAACTAAAGG	AGCCGTCGATACACTGGTCG	60ºC	109 bp
Galectin 7	ATGTCCAACGTCCCCCACAAG	TGACGCGATGATGAGCACCTC	60˚C	282 bp
Plakophilin 3	AGCCTGGAGGAGAAGGCTAAT	AGTGCTGGCTATCCCAAGATACT	60ºC	234 bp
Prosaposin	CCAGAGCTGGACATGACTGA	CAGTTCCCAACAAGGGCTTA	60ºC	999 bp
DGKα****	CGGATTGACCCTGTTCCTAACAC	TTTGAGGTGGTGCAGGATAATAAA TA	60˚C	1262 bp
Keratin 17	TGCAGGCCTTGGAGATAGAGCT	ACGCAGTAGCGGTTCTCTGTCT	60˚C	90 bp
Keratin 19	CAGATGAGCAGGTCCGAGGTTA	CGTTTCTGCCAGTGTGTCTTCC	60˚C	113 bp
Smurf 1*****	GTCCAGAAGCTGAAAGTCCTCAGA	CACGGAATTTCACCATCAGCC	64˚C	165 bp
β-2-microglobulin	CTCGCGCTACTCTCTCTTTCT	TGCTCCACTTTTTCAATTCTCT	60ºC	185 bp
β-actin	GTCAGAAGGATTCCTATGTGG	GCTCATTGCCAATGGTGATG	52ºC	628 bp

Table 1: Primer sequences. Primer sequences used for RT-PCR with corresponding used annealing temperatures and PCR fragment lengths. *) TFF= Trefoil Factor, **) CK7= Cytokeratin 7, ***) SOX4= SRY box 4, ****) DGKα= Diacylglycerol kinase α, *****) Smurf1= SMAD specific E3 ubiquitin protein ligase 1

Results

Patients

In this study, 34 BE patients were included, 29 were male, mean age was 64 years (range 33-83 years). The average length of the BE segment measured endoscopically was 3.9 cm (range 2-9 cm). Endoscopically, none of the patients had reflux esophagitis. All patients had proven incompletely differentiated intestinal type of columnar epithelium without dysplasia in the histological control biopsies with no signs of active or acute inflammation. Normal esophageal

squamous epithelium was also confirmed histologically, in all the pair wise taken control biopsies.

SAGE library characteristics

Two unique SAGE libraries were obtained. The SAGE library of the non transformed BE epithelial primary cell culture consisted of 44,991 tags containing 13,521 different transcripts. Of these transcripts, 1,753 (13%) were observed at least 5 times, and 756 transcripts (5.6%) were found to be at least 10 times present in the BE cell culture SAGE library (Table 2). The SAGE library of the non transformed squamous esophageal epithelial primary cell culture consisted of 43,402 tags, with 13,816 different transcripts. Sixteen hundred and sixty-four transcripts (11.9%) were at least 5 times present and 719 (5.2%) transcripts were observed at least 10 times in the squamous esophageal primary cell culture SAGE library (Table 2). About 26% of the BE tags and 27% of the squamous esophagus tags were observed only once in each library (Table 2). The complete SAGE libraries can be found on the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/; Table 2). When interpreting SAGE results, it has to be considered that polymorphisms, alternative splicing and alternative polyadenylation in the mRNA can cause that various different tags code for the same gene. For example the Expressed Sequence Tags (ESTs) of GGAACAAACA, CGAACAAAAG and GTCACACACT correspond to the same gene cluster of CD24 antigen, and the ESTs of TGCAGCACGA and GTGCGCTGAG correspond to the gene cluster of MHC class 1C.

Comparison of the gene expression profiles of the epithelial cell cultures

Comparing the SAGE libraries of BE cell culture with normal squamous esophageal primary cell cultures, 511 tags were found to be significantly differentially expressed ($p<0.05$, Pair-wise comparison, binominal approach). A total of 282 tags were significantly up-regulated in BE and 229 tags were down–regulated. Just 85 tags were more than 5 fold up-regulated in BE, from these 5 tags were more than 10 fold up-regulated. Eighty tags were more than 5 fold down-regulated in BE and from these 8 tags were more than 10 fold down-regulated. The 5 tags that were more than 10 fold up-regulated in the BE primary cell culture SAGE library correspond to the genes: glutamic-oxaloacetic transaminase 2, MHC class 1C, beta-1-glycoprotein 2, KDEL endoplasmic reticulum protein retention receptor 1 and interleukin 1 receptor antagonist (Table 3). The 8 tags

that were more than 10 fold down-regulated in the BE SAGE library correspond to serum amyloid A1, thioredoxin, protein tyrosine kinase 9, glutamate dehydrogenase, DEAD/H box polypeptide 3, epidermal growth factor receptor, MHC class 1C and an unknown gene (Table 3). Most highly expressed genes in both primary cell cultures were ribosomal proteins and calcium binding proteins.

	Squamous cell culture	**Barrett cell culture**
Total tags	44,991	43,402
Unique tags	13,521	13,816
Singletons	11,992 (26.7%)	11,471 (26.4%)
Tags 5-times present	1,753 (13%)	1,664 (11.9%)
Tags 10-times present	756 (5.6%)	719 (5.2%)
Accession code	GSM 110380	GSM 110378

Table 2: SAGE library characteristics. Number of total tags in the squamous and Barrett cell culture SAGE libraries, together with the corresponding accession code in the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/), the number of unique tags, the number of singletons and the number of tags at least 5 times and 10 times present in each of the libraries. Calculation of the percentages of singleton tags was based on the total number of tags present in the libraries.

Validation of the primary cell culture SAGE results

To validate the SAGE data, expression levels of Prosaposin, Plakophilin 3, Annexin A1, Annexin A10, Diacylglycerol kinase α, Galectin 7, Keratin 17, Keratin 19 and Lipocalin 2 were determined using RT-PCR on RNA samples of 16 squamous primary cell cultures and 16 Barrett primary cell cultures. Although, in the SAGE analysis several of these genes were differentially expressed, in all cases examined results showed equal expression of these genes in both squamous and Barrett primary cell cultures (Figure 1).

Gene expression comparison of squamous epithelial cell culture versus squamous biopsy

Comparing the SAGE libraries of squamous primary cell culture with the squamous biopsy[10], 1011 tags were found to be significantly different expressed ($p<0.05$; Pair-wise comparison, binominal approach); 628 tags were significantly up-regulated in the squamous cell culture SAGE library, 383 tags were significantly down-regulated. A total of 104 tags were more than 10 fold up-regulated in the squamous primary cell culture SAGE library and 132 tags were more

than 10 fold up-regulated in the squamous biopsy SAGE library. Genes corresponding to tags that were more than 10 fold up-regulated in the squamous primary cell culture were Apolipoprotein L2 (AAAGCAGTTT), Synaptogyrin 2 (GCTCCCAGAC), TGFβ-1 (GGGGCTGTAT) and Cytokeratin 7 (CCTGGTCCCA). Several genes corresponding to the tags that were more than 10-fold up-regulated in the squamous biopsy SAGE library were several CKs, i.e. keratin 1 (ACATTTCAAA), keratin 4 (GTGACAACCT; AGTGTGAAGC; AGTGTGGCTG), keratin 6C (ATGTAATCAC; GACAACAACC), keratin 13 (AAAGCGGGGC). Furthermore epithelial membrane protein 1 (TAATTTGCAT; AGATAAATAA), Fatty Acid Binding Protein 5 (AAGGAGCTAG) and Cadherin 12 (GACAGAGAAG) were genes corresponding to the tags that were more than 10 fold up-regulated in the squamous biopsy SAGE library.

tag	p SQ	p BE	down in BE	up in BE	gene ID
					glutamic-oxaloacetic transaminase 2,
TAGACTTATT	0,230	4,001		17,4	mitochondrial
GTGCGCTGAG	0,230	3,779		16,4	major histocompatibility complex, class I, C
GAAACTGTGA	0,230	3,779		16,4	pregnancy specific beta-1-glycoprotein 2
					KDEL (Lys-Asp-Glu-Leu) endoplasmic
TTTTTGTACA	0,230	2,667		11,6	reticulum protein retention receptor 1
ACTCGTATAT	0,461	4,890		10,6	interleukin 1 receptor antagonist
TGCAGCACGA	2,304	0,222	10,4		major histocompatibility complex, class I, C
GGAAAGATGT	4,608	0,445	10,4		Epidermal growth factor receptor
TCCCCGTACA	28,340	2,667	10,6		unknown
					DEAD/H (Asp-Glu-Ala-Asp/His) box
TGCTGTGCAT	2,534	0,222	11,4		polypeptide 3
GCTTAACCTG	2,765	0,222	12,4		glutamate dehydrogenase 1
CCTTCTGGTG	2,765	0,222	12,4		protein tyrosine kinase 9
TTTTCTGAAA	2,995	0,222	13,5		thioredoxin
CTCGGGGGAA	21,197	0,222	95,4		serum amyloid A1

Table 3: Tags more than 10 fold up- or down-regulated in Barrett primary cell culture comparing to normal squamous esophageal cell culture. Tags more than 10 fold up- or down-regulated in Barrett primary cell culture (BE) compared to normal squamous esophageal cell culture (SQ) along with their counts in percentage (p). The fold induction of each tag and their corresponding gene ID's are presented.

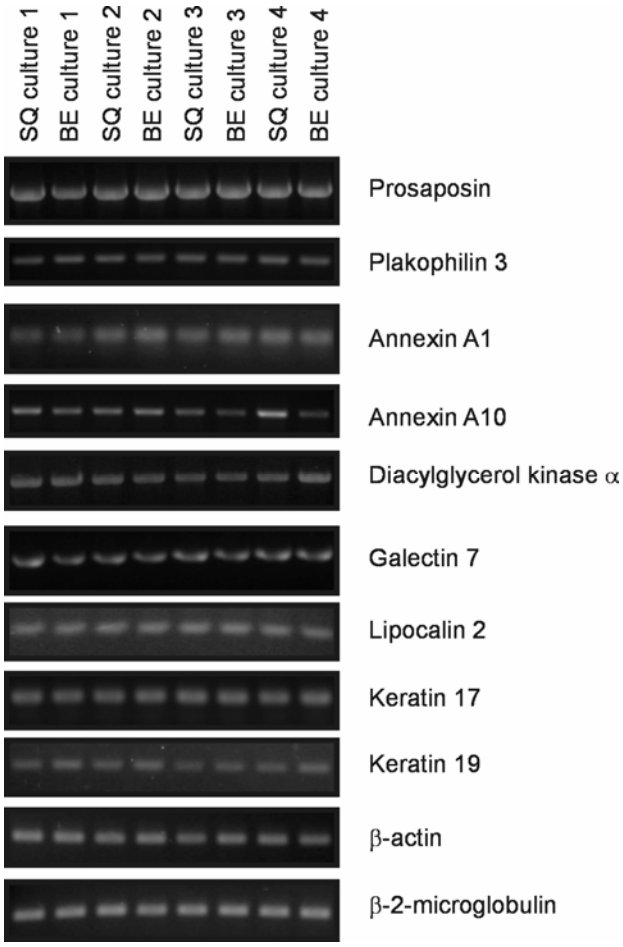

Figure 1: RT-PCR validation of SAGE results of Barrett's esophagus primary cell cultures and squamous esophagus primary cell cultures. RT-PCR on RNA from Barrett's esophagus primary cell cultures (BE culture) and squamous esophagus primary cell cultures (SQ culture) from different patients, demonstrates that Prosaposin, Plakophilin 3, Annexin A1, Annexin A10, Diacylglycerol kinase α, Galectin 7, Lipocalin 2, Keratin 17 and Keratin 19 are expressed in both Barrett's esophagus and squamous esophagus primary cell cultures. β-actin and β-2-microglobulin were used as control.

Gene expression comparison of BE epithelial cell culture versus BE biopsy

Analysis of the BE primary cell culture SAGE library and the BE biopsy SAGE library[10] revealed 1228 significantly differentially expressed tags; 667 tags were significantly up-regulated in the BE primary cell culture and 561 tags were significantly up-regulated in the BE biopsy SAGE library. From these 132 tags were more than 10 fold up-regulated in the BE culture and 122 tags were more than 10 fold up-regulated in the BE biopsy. Genes corresponding to these up-regulated tags in the BE primary cell culture SAGE library were Fibronectin 1 (ATCTTGTTAC), TGFβ-1 (GGGGCTGTAT), Dickkopf 3 (CTTTCTTTGA), keratin 14 (GTGCGCCGGA), and Claudin 4 (ATCGTGGCGG). Genes corresponding to tags that were more than 10 fold down-regulated in the BE primary cell culture SAGE library were BMP4 (CTGTACCTGG), Annexin A10 (ATGGAATAAT), TFF1 (CTGGCCCTCG), TFF2 (AAATCCTGGG), TFF3 (CTCCACCCGA), Gastric lipase (CAGTGCTTCT) and Galectin 4 (GGAAAACAGA).

Validation of SAGE results comparing primary cell cultures with biopsies

Expression levels of Cytokeratin 7 and Smurf 1 were verified using RT-PCR on RNA samples of squamous primary cell cultures and normal squamous biopsies. In all cases examined, the expression of genes represented by tags in the SAGE libraries was confirmed. Figure 2 show that indeed Cytokeratin 7 and Smurf 1 were significantly higher expressed in squamous primary cell cultures compared to the squamous esophageal biopsies.

In addition, expression levels of several genes, which were significantly differentially expressed comparing BE biopsy with Barrett primary cell culture were validated by RT-PCR on RNA samples of 16 BE biopsies and 16 Barrett primary cell cultures. Figure 3 shows that expression levels of TFF1, TFF2, and Annexin A10 were significantly higher expressed in BE biopsies compared to Barrett primary cell cultures, whereas Cyclin D1, Lipocalin 2, SOX 4, Cytokeratin 7 and Galectin 7 were significantly higher expressed in Barrett primary cell cultures compared to BE biopsies (Figure 3).

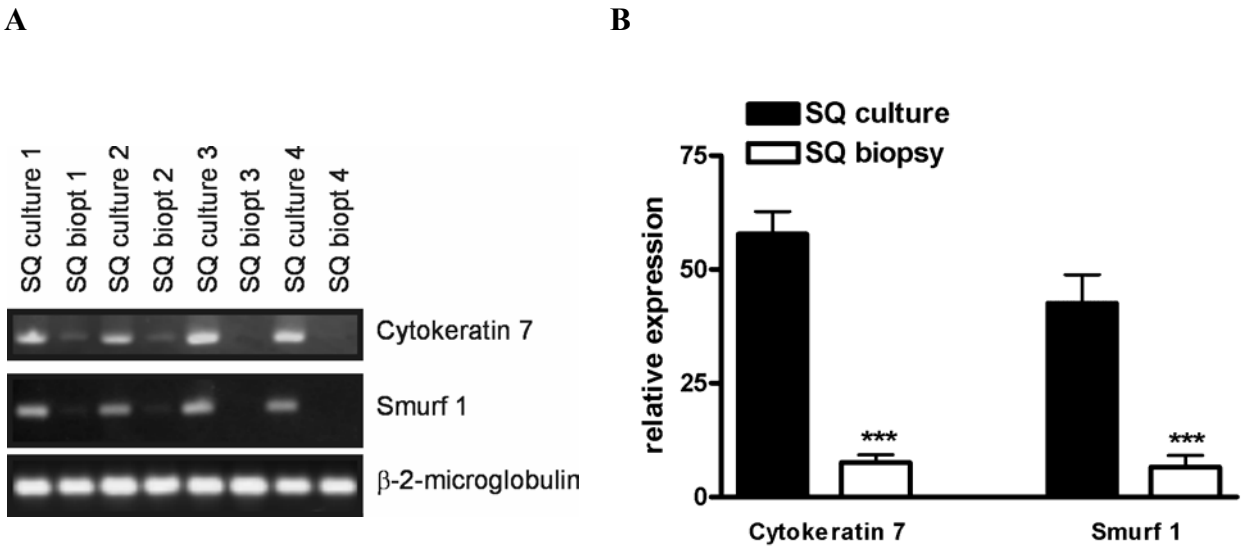

Figure 2: RT-PCR for validation of SAGE results of squamous esophagus primary cell cultures and normal squamous esophagus biopsies. RT-PCR on RNA from squamous esophagus primary cell cultures (SQ culture) and squamous esophagus biopsies (SQ biopsy) from different patients, demonstrates that Cytokeratin 7 and Smurf 1 are highly expressed in the squamous esophagus primary cell cultures, whereas no expression of these genes is seen in squamous biopsies. β-2-microglobulin was used as control (A). Quantification of RT-PCR results of 16 patients shows that Cytokeratin 7 and Smurf 1 are significantly higher expressed in squamous esophagus cell cultures compared to squamous esophagus biopsies (B; two-tailed *t*-tests; *** $p<0.001$). The gene expression levels were determined by the ratio of signal intensity of the mRNA to that of the β-2-microglobulin. Data are expressed as means ± SEM.

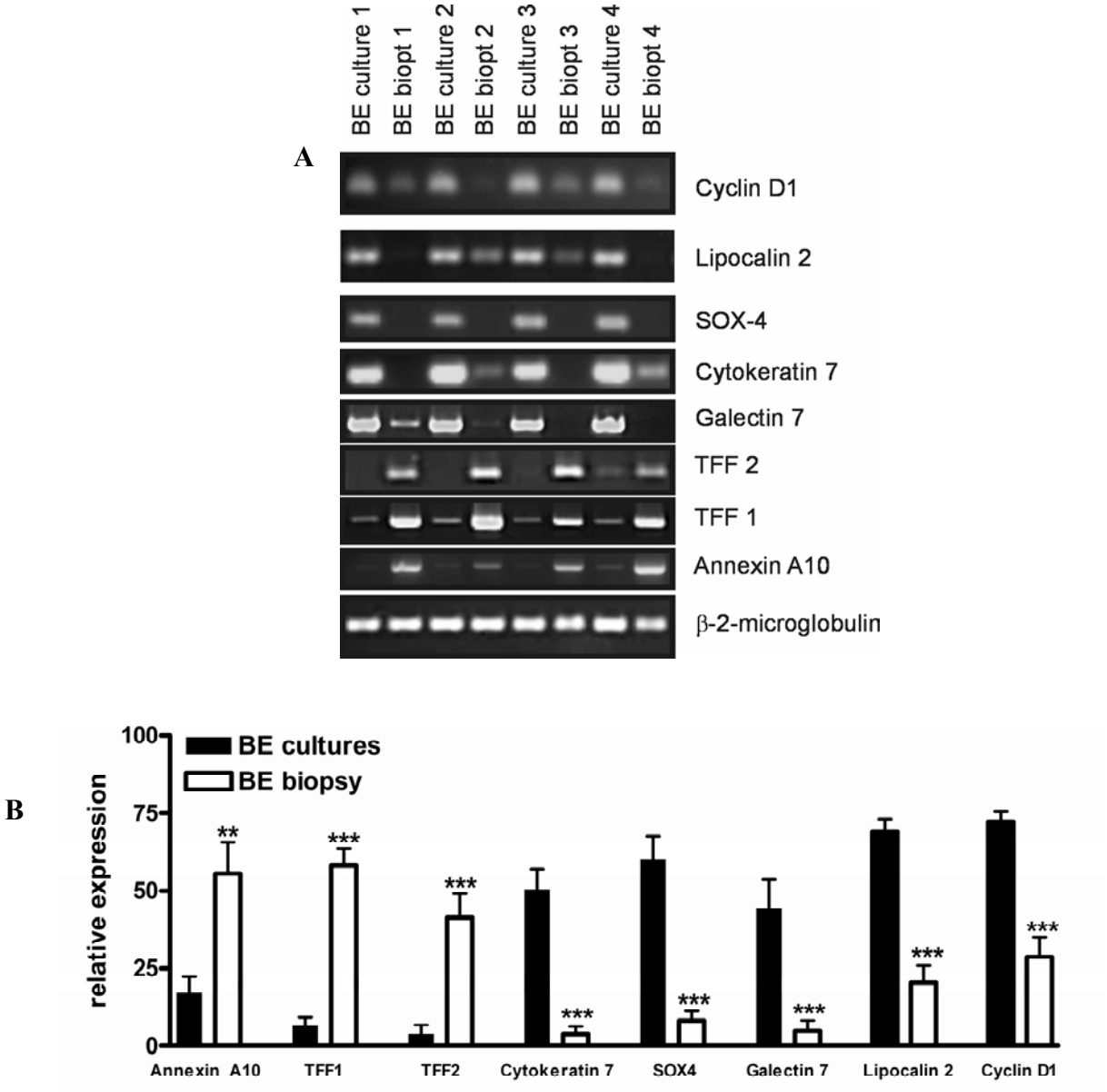

Figure 3: RT-PCR for validation of SAGE results of Barrett's esophagus primary cell cultures and Barrett's esophagus biopsies. RT-PCR on RNA from Barrett's esophagus primary cell cultures (BE culture) and Barrett's esophagus biopsies (BE biopsy) from different patients, demonstrates that Cyclin D1, Lipocalin 2, SOX4, Cytokeratin 7, Galectin 7 are highly expressed in Barrett's esophagus primary cell cultures, whereas TFF1, TFF2 and Annexin A10 are highly expressed in the Barrett's esophagus biopsies (A). β-2-microglobulin was used as control. Quantification of RT-PCR results of 16 patients shows that Cyclin D1, Lipocalin 2, SOX4, Cytokeratin 7, Galectin 7 are significantly higher expressed in Barrett's esophagus primary cell cultures compared to Barrett's esophagus biopsies (B; two-tailed *t*-tests; *** $p<0.001$). TFF1, TFF2 and Annexin A10 are significantly higher expressed in Barrett's esophagus compared to Barrett's esophagus cell cultures (B; two-tailed *t*-tests; ** $p<0.01$, *** $p<0.001$). The gene expression levels were determined by the ratio of signal intensity of the mRNA to that of the β-2-microglobulin. Data are expressed as means ± SEM.

Discussion

SAGE analysis was used to identify the transcriptome of Barrett and normal squamous esophageal primary epithelial cell cultures. A comparison of these transcriptomes with the transcriptomes of BE and normal squamous esophageal biopsies was made in order to identify factors specific for epithelial cells and those expressed by stromal tissue.

Previous studies that describe and compare the gene expression profiles of BE and esophageal carcinomas, or BE, normal squamous esophagus and gastric cardia utilized biopsies[7-10]. A biopsy however is a heterogeneous cell population, containing not only epithelial cells but also stromal tissue and inflammatory cells. Genes specifically expressed by these different types of cells are all expressed in these biopsies and can obscure the gene expression profile or even overrule genes expressed by the cells of interest. By employing primary non transformed epithelial cell cultures for SAGE analysis, the specific expression profile of epithelial cells is assessed. Comparison of the expression profile of squamous and BE epithelial cells could further improve our insight in the biological mechanisms involved in the metaplastic transition of normal squamous esophagus into columnar epithelium.

Because the transcriptome is variable and dependent 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 avoid confounding of the results by inflammatory factors. Histological confirmation, confirmed that none of the patients had severe or active inflammation in the pair wise taken control biopsies. Nevertheless, there will always be a certain amount of genes that are up- or down-regulated through constitutional differences between individuals that cannot be ruled. Therefore any gene identified as up- or down-regulated needs to be validated by conventional technologies to determine whether or not the expression is truly disease or tissue specific.

In this study over 85,000 tags were identified. Comparison of the SAGE generated tag expression profiles of BE and normal squamous esophageal primary cell cultures with previously published SAGE libraries of BE and squamous esophageal biopsies revealed hundreds of differentially expressed transcripts. The limitation of the technique, however is that some tags might be incorrectly assigned to a certain gene cluster due to sequence artefacts. Mapping of the SAGE tags that were found to be significantly differentially expressed comparing the primary cell

cultures with the biopsies to known genes and mRNAs in the SAGE Genie database revealed a large number of genes know to be expressed in BE and normal squamous esophagus, as well as many genes not previously recognized in BE. Moreover, this study revealed certain genes specifically expressed by BE and squamous epithelial cells or genes that are more likely expressed by the stroma. For example, Cyclin D1 was found to be specifically expressed by both squamous and Barrett epithelial cells but was not or less expressed in the biopsies indicating that this is an epithelial factor. Upon validation by RT-PCR Lipocalin 2, SOX4, Cytokeratin 7 and Galectin 7 were specifically expressed in the BE epithelial cells, whereas genes like Annexin A10, TFF1 and TFF2 were highly expressed in the BE biopsy and are more likely to be expressed in the BE stroma (Figure 3). Comparing squamous primary cell culture SAGE library with squamous esophagus biopsy we validated that Cytokeratin 7 and Smurf 1 were found to be specifically expressed by the epithelial squamous cells (Figure 2).

Comparing the different profiles of the epithelial cultured cells with the full biopsies, we may assume that genes exclusively or highly expressed in the BE epithelial cells are important and may be involved in the profound phenotypic changes as takes place during the process of metaplasia. Yet, stromal factors may as well have an important part in the transformation of epithelial cells through signalling cascades.

Of interest was the comparison of BE and squamous primary cell cultures, that revealed a relatively low number of significantly differentially expressed tags (511 tags). Only 13 tags were more than 10 fold up- or down-regulated, indicating a strong correlation between these cell types. RT-PCR as performed for several highly up-regulated genes in an additional 16 cases did not confirm the SAGE findings (Figure 1). Although, SAGE is a strongly quantitative technique and RT-PCR is semi-quantitative and might not detect subtle differences, it seemed that upon validation of the assumed highly differentially expressed genes could not be confirmed in cultures of other individuals, implicating that these differences may be constitutional individual differences and not related to the tissue itself. This observation indicates that culturing of these two epithelial cells leads to de-differentiation to a primordial cell type and presumably loss of the specific cellular phenotype. Since epithelial cell cultures are used in many fundamental studies to look into development and transformation of cells, it is important to realize the effects of culturing. Although BE has a columnar phenotype, we recently proved that at expression level BE has important similarities with its surrounding epithelia i.e., cardia and squamous epithelia[10].

It is therefore not surprising that culturing of BE and squamous epithelial cells may lead to a primordial cell type with a similar expression profile. For the analysis as performed in this study this observation implicates that several epithelial factors through this process of dedifferentiation may be missed in the SAGE expression profile. Moreover, it is possible that several specific factors will be underrepresented and only less differentially expressed in the SAGE library. As an alternative approach, a more precise analysis might be performed by literally dissecting epithelial cells from stroma for instance through micro-dissection. The main drawback of this methodology is however, that the mRNA necessary for the analysis will be partly degraded during the process of tissue processing and expression profiles may not be representative.

In conclusion, in this study we generated a transcriptome of a Barrett epithelial cell culture, made a comparison with the transcriptome of a BE biopsy and validated several factors as being specifically expressed by the epithelial cells or the stromal tissue. We as well describe the transcriptome of a squamous esophageal cell culture in comparison with a squamous esophageal biopsy. This SAGE analysis provides a wealth of information concerning the gene expression profiles of both Barrett and normal squamous primary epithelial cell cultures. This information can be used for further analysis of the underlying biological mechanisms involved in the metaplastic transformation of squamous cells into columnar cells in which there is an interaction between epithelial and stromal factors. Future manipulation of this interaction will ultimately help us to resolve the enigma of BE.

Acknowledgements

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Chapter 6

A comparative analysis by SAGE of gene expression profiles of esophageal adenocarcinoma and esophageal squamous cell carcinoma

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Abstract

Esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC) are the two main types of esophageal cancer. Research has been done concerning the pathophysiology of these cancers, however the exact molecular basis of these cancers remains largely unclear. Therefore we evaluated the transcriptome of EA in comparison to the expression profile of non dysplastic Barrett's esophagus (BE), the metaplastic epithelium that predisposes for EA, while the expression profile of ESCC was compared to normal esophageal squamous epithelium. The technique Serial analysis of gene expression (SAGE) was used for obtaining gene expression libraries of biopsies taken from an EA and an ESCC patient. Validation of results by RT-PCR and immunoblotting was performed using tissues of 10 extra EA and 13 ESCC patients. Over 58,000 tags were sequenced. Between EA and BE 1013, and between ESCC and normal squamous epithelium 1235 tags were significantly differentially expressed ($p<0.05$, Pair-wise comparison). The most up-regulated genes in EA compared to BE were SRY-box (SOX) 4 and Lipocalin 2, whereas the most down-regulated genes in EA were Trefoil factors and Annexin A10. The most up-regulated genes in ESCC compared to normal squamous epithelium were BMP4, Cyclin D1, E-Cadherin and TFF3. This study provides a comparison of the transcriptomes of EA and BE and furthermore a comparison of ESCC and normal squamous epithelium. In addition several uniquely expressed genes are identified. These results are a major advancement in understanding the process of cancer development in the esophagus.

Introduction

Two main types of esophageal cancer are esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC). EA is associated with Barrett's esophagus (BE), a metaplastic condition of the distal esophagus, in which through longstanding gastro-esophageal reflux disease, the normal squamous epithelium is replaced by columnar epithelium[1, 2]. Malignant degeneration of BE is thought to be a multi-step process in which metaplasia progresses through low grade and high grade dysplasia into an invasive adenocarcinoma[3]. BE patients have an increased risk of developing EA, with an estimated annual incidence varying from 0.4% to 1.8%[4-7]. Over the last 3 decades, the incidence of BE associated adenocarcinoma has increased in Western countries at a rate that exceed that of any other malignancy[8-10]. Abnormalities in oncogenes, tumor suppressor genes and growth factors play an important role in the development of EA. These factors have an influence on cell cycle progression and are critical in malignant transformation. For instance mutation of p16 and p53 can be found in EAs[11-13].

ESCC develops in a multi step, progressive process, as the result of a sequence of histopathological changes that typically involves esophagitis, atrophy, mild to severe dysplasia, carcinoma in situ and finally, invasive cancer. Worldwide ESCC is the predominant esophageal cancer and has a high mortality rate[14]. Several genetic changes are associated with the development of ESCC including mutations of the p53 gene, activation of oncogenes like EGFR and c-MYC, inactivation of several tumor suppressor genes and disruption of cell-cycle control in G1. The G1 phase of the cell cycle is controlled by several mechanisms that are disrupted in ESCC, like inactivation of p16, amplification of Cyclin D1 and alterations of RB[15].

Although both types of esophageal cancers have comparable clinical outcomes characterized by early metastasis and poor patient prognosis, the pathophysiology of these cancers seem to be different. For understanding and optimizing future treatments it is of high importance to understand the specific biology of these cancers. We hypothesized that generating large molecular data sets of EA and ESCC and quantitative analyzing these with non dysplastic BE and normal squamous epithelium, respectively, we will accurately classify the different phenotypes of these carcinomas and improve our insight in the biological pathways and mechanisms involved in these malignancies. In this study gene expression profiles were obtained using the technique Serial analysis of gene expression (SAGE). The method as first described by Velculescu *et al*.

allows rapid, quantitative and simultaneous analysis of thousands of genetic transcripts from tissue samples[16]. It is based on two principles: a nucleotide sequence of 10 bp, a tag, is produced. The location of the tags within the transcripts is precisely defined; therefore these tags contain adequate information to identify transcripts using public databases (SAGEgenie, http://cgap.nci.nih.gov). Additionally, by sequencing a large amount of transcripts can be identified efficiently, because the tags are serially cloned along with a restriction enzyme recognition sequence that serves as an anchor. This reveals the identity of thousands of tags and at the same time it quantifies their level of expression. In this study transcriptomes are made of RNA isolated from EA and ESCC biopsies with totally over 58,000 tags. These transcriptomes are analyzed and compared with already known transcriptomes of BE and normal squamous epithelium[17]. A panel of another 10 EA and 13 ESCC patients was used for validation by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and immunoblotting.

The unique gene expression profiles described in this study harbors a wealth of information and provides us the identity of several genes involved in several cell signaling pathways involved in EA and ESCC. These profiles will contribute to a better understanding of the molecular alterations and elucidate important biological processes involved in cancer development of the esophagus. Therefore this could improve tumor control and prevention and consequently could lead to a better disease management. Additionally, several unique genes that can be used as novel markers for distinguishing EA, ESCC, BE and normal squamous epithelium are identified.

Materials and Methods

Patients and Biopsy Specimens

All patients signed informed consent for the use of their biopsy material. Tissue samples were obtained during endoscopy of 11 patients with known EA (8 were male; mean age was 66 years, range 49-83 years) 4 patients had T1N0M0 stage of cancer, 2 patients T2N0M0, 4 patients T3N0M0 and 1 patient T3N1M0. No patient had received irradiation or chemotherapy before endoscopy. Additionally, during endoscopy, tissue samples of 14 patients with known ESCC were obtained (8 were male; mean age 62 years, range 50-80 years) 7 patients had T3N1M0 stage of cancer, 2 patients T3N1M1b, 3 patients T4N1M0 and 2 patients T4N1M1b. No patient had

received irradiation or chemotherapy before endoscopy. Paired biopsies, taken next to each other, were obtained from the cancer mass. Histological examination of the pair wise taken control biopsies confirmed presence of EA or ESCC.

Tissue samples were obtained during routine surveillance endoscopy of 16 patients with known BE but without dysplasia (13 were male; mean age was 62 years, range 41-83 years; average length of BE segment 3.6 cm, range 2-9 cm). All patients were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. BE was defined as histologically recognized incompletely differentiated intestinal type of metaplasia in the distal esophagus. Paired biopsies, taken next to each other, were obtained of the Barrett's segment and normal squamous esophagus. 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 columnar type of metaplasia. Normal squamous epithelium was taken at least 2 cm above the Barrett's segment. Endoscopically, none of the patients had reflux esophagitis. All patients had proven incompletely differentiated intestinal type of columnar epithelium without dysplasia in the histological control biopsies with no signs of active or acute inflammation. Normal squamous esophagus epithelium was also confirmed histologically, in all pair wise taken control biopsies.

RNA isolation

Total RNA was isolated from biopsies using TRIzol Reagent (Life Technologies Inc, Invitrogen, Breda, The Netherlands) according to manufacturer's instructions. In brief: tissues were lysed by adding 200 µl TRIzol. After phenol/chloroform extraction, RNA was precipitated with isopropanol, washed with 70% ethanol and air-dried. The RNA was then dissolved in RNase-free H_2O and stored at -80°C until required. Spectrophotometry was performed with 1 µl of total RNA to determine the concentration on the Nanodrop® (type ND-1000, Wilmington, USA).

SAGE procedure

The SAGE libraries were obtained essentially following the SAGE protocol as described by Velculescu *et al*. using the Life Technologies I-SAGE kit and following manufacturer's instructions[16, 17]. Five µg of total RNA was used per SAGE analysis. The isolated concatemers, consisting of serially ligated tags, were ligated into the pZErO-1 vector (Life Technologies) and

transformed in TOP10 Electrocompetent *Escherichia coli* cells (Life Technologies) by means of electroporation, following manufacturer's protocol (Biorad, Hercules, CA). PCR was performed on obtained colonies with specific primers Sp6-F (5'-GATTTAGGTGACACTATAG-3') and T7A-R (5'- TAATACGACTCACTATAGGG-3') and PCR products were analyzed by agarose gel electrophoresis. A total of 1920 clones were selected for DNA sequencing using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) and the T7A-R primers. Samples were run on an ABI3730 DNA Analyzer (Applied Biosystems) and analyzed with Sequence Analysis 5.1 software.

SAGE and Statistical Analysis

Statistical analysis of SAGE data was performed according to van Baal *et al*.[17]. Briefly, for analysis of the SAGE data the program USAGE V2 (Academic Medical Center, bioinformatics department) and the public databases of the NCBI-site and SAGE Genie (http://cgap.nci.nih.gov) were used[18, 19]. Statistical analyses and comparison of the SAGE libraries was done using a comparative *Z*-test (Pair-wise comparison, binominal approach) of the USAGE V2 program[20, 21].

Reverse-Transcription Polymerase Chain Reaction

cDNAs from biopsies were synthesized from 1 µg of total RNA using an oligo dT primer and Superscript II MMLV-reverse transcriptase according to manufacturer's instructions (Life Technologies). Primers for selected genes (Table 1) were derived from mRNA sequences as deposited in GenBank (NCBI-site). Subsequent PCR analyses were carried out in 25 µl reactions containing 1 µl cDNA, 23 µl Reddy Mix PCR Master Mix (Applied Biosystems), 200 ng Forward primer and 200 ng Reverse primer. The products were electrophoresed on 1% agarose gel. β-actin and β-2-microglobulin were used as control.

Immunoblotting

Immunoblotting was performed as described by Hardwick *et al*.[22]. Biopsies were lysed with 200 µl lysis buffer. Twenty mg of protein per lane was loaded onto SDS-PAGE. The blots were blocked with 2% BSA in Tris Buffered Saline supplemented with 0.1% Tween-20. The antibodies used and dilutions are summarized in Table 2.

Gene	Forward primer	Reverse primer	Annealing temperature	Fragment length
TFF1*	TTTGGAGCAGAGAGGAGG	TTGAGTAGTCAAAGTCAGAGCAG	60ºC	438 bp
TFF2	ATGGATGCTGTTTCGACTCC	GGCACTTCAAAGATGAAGTTG	55ºC	247 bp
TFF3	GTGCCAGCCAAGGACAG	CGTTAAGACATCAGGCTCCAG	58ºC	303 bp
Annexin A10	TTGTTCTCTGTGTTCGAGACAAACC	GTAGGCAAATTCAGGATAGTAGGC	52ºC	609 bp
SOX4**	CTTGACATGATTAGCTGGCATGATT	CCTGTGCAATATGCCGTGTAGA	64ºC	100 bp
FABP1***	TCATGAAGGCAATCGGTCTG	GTGATTATGTCGTCGCCGTTGAGT	55ºC	277 bp
BMP4	ACCTGAGACGGGGAAGAAAA	TTAAAGAGGAAACGAAAAGCA	62ºC	348 bp
Plakophilin 3	AGCCTGGAGGAGAAGGCTAAT	AGTGCTGGCTATCCCAAGATACT	60ºC	234 bp
Prosaposin	CCAGAGCTGGACATGACTGA	CAGTTCCCAACAAGGGCTTA	60ºC	999 bp
SBP1****	TCAGATGATCCAGCTCAGCCT	TCACAGCCTTCCCTGATGA	60ºC	109 bp
E-Cadherin	GACGCGGACGATGATGTGAAC	TTGTACGTGGTGGGATTGAAGA	56ºC	280 bp
Lipocalin 2	GGAGCTGACTTCGGAACTAAAGG	AGCCGTCGATACACTGGTCG	60ºC	109 bp
β-actin	GTCAGAAGGATTCCTATGTGG	GCTCATTGCCAATGGTGATG	52ºC	628 bp
β-2-microglobulin	CTCGCGCTACTCTCTCTTTCT	TGCTCCACTTTTTCAATTCTCT	60ºC	185 bp

Table 1: Primer sequences. Primer sequences used for RT-PCR with corresponding annealing temperatures and PCR fragment lengths. *) TFF= Trefoil Factor **) SOX4= SRY box 4 ***) FABP1= Fatty Acid Binding Protein 1 ****) SBP1= Selenium Binding Protein 1

Antibody	Species	Company	Country	Dilution
Cytokeratin 5/6	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 8	Mouse monoclonal	Chemicon	USA	1:500
Cytokeratin 10/13	Mouse monoclonal	Dako	Denmark	1:500
Cytokeratin 20	Mouse monoclonal	Progen	Germany	1:500
PKC β1	Rabbit polyclonal	Santa Cruz	Germany	1:500
BMP 4	Mouse monoclonal	R&D	United Kingdom	1:500
ID 2	Rabbit polyclonal	Santa Cruz	Germany	1:1000
Cyclin D1	Mouse monoclonal	Neomarkers	USA	1:1000
TGF-β	Rabbit polyclonal	Santa Cruz	Germany	1:500
EGF-receptor	Rabbit polyclonal	Cell Signaling	USA	1:500
p 19	Mouse monoclonal	Neomarkers	USA	1:1000
p 27	Mouse monoclonal	Santa Cruz	Germany	1:1000
PCNA	Mouse monoclonal	Santa Cruz	Germany	1:2000
β-Actin (I-19)	Goat polyclonal	Santa Cruz	Germany	1:2000

Table 2: Antibodies as used for immunoblot analysis.

Results

SAGE library characteristics

Two unique SAGE libraries were made, totally consisting of over 58,000 tags. The SAGE library characteristics are described in Table 3 and in addition the complete SAGE libraries can be found on the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/; Table 3). A minority of the identified tags correspond with different genes due to the presence of conserved sequences and common repeats in the 3' un-translated mRNA transcript. An example is the tag GGTGAGACAC which matches with several genes, namely Solute carrier family 25 member 6, WD repeat domain 27 and Golgi autoantigen 8A. Various different tags can represent the same gene, for instance the Expressed Sequence Tags (ESTs) of CAGGACAGCG and GTGACAACCT correspond to the gene cluster of Keratin 4. This variation may be the result of alternative splicing, alternative polyadenylation, or polymorphisms in the mRNA from which these tags are derived.

	Esophageal squamous cell carcinoma	**Esophageal adenocarcinoma**
Total tags	24,922	33,666
Unique tags	8,636	10,794
Singletons	5,994 (24%)	7,188 (21%)
Tags 5-times present	666	945
Tags 10-times present	298	351
Accession code	GSM 110381	GSM 110379

Table 3: SAGE library characteristics. Number of total tags in the esophageal squamous cell carcinoma and esophageal adenocarcinoma, together with the corresponding accession code in the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/), the number of unique tags, the number of singletons and the number of tags at least 5 times and 10 times present in each of the libraries. Calculation of the percentages of singleton tags was based on the total number of tags present in the libraries.

Comparison of the expression profiles of esophageal adenocarcinoma and Barrett's esophagus

Between the EA and BE SAGE library 1013 tags were significantly differentially expressed ($p<0.05$), 673 tags were significantly up-regulated and 340 tags were significantly down-regulated in EA. From these 55 tags were more than 10 fold up-regulated and 42 more than 10 fold down-regulated in EA (supplemental data, Table 1).

Genes corresponding to tags that were more than 10 fold up-regulated in EA are Insulin-like growth factor binding protein 7, Small nuclear ribonucleoprotein D3 polypeptide, Nucleolin and Trf-proximal homolog. Genes corresponding to tags that were more than 10 fold down-regulated in EA are Gastric lipase, Intelectin 1, Fatty acid binding protein (FABP) 1, Galectin 7, Trefoil factor (TFF) 1 and Tumor rejection antigen 1. Genes corresponding to tags that were significantly differentially expressed comparing EA versus BE, were also clustered in groups of biological processes according to the Gene Ontology of the European Bioinformatics Institute (http://cgap.nci.nih.gov). A higher expression level of genes in the groups of nucleobase/ nucleoside/ nucleotide and nucleic acid metabolism, cell division, cell growth, response to stimulus and signal transduction was found in EA compared to BE (Figure 1A). Comparing EA with BE these biological processes were more than 2 fold higher in EA (Table 4).

Comparison of the expression profiles of esophageal squamous cell carcinoma and normal squamous esophagus

Between the ESCC and normal squamous esophagus SAGE library 1235 tags were significantly differentially expressed ($p<0.05$), 1022 tags were significantly up-regulated and 213 tags were significantly down-regulated in ESCC. From these, 129 tags were more than 10 fold up-regulated and 41 more than 10 fold down-regulated in ESCC (supplemental data, Table 2).

Genes corresponding to tags that were more than 10 fold up-regulated in ESCC are E-Cadherin, Tetraspanin 3, TFF1, Keratin 8, Claudin 18 and Galectin 4. Genes corresponding to tags that were more than 10 fold down-regulated in ESCC are Epithelial membrane protein 1, Annexin A1, Calponin 2, Keratin 13 and S100 calcium binding protein A9. Genes, corresponding to tags that were significantly differentially expressed comparing ESCC with normal squamous epithelium, were clustered in groups of different biological processes according to the Gene Ontology of the European Bioinformatics Institute (http://cgap.nci.nih.gov). Genes in the biological clusters cell cycle, nucleobase/ nucleoside/ nucleotide and nucleic acid metabolism, metabolism, cell division, cell communication were more abundantly expressed in ESCC compared to normal squamous epithelium (Figure 1B). These clusters were 10 fold or more increased in ESCC (Table 5). Genes in the cluster cell-cell signaling were predominantly expressed in normal squamous epithelium compared to ESCC (Figure 1B and Table 5).


response to stress
other
cell division
localization
cell communication
cell homeostasis
cell growth
cell motility
morphogenesis
cell differentiation
cell adhesion
cell proliferation
immune response
cell death
cell cycle
response to stimulus
metabolism
development
cell organization and biogenesis
transport
signal transduction
nucleobase, nucleoside, nucleotide and nucleic acid metabolism
cellular metabolism
unknown
Esophageal adenocarcinoma
Barrett's esophagus
0
5
10
15
20
relative expression
A

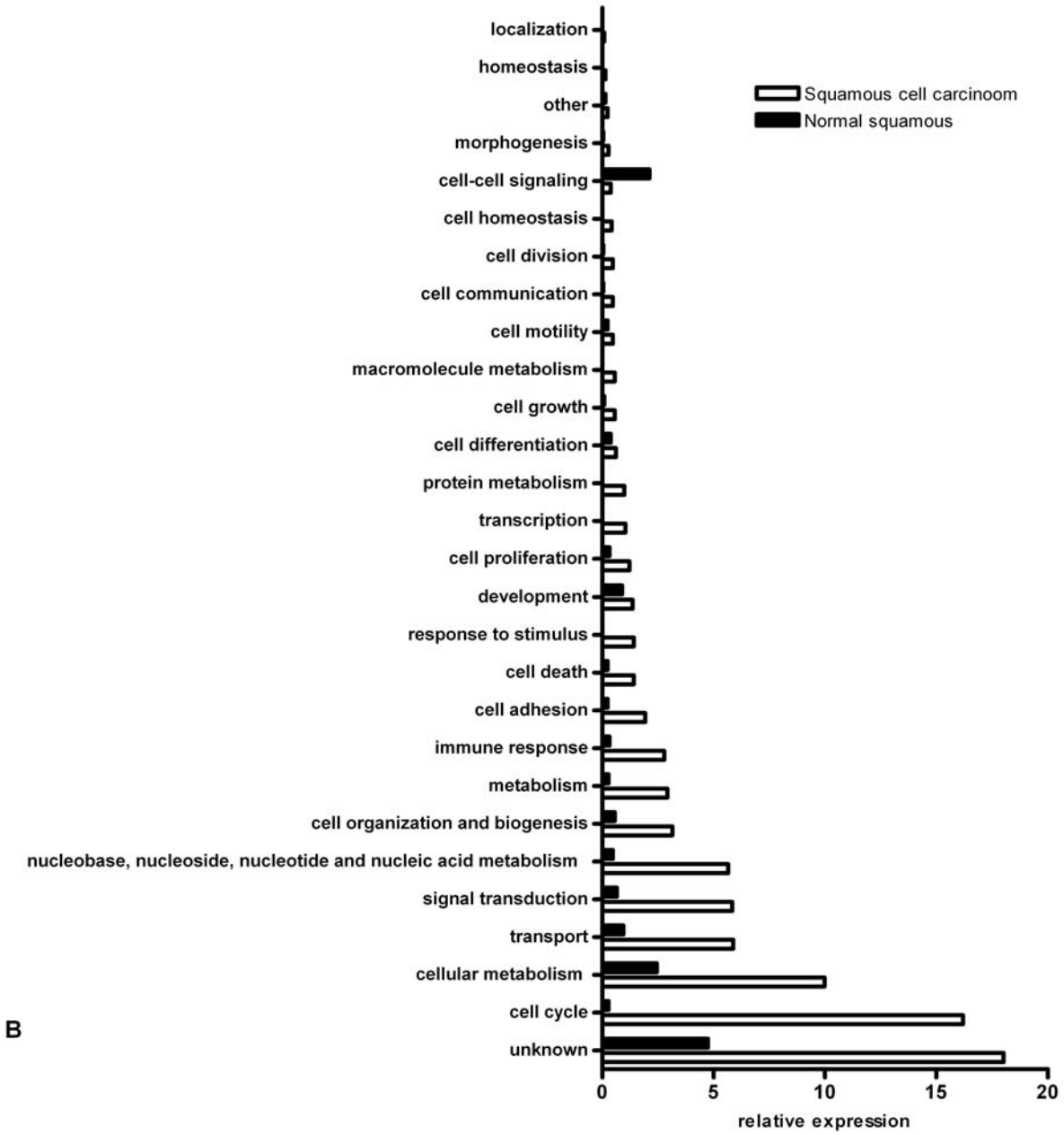

Figure 1: Clustering of genes in biological processes. Clustering of genes corresponding to tags significantly differentially expressed comparing esophageal adenocarcinoma with Barrett's esophagus in groups of biological processes according to the Gene Ontology of the European Bioinformatics Institute (http://cgap.nci.nih.gov) shows that genes in the clusters nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, cell division, cell growth, response to stimulus and signal transduction are predominantly expressed in esophageal adenocarcinoma (A). Genes in the clusters cell communication and cell differentiation are more expressed in Barrett's esophagus (A). Clustering of genes corresponding to tags significantly differentially expressed comparing esophageal squamous cell carcinoma with normal squamous esophagus shows that genes in the clusters cell cycle, metabolism, signal transduction, immune response and cell growth are more abundantly expressed in esophageal squamous cell carcinoma (B). Only genes in the cluster cell-cell signaling were predominantly expressed in normal squamous epithelium (B).

biological process	p EA	p BE	up in EA	down in EA
nucleobase, nucleoside, nucleotide & nucleic acid metabolism	8,04	1,56	5,17	
localization	0,32	0,06	5,00	
unknown	19,52	6,23	3,14	
signal transduction	5,06	2,01	2,52	
cell growth	0,45	0,19	2,33	
response to stimulus	1,75	0,78	2,25	
cell homeostasis	0,39	0,19	2,00	
cell cycle	1,49	0,78	1,92	
cell motility	0,45	0,26	1,75	
metabolism	1,88	1,23	1,53	
transport	4,60	3,11	1,48	
immune response	1,30	0,91	1,43	
morphogenesis	0,65	0,45	1,43	
development	2,27	1,62	1,40	
cell organization and biogenesis	3,11	2,27	1,37	
cell division	0,26	0,19	1,33	
cell adhesion	0,91	0,78	1,17	
cellular metabolism	8,88	8,04	1,10	
cell proliferation	1,04	0,97	1,07	
other	0,13	0,19		1,50
cell communication	0,32	0,45		1,40
cell differentiation	0,84	0,97		1,15
cell death	1,36	1,43		1,05
response to stress	0,00	0,26		

Table 4: Clustering of genes in biological processes comparing esophageal adenocarcinoma with Barrett's esophagus. Clusters of biological processes with the corresponding proportion (p) in esophageal adenocarcinoma (EA) and Barrett's esophagus (BE). The fold up and down regulation of each cluster is presented.

Validation of esophageal adenocarcinoma SAGE results

Expression levels of TFF1, TFF2, TFF3, Annexin A10, Selenium Binding Protein (SBP) 1, Lipocalin 2, SOX4 and FABP1 were verified by RT-PCR to validate the EA SAGE results. In all cases examined, the expression of genes represented by tags in either SAGE library was confirmed. TFF1, TFF2, TFF3, Annexin A10 and FABP1 were significantly lower expressed in all EA samples compared to all BE samples, whereas Lipocalin 2, SBP1 and SOX4 were significantly higher expressed in all EA samples compared to all BE samples (Figure 2).

Furthermore, protein expression levels were validated using immunoblotting. Results indicated that Cytokeratin (CK) 8, CK10/13 and BMP4 were lower expressed in EA compared to BE, whereas CK20 and EGF-receptor were higher expressed in EA compared to BE (Figure 3).

biological process	p ESCC	p SQ	up in ESCC	down in ESCC
cell cycle	16,19	0,28	57,33	
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	5,65	0,47	12,00	
metabolism	2,92	0,28	10,33	
cell communication	0,47	0,05	10,00	
cell division	0,47	0,05	10,00	
signal transduction	5,84	0,66	8,86	
immune response	2,78	0,33	8,43	
cell adhesion	1,93	0,24	8,20	
transport	5,88	0,94	6,25	
cell death	1,41	0,24	6,00	
cell growth	0,56	0,09	6,00	
morphogenesis	0,28	0,05	6,00	
cell organization and biogenesis	3,15	0,56	5,58	
cellular metabolism	9,98	2,45	4,08	
unknown	18,02	4,75	3,79	
cell proliferation	1,22	0,33	3,71	
cell motility	0,47	0,24	2,00	
other	0,24	0,14	1,67	
cell differentiation	0,61	0,38	1,63	
development	1,36	0,89	1,53	
cell-cell signaling	0,38	2,12		5,63
response to stimulus	1,41	0,00		
transcription	1,04	0,00		
protein metabolism	0,99	0,00		
macromolecule metabolism	0,56	0,00		
cell homeostasis	0,42	0,00		
homeostasis	0,14	0,00		
localization	0,09	0,00		

Table 5: Clustering of genes in biological processes comparing esophageal squamous cell carcinoma with normal squamous epithelium. Clusters of biological processes with the corresponding proportion (p) in esophageal squamous cell carcinoma (ESCC) and normal squamous esophagus (SQ). The fold up and down regulation of each cluster is presented.

Validation of esophageal squamous cell carcinoma SAGE results

To validate the ESCC SAGE results, expression levels of TFF3, Annexin A10, Prosaposin, BMP4, E-Cadherin and Plakophilin 3 were verified by RT-PCR. In all cases examined, the expression of genes represented by tags in each SAGE library was confirmed. TFF3, BMP4, Annexin A10, Prosaposin and E-Cadherin were significantly higher expressed in all ESCC samples, whereas Plakophilin 3 was significantly lower expressed in ESCC compared to normal squamous esophagus epithelium (Figure 4).

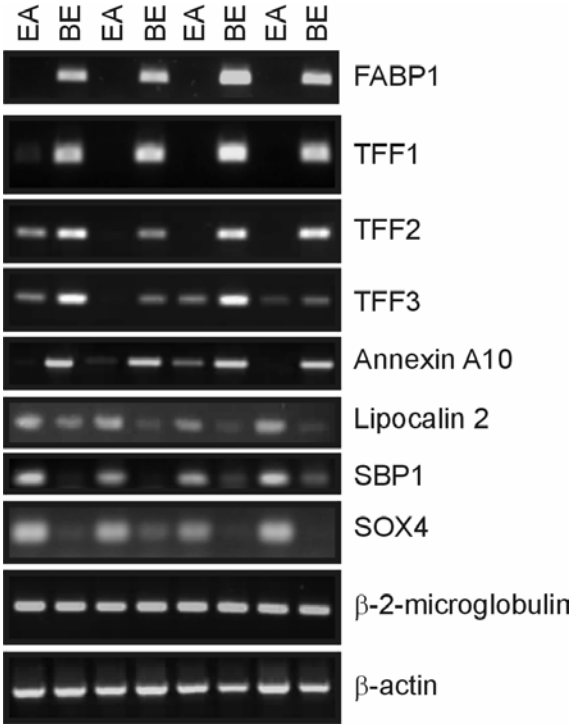

Figure 2: RT-PCR to validate SAGE results of esophageal adenocarcinoma and Barrett's esophagus. RT-PCR on RNA from esophageal adenocarcinoma (EA) and Barrett's esophagus (BE) biopsies from different patients, demonstrates that Fatty Acid Binding Protein (FABP) 1, Trefoil Factor (TFF) 1, TFF2, TFF3, and Annexin A10 are highly expressed in the Barrett biopsies, but virtually absent in esophageal adenocarcinoma. Furthermore Lipocalin 2, Selenium Binding Protein (SBP) 1 and SRY box (SOX) 4 are highly expressed in esophageal adenocarcinoma biopsies and lower expressed in Barrett biopsies. β-actin and β-2-microglobulin were used as a control. Pictures are representative for results of 10 patients.

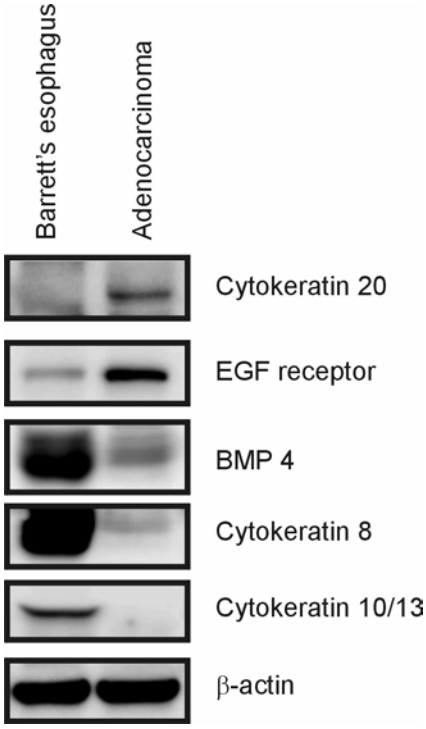

Figure 3: Immunoblot validation of SAGE results of esophageal adenocarcinoma and Barrett's esophagus. Immunoblot analysis on protein samples from esophageal adenocarcinoma and Barrett's esophagus biopsies from different patients reveals that Cytokeratin 20 and EGF-receptor are highly expressed in esophageal adenocarcinoma and lower in Barrett's esophagus. Furthermore BMP4, Cytokeratin 8 and Cytokeratin 10/13 are highly expressed in Barrett's esophagus and lower in esophageal adenocarcinoma. Pictures are representative for results of 10 patients.

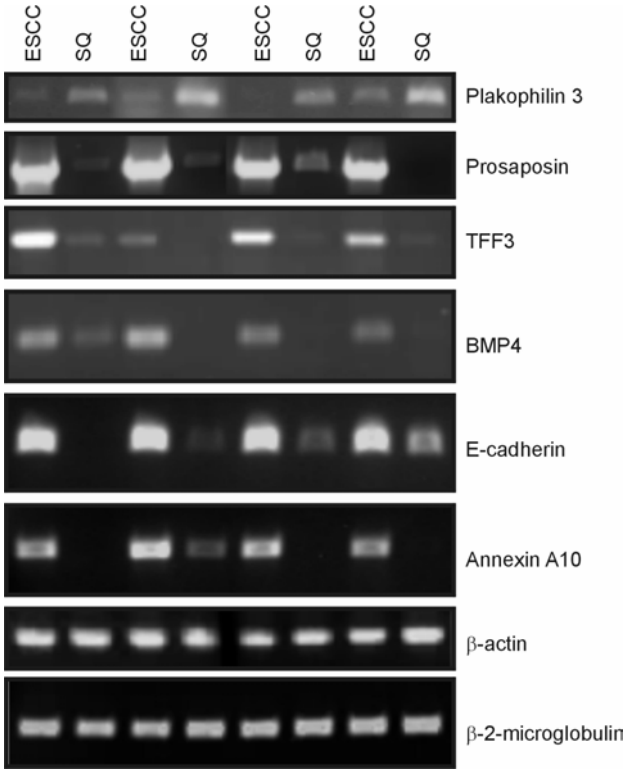

Figure 4: RT-PCR to validate SAGE results of esophageal squamous cell carcinoma and normal squamous epithelium. RT-PCR on RNA from esophageal squamous cell carcinoma (ESCC) and normal squamous esophagus (SQ) biopsies from different patients, demonstrates that Prosaposin, Trefoil Factor (TFF) 3, Bone Morphogenetic Protein (BMP) 4, E-Cadherin and Annexin A10 are highly expressed in esophageal squamous cell carcinoma, whereas Plakophilin 3 is highly expressed in normal squamous esophagus biopsies. β-actin and β-2-microglobulin were used as a control. Pictures are representative for results of 13 patients.

In addition immunoblotting was performed for validation of certain genes on protein level. CK5/6, CK10/13 and EGF-receptor were lower expressed in ESCC compared to normal squamous epithelium (Figure 4). CK 8, PKC-β1, Cyclin D1, TGF-β, BMP4, ID2, p19 and p27 were higher expressed in ESCC compared to normal squamous epithelium (Figure 5).

Discussion

In this study, SAGE technology was used to identify the gene expression profile of EA and ESCC, subsequently these expression profiles were compared to the gene expression profiles of BE and normal squamous epithelium. The specific information gained from this study helps to identify factors involved in the neoplastic lesions in the esophagus and to identify uniquely expressed tissue specific genes. Furthermore this information can be used to gain insight in the biology of these neoplastic lesions that may ultimately lead to a better disease management.

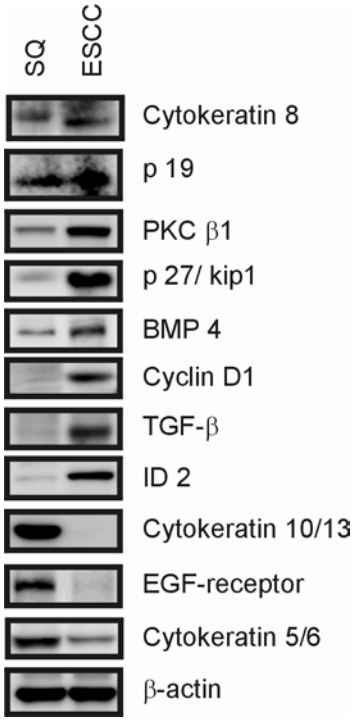

Figure 5: Immunoblot validation of SAGE results of esophageal squamous cell carcinoma and normal squamous esophagus. Immunoblot analysis on protein samples from esophageal squamous cell carcinoma and normal squamous esophagus biopsies from different patients reveals that Cytokeratin 8, p19, PKC-β1, p27/kip, BMP4, Cyclin D1, TGF-β and ID2 are highly expressed in esophageal squamous cell carcinoma (ESCC) and lower in normal squamous esophagus (SQ). Furthermore Cytokeratin 10/13, EGF-receptor and Cytokeratin 5/6 are highly expressed in normal squamous esophagus and lower in esophageal squamous cell carcinoma. Pictures are representative for results of 13 patients.

The major advantage of the SAGE technology compared to microarray is that using SAGE a library of thousands of expressed genes is generated without any previous knowledge of the cell's repertoire. The transcriptome obtained using SAGE technology conveys not only the identity of each expressed gene but also quantifies its level of expression. In this study over 58,000 tags were analyzed. Comparison of the SAGE-generated gene expression profiles of EA and BE identified over a thousand differentially expressed transcripts. The comparison of the transcriptomes of ESCC and normal squamous epithelium revealed over 1200 differentially expressed genes. Yet, it should be noted that because of sequence artefacts, some tags could incorrectly be allocated to a certain gene cluster. Primarily, tags expressed only once should be carefully considered, even though these generally correspond to mRNAs expressed at very low levels, some may be due to sequencing errors. Singleton tags were found in approximately 21-24% of each library (Table 3), which is in accordance with previously published other SAGE libraries[17, 23].

Several gene expression profile and microarray studies have been performed, for example, SAGE and microarray analysis indicated that BE has strong similarities with the surrounding normal

epithelium[17, 24]. In addition, transcriptome analysis studies have been performed through comparing BE and esophageal carcinomas, or through comparison of the gene expression profiles of BE and intestinal metaplasia of the cardia to get a better overview of genes involved in BE transition[25, 26]. In a microarray analysis study, Selaru *et al*. reported that EA clustered more closely to ESCC than to BE and they therefore concluded that the global gene expression profile fundamentally changed during the neoplastic progression of BE to EA[25]. Dahlberg *et al*. showed that normal squamous esophagus and gastric cardia are clustered closer together than to EA[27]. Additionally Fox *et al*. reported that BE has a unique expression profile distinct from normal and EA specimens, whereas Wang *et al*. reported that the expression pattern of BE was significantly more similar to EA than to normal squamous epithelium[28, 29].

We preferred to use tissue samples of one male individual known with EA and one male individual known with ESCC, for making the SAGE libraries. It is mandatory to verify RNA expression levels on a larger panel of samples for confirmation of the SAGE results, therefore tissue samples of 10 EA and 13 ESCC patients were included in this study to validate differential expression of several genes by RT-PCR and proteins by immunoblotting.

Mapping the SAGE tags to known genes and mRNAs in the SAGE Genie database revealed a large number of genes known to be expressed in EA and ESCC, as well as many genes not previously recognized in EA and ESCC. For instance, the EA SAGE library confirmed high expression of Mucin 5 (TGCACAATAT), Mucin 1 (CCTGGGAAGT), Chemokine (C-X-C motif) ligand 3 (ATAATAAAAG) and Insulin-like growth factor binding protein 7 (CATATCATTA)[27, 29-31]. In the ESCC SAGE library high number of tags were found for instance for TFF1 (CTGGCCCTCG) and TFF3 (CTCCACCCGA), known to be highly expressed in ESCC[32]. Furthermore in ESCC low numbers of tags were found for instance for Keratin 13 (GCAGAGAGGA) and Keratin 4 (GTGACAACCT), these keratins are known to be expressed on protein level in normal squamous epithelium[33, 34].

The expression of TFF1, TFF2, TFF3, Lipocalin 2, SBP1, FABP1, Galectin 4 and Annexin A10 was validated for the comparison of EA with BE (Figure 2). For the comparison of ESCC with normal squamous esophagus, the expression of TFF3, Annexin A10, Prosaposin, BMP4, E-Cadherin and Plakophilin 3 was validated (Figure 4). Several proteins were validated using immunoblot, for example the expression of CK8, CK10/13, CK20, EGF-receptor and BMP4 were validated for the comparison of EA with BE and CK5/6, CK8, CK10/13, EGF-receptor,

BMP4, ID2, TGF-β, Cyclin D1, p27, p19, PKC-β1 and PCNA were validated for the comparison of ESCC with normal squamous epithelium (Figures 3 and 5). These validations indicate that the SAGE data presented here is representative for EA and ESCC biopsies.

Plakophilin 3 is a member of the Armadillo protein family and is recently found to be a potential molecular target for treatment of lung cancer[35]. Plakophilins are one of the types of proteins found in the cytoplasmic desmosomal plaque, which connect intermediate filaments to adhesive desmosomal transmembrane proteins. Therefore plakophilin 3 is important in cell-cell adhesion complexes[36]. Prosaposin is the precursor of four glycoprotein activators (saposins) for lysosomal hydrolases. Before delivery to endosomes, portions of prosaposin and procathepsin D are assembled into complexes. Recently secretion of prosaposin by breast cancer cells has been reported, furthermore amplification and overexpression of the gene in prostate cancer has been shown[37, 38]. SBP1 is mapped at chromosome 1q21-22[39]. Reduced expression of SBP1 in gastric and lung adenocarcinomas has been reported[40-42]. However the molecular mechanism of down-regulation of SBP1 in cancer cells is not clear at present. SOX4 is found to be expressed in several human cancers and is proposed to be a transforming oncogene in prostate cancer[43].

In order to get a better overview of biological events occurring in EA and ESCC in comparison to respectively BE and normal squamous epithelium, genes corresponding to tags significantly differentially expressed between the two SAGE libraries were clustered into groups of biological processes using the website for Gene Ontology of the European Bioinformatics Institute (http://cgap.nci.nih.gov). Interestingly, in EA an abundance of genes situated in the clusters nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, cell division, cell growth, response to stimulus and signal transduction were found (Figure 1A), indicating that these processes play a major role in EA given that cell division and cell growth are related to each other. Of interest is that the cluster analysis indicates that cell-cell signalling is five fold decreased in ESCC compared to normal squamous epithelium (Table 5). In contrast, the clusters cell cycle, nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, metabolism, cell division and cell communication were important in ESCC, all these clusters were more than 10 fold increased. In more detail, 13 main clusters of biological processes were more than 5 fold increased in ESCC in contrast to 1 cluster in normal squamous epithelium. This indicates that ESCC is a highly active epithelium in several types of processes, like cell adhesion, signal transduction, cell death, immune response and cell growth.

In summary, the comparison of the gene expression profiles of EA and ESCC with BE and normal squamous esophagus, respectively, shows that EA and ESCC have their own specific characteristics. The identification of genes exclusively expressed in the EA and ESCC compared to metaplastic BE and normal squamous esophagus may have important clinical implications, as these may be useful as tissue markers. To further improve our insight in the tumorigenesis of these cancers, transcriptome analysis analyzing the different stages of dysplasia in the development of EA and ESCC is necessary. Nevertheless, the present analysis provides a wealth of information and the identification of novel genes that may be involved in the neoplastic process in the esophagus or can be used as tissue identification markers.

Acknowledgements

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Supplemental Information

Tag	p EA	p BE	up	gene ID
TCCCTATTAA	1.809	0.032	56	Protein kinase, DNA-activated, catalytic polypeptide
CTTACAAGCA	0.104	0.000	48	unknown
TGAAGTAACA	0.062	0.000	29	Eukaryotic translation initiation factor 1
TTTTGAAATA	0.062	0.000	29	TBC1 domain family, member 3
AATATTGCAC	0.050	0.000	23	unknown
TTGATGTACA	0.048	0.002	22	Splicing factor, arginine/serine-rich 11
GTTTTTGCTT	0.048	0.002	22	Nucleolin
CCTGCTGCAG	0.045	0.002	21	Trf-proximal homolog
GTTTTCCATA	0.039	0.000	18	ATPase, Class V, type 10B
CCTGTGTTGG	0.039	0.002	18	unknown
CCCTACCCTG	0.033	0.000	15	Apolipoprotein D
TTTTCTATCA	0.033	0.000	15	Six transmembrane epithelial antigen of the prostate 2

AAAGGAATAA	0.033	0.000	15	Pro-oncosis receptor inducing membrane injury gene
TCATATAAGT	0.033	0.000	15	unknown
AAAAATAAAA	0.033	0.002	15	Small nuclear ribonucleoprotein D3 polypeptide
ACCCTTGGCC	0.131	0.009	15	unknown
CATTTGGTAT	0.190	0.013	15	unknown
AGCTGGAGTC	0.030	0.000	14	unknown
TGCTCTGAAT	0.030	0.000	14	Hypothetical protein FLJ22795
AAAAGGTTAT	0.030	0.000	14	Sorting nexin 1
TTTTAAATTA	0.030	0.002	14	Heterogeneous nuclear ribonucleoprotein A2/B1
AAACTGTGGT	0.030	0.002	14	unknown
GATGACCCCC	0.238	0.017	14	unknown
TCCCCGTACA	2.165	0.169	13	unknown
TGAGCTACCC	0.027	0.000	12	Fer-1-like 4
TAATTTTTGC	0.027	0.000	12	Olfactomedin 4
TCCCTGTACA	0.027	0.000	12	unknown
TAATTTGCGT	0.027	0.000	12	unknown
AAATTCTGTT	0.027	0.000	12	AHA1, homolog 2
CCCATCGCCC	0.027	0.002	12	unknown
TCCTTGCTTC	0.027	0.002	12	Hypothetical protein FLJ20297
ATCACTAAAG	0.027	0.002	12	Chromosome 11 open reading frame 24
TCCCCGCACA	0.027	0.002	12	unknown
TTCCCGTACA	0.027	0.002	12	unknown
GATTCCTCTA	0.027	0.002	12	Vacuolar protein sorting 13C
CATATCATTA	0.027	0.002	12	Insulin-like growth factor binding protein 7
AAATGTGAAT	0.027	0.002	12	Nucleoporin 50kDa
AACCCAGGAG	0.050	0.004	12	Hemochromatosis
AAGGAGTTAC	0.024	0.000	11	unknown
ACACTTGGAG	0.024	0.000	11	unknown
AATACTTAAA	0.024	0.000	11	WAS protein family, member 2
TTTTAAAATA	0.024	0.000	11	Tweety homolog 3
AACGTTATTA	0.024	0.000	11	Endothelial PAS domain protein 1
CATTTCTCAT	0.024	0.000	11	Adult retina protein
AATAAACTTT	0.024	0.000	11	VAMP-associated protein A
GATTTTGCAC	0.024	0.002	11	Non-metastatic cells 7
TTACTTATAC	0.024	0.002	11	unknown
ATGGCAGGAG	0.024	0.002	11	RNA terminal phosphate cyclase-like 1
AATATATCCA	0.024	0.002	11	Chromosome 3 open reading frame 1
GAAGAACAGA	0.024	0.002	11	Chromosome 20 open reading frame 81
TGTGAGGGAA	0.024	0.002	11	FK506 binding protein 10, 65 kDa
TAATTAAAAA	0.024	0.002	11	Protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1
TTATAATAAA	0.024	0.002	11	Non imprinted in Prader-Willi/Angelman syndrome 2
TTGTGATGTA	0.024	0.002	11	Metastasis associated lung adenocarcinoma transcript 1
ATTTGAGAAG	0.713	0.069	10	RAD23 homolog B

Table 1A: Tags more than 10 fold up-regulated in esophageal adenocarcinoma comparing to Barrett's esophagus. Tags more than 10 fold up-regulated in esophageal adenocarcinoma compared to Barrett's esophagus along with their counts in percentage (p) in esophageal adenocarcinoma (EA) and Barrett's esophagus (BE). The fold induction (indicated in the column named up) of each tag and their corresponding gene ID's are presented.

Tag	p EA	p BE	down	gene ID
GGAAGGTTTA	0.000	0.568	191	Regenerating islet-derived family, member 4
CTGGCCCTCG	0.009	0.746	84	Trefoil factor 1
GCCCAGGTCA	0.003	0.151	51	CDNA clone IMAGE:5759225
CAGTGCTTCT	0.000	0.102	34	Lipase, gastric
GGGGACTGAA	0.003	0.084	28	Ubiquinol-cytochrome c reductase, complex III subunit VII
AAATCCTGGG	0.015	0.387	26	Trefoil factor 2
TCATTCTGAA	0.000	0.076	25	Gastrokine 1
GAGGAAGAAG	0.003	0.071	24	Tumor rejection antigen (gp96) 1
AAATAAAAGA	0.000	0.063	21	EF-hand calcium binding domain 1
AGATCCCAAG	0.000	0.061	20	Intelectin 1
TTTATAAAGG	0.000	0.058	20	Diffuse panbronchiolitis critical region 1
CACCTGCAGA	0.000	0.048	16	G protein-coupled receptor kinase 1
AAGAAAGCTC	0.027	0.419	16	Chromosome 1 open reading frame 22
TTGGCAGCCC	0.003	0.043	15	unknown
ACGTGTGTAA	0.000	0.041	14	unknown
TGTGAAGCCT	0.000	0.041	14	unknown
CAGCAGAAGC	0.003	0.039	13	Small EDRK-rich factor 2
TTGGCCCTCG	0.000	0.037	12	Ribosomal protein S17
ACCTGGAGGG	0.000	0.037	12	Suprabasin
GCCAATCCAG	0.000	0.037	12	Cornulin
GGTGGGAACA	0.000	0.037	12	Regenerating islet-derived family, member 4
TGTGAGGTCT	0.000	0.037	12	Serine peptidase inhibitor, Kazal type 5
GAGAGCTTTG	0.000	0.035	12	Aldo-keto reductase family 1, member C3
TTCCTGCTCT	0.000	0.035	12	unknown
GCAAATCCAA	0.003	0.035	12	CCR4-NOT transcription complex, subunit 6-like
GGGGGTCACC	0.000	0.032	11	ATP synthase, mitochondrial F0 complex, subunit c, isoform 1
AACTAATCTG	0.000	0.032	11	unknown
TACCTAATTG	0.000	0.032	11	unknown
TTTAGGATGA	0.000	0.032	11	Down-regulated in gastric cancer GDDR
TACCTGCAAA	0.003	0.032	11	Neutrophil cytosolic factor 1
TGGCAACCTT	0.003	0.032	11	Glutathione S-transferase kappa 1
TCTCCTGCTC	0.000	0.030	10	unknown
TGTAAGGCAG	0.000	0.030	10	unknown
GCTGTGCCTG	0.000	0.030	10	Protease, serine, 3
GTCCTTGCTG	0.000	0.030	10	unknown
GCAATAAGTG	0.000	0.030	10	LAG1 longevity assurance homolog 2
CCACCCCGAA	0.000	0.030	10	Testis enhanced gene transcript
GTGGCTGCTG	0.003	0.030	10	Mitochondrial ribosomal protein S36
TGCTTTGGGA	0.003	0.030	10	Tetratricopeptide repeat domain 11
CCAAGTTTTT	0.003	0.030	10	Transmembrane emp24 domain trafficking protein 2
CTTCCTGCTC	0.006	0.061	10	Aconitase 2, mitochondrial
CTCCACCCGA	0.021	0.212	10	Trefoil factor 3

Table 1B: Tags more than 10 fold down-regulated in esophageal adenocarcinoma comparing to Barrett's esophagus. Tags more than 10 fold down-regulated in esophageal adenocarcinoma compared to Barrett's esophagus along with their counts in percentage (p) in esophageal adenocarcinoma (EA) and Barrett's esophagus (BE). The fold down regulation of each tag and their corresponding gene ID's are presented.

Tag	p ESCC	p SQ	up	gene ID
TGGAAAGTGA	0.253	0.000	126	V-fos FBJ murine osteosarcoma viral oncogene homolog
GAAATAAAGC	0.201	0.000	100	Immunoglobulin heavy constant gamma 1
GTACGTATTC	0.140	0.002	71	Zinc finger homeobox 1b
CTCCCCCAAG	0.140	0.000	70	Hypothetical protein MGC27165
GGATATGTGG	0.136	0.002	69	Early growth response 1
TAATTTGCGT	0.128	0.002	65	unknown
GAATGATTTC	0.096	0.002	49	Putative nuclear protein ORF1-FL49
AAGGGAGCAC	0.068	0.000	34	Immunoglobulin lambda joining 3
GTGCACTGAG	0.257	0.008	32	Major histocompatibility complex, class I, A
TCCCGTACAT	0.060	0.002	30	unknown
GACCCAAGAT	0.060	0.000	30	Polymeric immunoglobulin receptor
TCAAAAGACC	0.056	0.000	28	V-fos FBJ murine osteosarcoma viral oncogene homolog
AAACCCCAAT	0.056	0.000	28	Immunoglobulin lambda joining 3
TCCCTATTAA	0.758	0.030	26	Protein kinase, DNA-activated, catalytic polypeptide
AATATTTATA	0.048	0.002	24	Carcinoembryonic antigen-related cell adhesion molecule 5
TAATTTTTGC	0.048	0.000	24	Olfactomedin 4
CTGGCCCTCG	0.048	0.000	24	Trefoil factor 1
GCCAGTCTGT	0.048	0.000	24	unknown
ATTTTCTAAA	0.088	0.004	22	Anterior gradient 2 homolog
ATGAAACCCC	0.044	0.002	22	Chromosome 9 open reading frame 90
CTCTAAGAAG	0.044	0.002	22	Complement component 1, q subcomponent, alpha polypeptide
CTCCCCCAAA	0.044	0.000	22	Hypothetical protein MGC27165
GTGCCCTGTT	0.040	0.002	20	NCK-associated protein 1
AATCTGCGCC	0.040	0.000	20	Interferon, alpha-inducible protein
ATGGAATAAT	0.040	0.000	20	Annexin A10
TTTGCACCTT	0.040	0.000	20	Connective tissue growth factor
CGCCGACGAT	0.273	0.014	20	Interferon, alpha-inducible protein
ATGTAAAAAA	0.148	0.008	19	Lysozyme
TCCCCGTACA	1.717	0.093	18	unknown
TTACGAGGAA	0.036	0.002	18	SEC13-like 1
ACTGCTTGCC	0.036	0.000	18	Methionine adenosyltransferase II, alpha
AAGGTAACAG	0.036	0.000	18	Serine peptidase inhibitor, Kazal type 1
TTGTGGTTAA	0.036	0.000	18	unknown
CAACAATAAT	0.036	0.000	18	Chromosome 8 open reading frame 4
ATAAATTTAT	0.036	0.000	18	Potassium voltage-gated channel, Isk-related family, member 3
CCTCCAGCTA	0.064	0.004	16	Keratin 8
ATCAAGAATC	0.032	0.002	16	Interferon, gamma-inducible protein 30
TAATAAACAG	0.032	0.002	16	N-acylsphingosine amidohydrolase 1
ACAAAGCATT	0.032	0.002	16	unknown
AAGAAAGCTC	0.032	0.000	16	Chromosome 1 open reading frame 22
ATGTGAAGAG	0.032	0.000	16	Secreted protein, acidic, cysteine-rich
CCTTTGTAAG	0.032	0.000	16	V-jun sarcoma virus 17 oncogene homolog
CTGTTGTGTG	0.032	0.000	16	Dual oxidase 2
TCCCTGTACA	0.032	0.000	16	SH3-domain GRB2-like 3
GAGGCCAAGA	0.032	0.000	16	Glutathione S-transferase A1
CCAGGGGAGA	0.124	0.008	16	Interferon, alpha-inducible protein 27
CTGACCTGTG	0.112	0.008	14	Major histocompatibility complex, class I, B
TCCCTGGCTG	0.028	0.002	14	Prosaposin

AAGCTCTGTG	0.028	0.002	14	Hypothetical protein AF447587
GAGTTTGTTA	0.028	0.002	14	Claudin 18
CAAACTAACC	0.028	0.000	14	Immunoglobulin heavy constant mu
TTCTGTGTGG	0.028	0.000	14	Protease, serine, 2 (trypsin 2)
GGAAAACAGA	0.028	0.000	14	Lectin, galactoside-binding, soluble, 4 (galectin 4)
ACTGATGCAA	0.028	0.000	14	unknown
AAAGGAAAGT	0.028	0.000	14	Hematological and neurological expressed 1
CCAAAGCTAT	0.028	0.000	14	Tetraspanin 8
CAAAACTGTT	0.028	0.000	14	Myosin X
AGAAATGTAT	0.052	0.004	13	SNF1-like kinase
ATAAAGTAAC	0.024	0.002	12	Serine/threonine kinase receptor associated protein
AGCACATTTG	0.024	0.002	12	Coactosin-like 1
GAATTCAGCA	0.024	0.002	12	Sterile alpha motif domain containing 9
AAAGGAATAA	0.024	0.002	12	Pro-oncosis receptor inducing membrane injury gene
GGCAGGAGTA	0.024	0.002	12	Guanylate binding protein 1, interferon-inducible, 67kDa
AGAACTTCCT	0.024	0.002	12	Defensin, beta 1
GTAAAACAAT	0.024	0.002	12	Protein tyrosine phosphatase, receptor type, G
GATTTTGCAC	0.024	0.000	12	Non-metastatic cells 7
TCCCCGGTAC	0.024	0.000	12	unknown
GAGGGCTTTG	0.024	0.000	12	Aldo-keto reductase family 1, member C3
CTTTTCATCA	0.024	0.000	12	Tumor necrosis factor superfamily, member 5-induced protein 1
TCCCCGTTCA	0.024	0.000	12	unknown
CTCCACCCGA	0.024	0.000	12	Trefoil factor 3
CTTTTCAAGA	0.024	0.000	12	CD46
GAGACTGCAA	0.024	0.000	12	Solute carrier family 40, member 1
TAGATTTCAA	0.024	0.000	12	Hypoxia-inducible factor 1, alpha subunit
TGATCACCTA	0.024	0.000	12	Splicing factor 3B, 14 kDa subunit
TGAATAAGTA	0.024	0.000	12	Palmdelphin
TCCCCGTATA	0.024	0.000	12	unknown
TACTAGTCCT	0.024	0.000	12	Heat shock 90kDa protein 1, alpha
TAAACTGTAT	0.024	0.000	12	Zinc finger, FYVE domain containing 9
AATAAACTTT	0.024	0.000	12	Hypothetical protein LOC129607
ACCCTTGGCC	0.044	0.004	11	unknown
TTTTTCAAGA	0.044	0.004	11	DMC
AATTTTATTT	0.044	0.004	11	Poly(rC) binding protein 1
CCTGGGAAGT	0.064	0.006	11	unknown
TACATAATTA	0.124	0.012	10	Trophoblast-derived noncoding RNA
TAAGCAGATG	0.040	0.004	10	Inhibitor of Bruton agammaglobulinemia tyrosine kinase
TCAATAAATG	0.040	0.004	10	Serine carboxypeptidase 1
CCCGACGTGC	0.020	0.002	10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa
TGGACAGTGC	0.020	0.002	10	Eyes absent homolog 2
GAGAAATATC	0.020	0.002	10	Zinc finger protein 638
TGGCTGTGTG	0.020	0.002	10	Hypothetical protein LOC284361
GAGGCCAATG	0.020	0.002	10	Pumilio homolog 2
AAGGAACTTG	0.020	0.002	10	Chemokine-like factor superfamily 4
AAATAAAGCA	0.020	0.002	10	Inhibitor of DNA binding 2
TAGACTAGCA	0.020	0.002	10	Tetraspanin 3
GAAAGGCAAA	0.020	0.002	10	ST6-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1
TAGCTGGAAA	0.020	0.002	10	Ataxin 10

ATTGCTGTAA	0.020	0.002	10	Replication factor C (activator 1) 5
CCTCTTGTAA	0.020	0.002	10	SET and MYND domain containing 2
GTTTCAGGTA	0.020	0.002	10	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
TGTGGGTGCT	0.020	0.002	10	E-cadherin
TCCCCTCTCT	0.020	0.002	10	Anaphase promoting complex subunit 5
GTTACTTTTT	0.020	0.002	10	Receptor tyrosine kinase-like orphan receptor 2
ACCTTGTGCC	0.020	0.000	10	L-iditol 2-dehydrogenase
CCACCTTTCC	0.020	0.000	10	C-Maf-inducing protein
AATTTGCAAC	0.020	0.000	10	H2A histone family, member Y
AATATTGTAC	0.020	0.000	10	unknown
GCTAGGGTTC	0.020	0.000	10	Transmembrane 4 L six family member 4
TCCCCGTACG	0.020	0.000	10	unknown
AATGGAATGG	0.020	0.000	10	Melanophilin
GTTCCAGCAG	0.020	0.000	10	Arginine vasopressin-induced 1
TATGGGGAAG	0.020	0.000	10	Trophoblast-derived noncoding RNA
AAAGAGAAAG	0.020	0.000	10	Adrenomedullin
AGTTTGTTAG	0.020	0.000	10	Tumor-associated calcium signal transducer 1
GAGTCTCCTG	0.020	0.000	10	Retinoic acid receptor, beta
CTGACTTGTG	0.020	0.000	10	unknown
AAATTTCTCA	0.020	0.000	10	Tetraspanin 13
AGCTCTTGGA	0.020	0.000	10	Selenium binding protein 1
TAGTTGGAAA	0.020	0.000	10	Nuclear receptor subfamily 4, group A, member 1
CTTTAAGAAA	0.020	0.000	10	CAMP responsive element binding protein 3-like 1
GGAAGGTTTA	0.020	0.000	10	Regenerating islet-derived family, member 4
GTGTGATGCT	0.020	0.000	10	unknown
CCCAAGGTCT	0.020	0.000	10	Aftiphilin protein
CAGATTAAGT	0.020	0.000	10	unknown
AATAGCCTGT	0.020	0.000	10	unknown
ACTGATTGAT	0.020	0.000	10	unknown
CCACAGAAAT	0.020	0.000	10	unknown
AGAATTGCTT	0.020	0.000	10	Phosphoribosylaminoimidazole carboxylase
CGCTGTTTTT	0.020	0.000	10	Flap structure-specific endonuclease 1

Table 2A: Tags more than 10 fold up-regulated in esophageal squamous cell carcinoma comparing to normal squamous esophagus. Tags more than 10 fold up-regulated in esophageal squamous cell carcinoma compared to normal squamous esophagus along with their counts in percentage (p) in esophageal squamous cell carcinoma (ESCC) and normal squamous esophagus (SQ). The fold induction (indicated in the column named up) of each tag and their corresponding gene ID's are presented.

Tag	p ESCC	p SQ	down	gene ID
CACCTGCAGA	0.000	0.198	49	G protein-coupled receptor kinase 1
TGAAAGATGT	0.000	0.121	30	unknown
TGAAATACTT	0.000	0.101	25	Zinc finger and BTB domain containing 33
TTGGCCACGG	0.000	0.097	24	Hypothetical protein FLJ25976
GCCAATCCAG	0.008	0.180	22	Cornulin
TCTCCATACC	0.000	0.087	22	unknown
ACACAGCAAG	0.000	0.085	21	unknown
TTTCCTGCAG	0.000	0.083	21	F-box and WD-40 domain protein 8
GCAATAAGTG	0.000	0.083	21	LAG1 longevity assurance homolog 2
GGGGCCACGG	0.004	0.079	20	Pericentrin 2
TACCTGCTCT	0.000	0.077	19	T-cell leukemia/lymphoma 1B
TGCAGAGAAG	0.004	0.077	19	Chromosome 1 open reading frame 73
GTCCTTGCTG	0.000	0.075	19	unknown
TATCTCTTGG	0.000	0.073	18	unknown
ACGTGTGTAA	0.008	0.139	17	unknown
AACCTGCAGA	0.004	0.069	17	Neurogenin 2
CTCCTTGCTG	0.000	0.061	15	unknown
CAGAGAAATG	0.008	0.117	15	Dystrobrevin, beta
TAAGCACAAG	0.000	0.057	14	unknown
TATTGTTACT	0.004	0.057	14	Annexin A1
CGAAAGATGT	0.004	0.055	14	unknown
TCCCTGCAGA	0.004	0.053	13	Calponin 2
TGTGAGGTCT	0.008	0.103	13	unknown
TTCCTTGCTG	0.000	0.049	12	unknown
TCCACCAAGT	0.000	0.049	12	Kv channel interacting protein 4
ACCCGCCGGG	0.000	0.048	12	unknown
TTCCTGCAGA	0.000	0.048	12	unknown
CATTGTAAAT	0.000	0.048	12	Serpin peptidase inhibitor, clade B, member 5
AGCGCTGATT	0.000	0.048	12	unknown
TGTCAAGTCT	0.004	0.048	12	unknown
TTCCTGCTCT	0.000	0.046	11	Leucine zipper, putative tumor suppressor 2
GGAGGTGAGA	0.004	0.046	11	Solute carrier family 28, member 1
GCAGAGAGGA	0.000	0.044	11	Keratin 13
ATCCTGCAGA	0.000	0.042	10	Fizzy/cell division cycle 20 related 1
AGATAAATAA	0.000	0.042	10	Epithelial membrane protein 1
TGTCAAGCCT	0.000	0.042	10	Serine peptidase inhibitor, Kazal type 5
ATAAAGATGT	0.000	0.042	10	Ubiquitin-conjugating enzyme E2B (RAD6 homolog)
CAGGACCTGG	0.004	0.042	10	Zinc finger, DHHC-type containing 13
TACCCTGCAG	0.004	0.042	10	Phosphatidylinositol glycan, class F
AAAAAGATGT	0.004	0.042	10	unknown
CTGATGGCGA	0.012	0.125	10	S100 calcium binding protein A9 (calgranulin B)

Table 2B: Tags more than 10 fold down-regulated in esophageal squamous cell carcinoma comparing to normal squamous esophagus. Tags more than 10 fold down-regulated in esophageal squamous cell carcinoma compared to normal squamous esophagus along with their counts in percentage (p) in esophageal squamous cell carcinoma (ESCC) and normal squamous esophagus (SQ). The fold down regulation of each tag and their corresponding gene ID's are presented.

Chapter 7

Cytokeratin and CDX-2 expression in Barrett's esophagus

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Abstract

Barrett's esophagus (BE) is a premalignant condition in which normal lined squamous epithelium of the lower esophagus is replaced by columnar epithelium containing intestinal metaplasia. For diagnostic purposes it is important to find biomarkers that can specifically identify BE, for instance to differentiate Barrett epithelium from gastric cardia. Several bio-markers, including certain Cytokeratins (CKs) are specifically expressed in BE. Recently the homeobox gene CDX-2 has been suggested to be highly expressed in BE. Our aim was to determine the specificity of CDX-2 and a set of CKs as specific markers for BE as compared to normal squamous esophageal and gastric cardia tissue. Immunohistochemistry with specific antibodies against CDX-2, CK7, CK8, CK10/13, CK18 and CK20 was performed on fresh frozen consecutive tissue sections of normal squamous, gastric cardia and non dysplastic BE of 80 patients. Immunohistochemistry results showed CK8, CK18 and CK20 expression in both BE and gastric cardia, while CK7 was seen in all BE but as well in 26% of gastric cardia biopsies. CK10/13 was only expressed in normal squamous epithelium. CDX-2 nuclear staining was found in 87.5% of the BE biopsies, whereas normal squamous esophagus and cardia were negative. CDX-2 in combination with a set of CKs can be used as biomarkers to distinguish between Barrett and normal squamous esophagus. In order to distinguish Barrett from cardia tissue, a combination of CDX-2 and CK7 is most informative.

Introduction

The pre-malignant condition of the distal esophagus in which normal lined squamous epithelium is replaced by a metaplastic columnar lined epithelium is called Barrett's esophagus (BE). BE is a complication of chronic gastroesophageal reflux disease (GERD), the key feature of GERD is reflux of gastric and/or bile contents into the esophagus[1-3]. Patients, who have BE have a 30 to 125 fold increased risk of developing an esophageal adenocarcinoma (EA), with an estimated annual incidence varying from 0.4% to 1.8%[4-7]. In Western countries, the incidence of BE and the associated EA has increased at a rate that exceeds that of any other malignancy[8-10]. Of major concern is the increasing prevalence of BE particularly in Western countries. Several studies show that in white Caucasian males the prevalence of BE is increasing[11-14]. From these data it is clear that the majority of BE patients are asymptomatic or only have mild subclinical GERD symptoms. As a consequence, a large BE population is not identified and is not included in surveillance programs. These patients may develop EA and at presentation may already have advanced stage disease with poor prognosis. Indeed the majority of cases of EA are detected in patients who were not included in surveillance programs and at diagnosis presented with severe dysphagia and advanced stage disease[15]. From these facts one may postulate that for effective prevention of EA, screening for BE in high risk populations will be necessary. Currently for diagnosing BE, endoscopy with biopsy above the gastroesophageal junction to detect even short segments of intestinal type of metaplasia is the gold standard. Since it has been proven that any length of BE may subsequently progress into EA, it is of major importance to be able to distinguish BE from its surrounding epithelia. Research has mostly been focused to find biomarkers for malignant transformation of BE into EA as an alternative for the classical histopathological grading for dysplasia. Biomarkers that were investigated include p53, Cyclin-D, DAS-1, PCNA, villin and Ki-67[16]. There has been less interest for markers that specifically identify BE and distinguishes BE from its surrounding epithelial cell types such as gastric cardia and squamous epithelium. In literature CKs are reported to be an important marker for the differentiation of normal squamous esophageal epithelium to metaplastic and dysplastic epithelium[17-19]. CKs are important subunits of the intermediate filaments of the cytoskeleton of epithelial cells. There are 20 distinct forms of CKs and the expression pattern in epithelial cells is dependent on the type, location and differentiation of these cells[19]. CKs 1-6 and 9-17 are

expressed mainly in stratified squamous epithelium like the epidermis, epithelium of the mouth, esophagus and anal canal while CKs 7, 8, 18-20 are expressed mainly in 'simple' or columnar epithelium like epithelia of the stomach and the small and large intestine, yet each of these tissues have their own CK expression pattern[20]. The CK expression patterns in normal tissues and in BE is still a point of debate. Ormsby *et al*. describes a unique pattern of immunoreactivity, the Barrett CK7/20 pattern, with a sensitivity and specificity of 94% and 100% respectively for the diagnosis of BE when compared with intestinal metaplasia of the cardia of the stomach[18]. Others, such as El-Zimaity *et al*. do not agree and find a sensitivity and specificity of 45% and 65% respectively[17]. Recently the caudal-type homeobox gene, CDX-2, is reported as biomarker for BE[21]. CDX-2 is a transcription factor involved in intestinal mucosal cell proliferation and differentiation and important in the early differentiation and maintenance of the epithelium of the intestine[22-25]. CDX-2 can induce MUC2 transcription, sucrase-isomaltase, alkaline phosphatase, lactase-phlorizin hydrolase, intestine phospholipaseA/lysophospholipase and Claudin-2[22, 25-35]. Bai *et al*. reports that CDX-2 serves as a sensitive marker of intestinal metaplasia in the upper gastrointestinal tract and Eda A *et al*. found expression of CDX-2 in BE, but not in squamous esophagus[36, 37]. The present study looked into the specificity of CDX-2 and CKs as a set of markers for distinguishing BE from its adjacent squamous and cardia epithelia. To this end immunohistochemistry was performed on samples of BE, normal squamous esophagus and gastric cardia tissues of 80 BE patients. Our results indicate that CDX-2 is expressed in a high percentage of the BE cases, yet in a significant number of cases only a low amount of cells showed positive nuclear staining. CK7, CK8, CK18 and CK20 were positively stained in subsets of BE, whereas CK8, CK18 and CK20 were positively stained for gastric cardia epithelium and CK10/13 for normal squamous epithelium. From these observations we may conclude that for distinguishing BE from the surrounding epithelia a combination of CK7, CK20 and CDX-2 is most informative.

Materials and Methods

Patients and Biopsy Specimens

Tissue samples of 80 BE patients taken during routine surveillance endoscopy were used. The mean age was 62 years (range 30-87 years), 66 patients were male and the average length of the

BE segment measured endoscopically was 5 cm (range 2-13 cm). All patients had known BE without dysplasia and were on long term proton pump inhibition of 40 to 80 mg daily to prevent reflux esophagitis. 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 segment was biopsied at least 2 cm above the gastroesophageal junction yet within the Barrett segment, recognized endoscopically as typically pink colored mucosa. Normal squamous epithelium was biopsied at least 2 cm above the Barrett segment and gastric cardia was taken within 2 cm below the gastroesophageal junction. Of each set of biopsies, one biopsy was used for histopathological confirmation whereas the other was snap-frozen in liquid nitrogen for subsequent immunohistochemical analysis. All patients signed informed consent for the use of their biopsy material.

All patients had proven incompletely differentiated intestinal type of columnar epithelium with the presence of goblet cells in the histological control biopsies with no signs of active or acute inflammation. Normal gastric cardia and normal esophageal squamous epithelia were confirmed histologically in all the pair wise taken control biopsies.

Fluorescent immunohistochemistry

Sections of 4 μm thickness were cut using a microtome, sections were mounted on Superfrost + (Menzel Glaser, Braunschweig, Germany) glass slides. Slides were fixed for 20 minutes in Phosphate buffered Saline (PBS) with 4% Paraformaldehyde (PFA) and 0.1% Triton and washed in PBS. Blocking of a-specific antigens was performed by incubating slides for 45 minutes with PBS with 1% Bovine Serum Albumin (BSA) with 10% Fetal Calf Serum (FCS). Slides were washed with PBS and incubated overnight at 4ºC with the appropriately diluted primary antibody in PBS with 1% BSA with 0.1% Triton. After incubation, the slides were washed with PBS and incubated with the secondary antibody FITC conjugated (Dako, Denmark) 1:500 diluted in PBS. Slides were washed and mounted with DAPI (Roche, Mannheim, Germany)/vectashield (Vector laboratories Inc, Burlingame, CA, USA) 1:1000. The antibodies used and dilutions are summarized in Table 1.

Evaluation of stainings

Staining was evaluated using a fluorescent microscope. Independent assessment of stainings was blindly performed by two independent observers. For CDX-2 only nuclear staining was considered as positive, while CK stainings were cytoplasmic. The distribution of the IHC staining in BE and gastric cardia was scored as localized in the glandular, crypt, or villus compartments of the epithelium. For squamous epithelium the basal and superficial layers were distinguished. The intensity of the stainings was scored as negative, moderate or strong. In case of CDX-2 staining the number of positive cells was determined in a semi-quantitative manner. Any discrepancies between the two observations were resolved by concordance at a double-headed microscope after independent review.

Antibody	Species	Company	Country	Dilution
Cytokeratin 7	Mouse monoclonal	Chemicon	USA	1:1000
Cytokeratin 8	Mouse monoclonal	Chemicon	USA	1:600
Cytokeratin 10/13	Mouse monoclonal	Dako	Denmark	1:1000
Cytokeratin 18	Mouse monoclonal	Sigma	USA	1:1000
Cytokeratin 20	Mouse monoclonal	Progen	Germany	1:1000
CDX-2	Mouse monoclonal	Biogenex	The Netherlands	1:1000

Table 1: Antibodies as used for immunohistochemistry.

Results

Results are summarized in Table 2. A total of 240 biopsies of 80 patients diagnosed with BE were evaluated for immunohistochemical CDX-2 and CK expression pattern in BE, squamous and gastric cardia epithelium.

Barrett's esophagus

CK8 and CK18 positively stained all BE biopsies (Figure 1). The BE epithelium showed strong positive cytoplasmic staining for CK8 and CK18 at the villi and moderately positive staining at the superficial and deep glands (Figure 1). The typical Barrett CK7/20 expression pattern was found in 79 out of 80 patients (99%). CK7 was strongly positive in the villus compartments and the upper and deeper crypts of the Barrett epithelium (Figure 2). Strong CK20 staining of the villus compartment and upper part of the crypts was seen, whereas the deeper crypts and the glandular compartment did not stain for CK20 (Figure 1). No CK10/13 expression was found in any of the BE biopsies (Figure 1).

Ten (12.5%) BE biopsies showed negative CDX-2 staining and 70 (87.5%) showed positive CDX-2 staining (Figure 3). However, 52 (65%) BE biopsies showed positive CDX-2 staining in less then 10% of the cells, 18 (22.5%) showed positive staining in 10-30% of the cells. In all cases staining for CDX-2 was predominantly found at the tops of the villi.

Normal squamous esophagus

All normal squamous esophagus biopsies were positively stained for CK10/13, whereas CK7, CK8, CK18, CK20 and CDX-2 were negatively stained (Figure 2, 3 and 4).

	Squamous	**Barrett's esophagus**	**Gastric cardia**
Cytokeratin 7	-	+	74% -
Cytokeratin 8	-	+	+
Cytokeratin 10/13	+	-	-
Cytokeratin 18	-	+	+
Cytokeratin 20	-	+	+
CDX-2	-	22.5% more than 10% + *	+

Table 2: Immunofluorescent staining results. * 22.5% of the BE biopsies showed positive staining in 10-30% of the cells, whereas 65% showed positive staining in less than 10% of the cells.

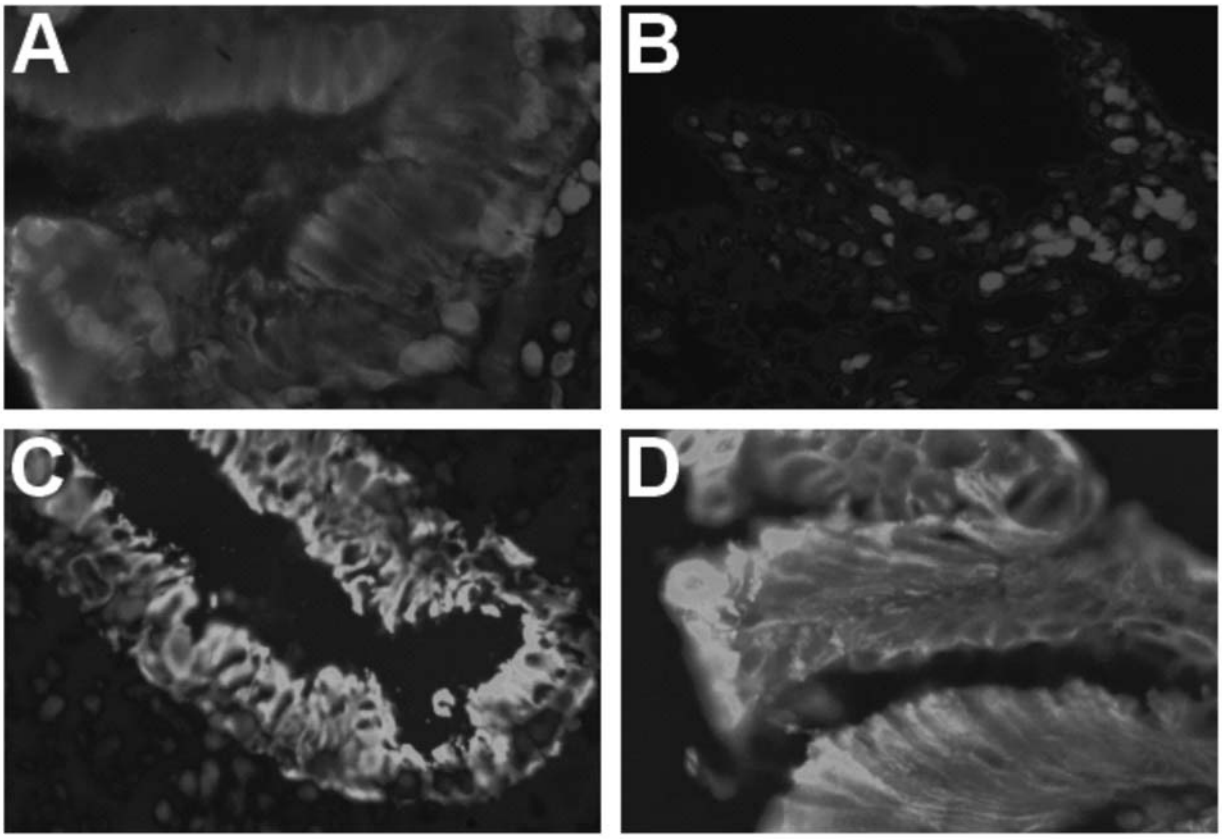

Figure 1: Cytokeratin immunofluorescent staining in Barrett's esophagus. Immunofluorescent staining of Barrett's esophagus showing positive staining for Cytokeratin 8 (A), Cytokeratin 18 (C) and Cytokeratin 20 (D), and negative staining for Cytokeratin 10/13 (B). Pictures are representative for results of 80 patients.

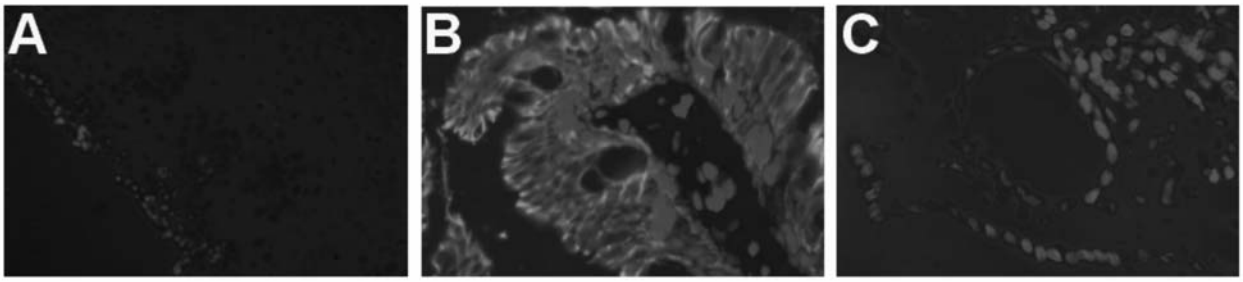

Figure 2: Cytokeratin 7 staining in normal squamous epithelium, Barrett's esophagus and gastric cardia. Immunofluorescent staining of normal squamous esophagus (A), Barrett's esophagus (B) and gastric cardia (C) stained for Cytokeratin 7, with only positive Cytokeratin 7 staining in Barrett's esophagus (B). Pictures are representative for results of 80 patients.

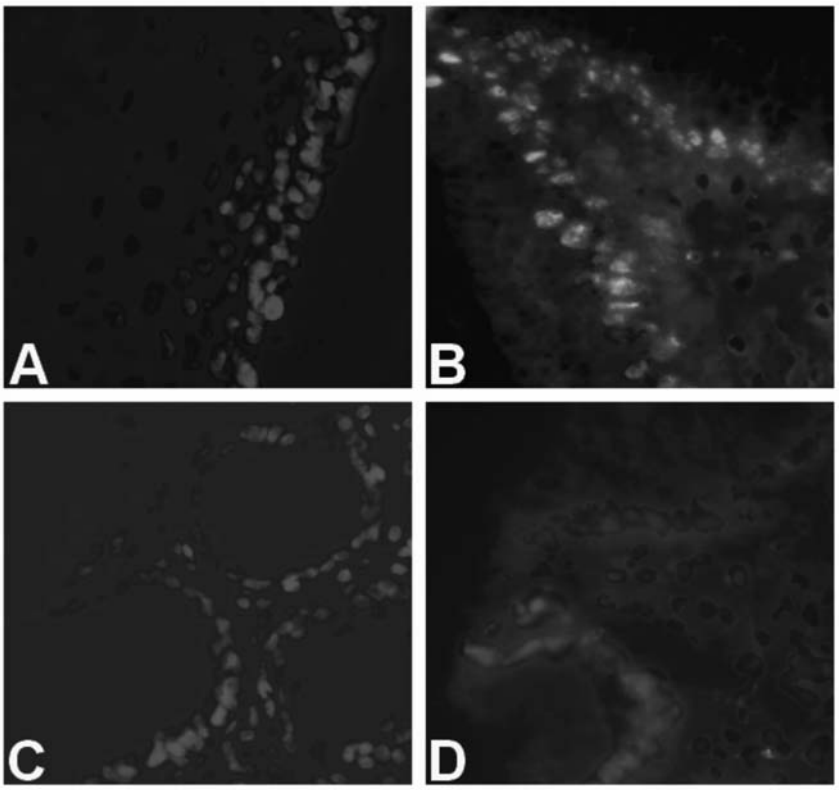

Figure 3: CDX-2 immunofluorescent staining in normal squamous esophagus, Barrett's esophagus and gastric cardia. Immunofluorescent staining of normal squamous esophagus (A), Barrett's esophagus (B and D) and gastric cardia (C) stained for CDX-2. CDX-2 staining is negative in normal squamous esophagus and gastric cardia biopsies, whereas Barrett's esophagus stains positive in B but negative in D. Pictures are representative for results.

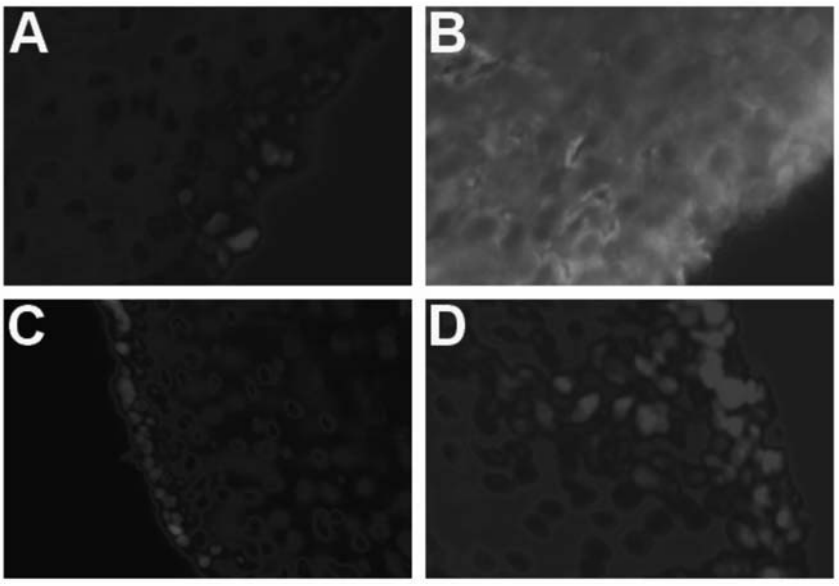

Figure 4: Cytokeratin immunofluorescent staining in normal squamous esophagus. Immunofluorescent staining of normal squamous esophagus stained for Cytokeratin 8 (A), Cytokeratin 10/13 (B), Cytokeratin 18 (C) and Cytokeratin 20 (D), showing only positive staining for Cytokeratin 10/13. Pictures are representative for results of 80 patients.

Gastric cardia

Gastric cardia showed negative staining for CK10/13, whereas CK8 and CK18 showed strongly positive staining of the villus compartments (Figure 5). The deeper layers of the epithelium were strongly positively stained for CK8, while CK18 staining was moderately positive (Figure 5). All 80 gastric cardia tissues showed positive staining for CK20 at the villus compartments and the upper part of the crypts, the deeper part of the crypts showed negative staining for CK20 (Figure 5). CK7 staining was negative in 67 (74%) of the gastric cardia biopsies; 23 (26%) showed positive staining at the villus regions and the upper part of the crypts (Figure 2). All gastric cardia biopsies showed negative staining for CDX-2 (Figure 3).

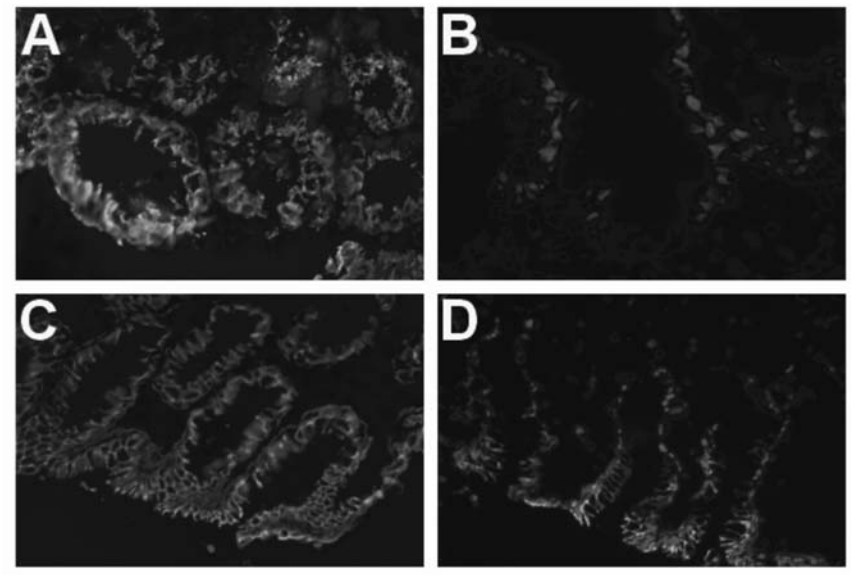

Figure 5: Cytokeratin immunofluorescent staining in gastric cardia. Immunofluorescent staining of gastric cardia showing positive staining for Cytokeratin 8 (A), Cytokeratin 18 (C) and Cytokeratin 20 (D), while staining for Cytokeratin 10/13 (B) is absent. Pictures are representative for results of 80 patients.

Discussion

CKs are subunits for intermediate filaments as a part of the cytoskeleton. The CK expression pattern is variable in epithelial cells and this depends on the type, differentiation and location op the epithelium[19]. CDX-2 is a transcription factor important in early differentiation and maintenance of intestinal type of epithelium[22-25]. CK7 and CK20 are debated in literature for their expression in BE and recently CDX-2 has been proposed as a highly specific marker for BE with intestinal type of differentiation. In this study we investigated several CKs and CDX-2 as marker specific for intestinal type of metaplasia. Immunofluorescent stainings of BE epithelium were

compared with stainings of normal squamous esophagus and gastric cardia. All BE cases showed CK7, CK8, CK18 and CK20 staining, whereas staining for CK10/13 was absent. BE showed a strong positive cytoplasmic CK7 staining of the surface epithelium and the superficial and deeper glands, CK20 demonstrated also a strong positive cytoplasmic staining of the surface epithelium and of the superficial glands, whereas the deeper glands were negatively or weakly positively stained. This indicates that in this study all BE biopsies showed the typical Barrett CK7/20 expression pattern. We also found 26% of the gastric cardia biopsies positive for CK7. Since all cardia biopsies are also positively stained for CK20, 26% of the cardia biopsies have the typical CK7/20 expression pattern. These results are in concordance with previously published papers that describes this specific CK7/20 pattern in BE[18], but as well in a subset of gastric cardia. Ormsby, however describes another pattern in which there is strong superficial CK20 staining and diffuse CK7 staining in BE. Mohammed et al. describes the typical CK7/20 pattern in 65% of the investigated BE biopsies while the remainder showed a variety of immunohistochemical staining patterns for CK7 and CK20[38]. He furthermore found a positive CK7/20 pattern in 4 of the 13 cardiac intestinal metaplasia biopsies and in 55% of the normal cardia or inflamed cardia. He concluded that for diagnosing of BE versus cardiac intestinal metaplasia the sensitivity was 65%, specificity 69% and negative predictive value 35%. Glickman *et al*. found in 91% of the classic long segment BE the specific CK7/20 pattern, but as well in 88% of the short segment BE cases and 88% of the cases with intestinal metaplasia of the gastroesophageal junction[39]. El-Zimaity *et al*. reported the specific CK7/20 pattern in 45% of the BE cases and 35% of the patients with intestinal metaplasia of the cardia[17].

In this study we demonstrated that all normal squamous biopsies were negative for CDX-2, which is in concordance with previous studies that reported a lack of CDX-2 in normal squamous epithelium. In our study we described as well negative CDX-2 expression in all 80 cases of gastric cardia, which is in accordance with a previously published paper where no CDX-2 mRNA expression in cardiac mucosa was reported[41]. Yet in several other papers gastric cardia was found to be positive for CDX-2 in 38%, 67% and 77% of the samples[21, 37, 40].

The positive expression of CDX-2 found in 87.5% of the BE cases in the present study, confirms previously published reports describing an increased CDX-2 expression in BE and EA[21, 37, 40-45]. However we also noticed that 12.5% of the BE patients are negative for CDX-2. And an important observation in our study is that in general less than 30% of the cells in the BE

epithelium express CDX-2, moreover in 65% of the positive cases CDX-2 staining was seen in less than 10% of the cells. Positive staining for CDX-2 was predominantly seen in the top of the villi of the BE epithelium. This is in contrast to a previously published paper were 4 out of 10 intestinal metaplasia samples were intensively stained[43]. Another discrepancy with this report is that they reported a more intense staining in the deeper glandular cells and crypt bases, compared with surface cells[43]. There are several explanations why several groups may find dissimilar results. The sample size of the material, sampling errors, the differences in immunohistochemical techniques, differences in used antibodies, fixation differences, inter-observer variability and patient populations may importantly influence the results. One may also speculate that intestinal type of metatplasia in gastric cardia may as well express CDX-2. In our study patients with metaplasia of the cardia were not included. Although, staining results of CKs and CDX-2 are not quite comparable between different groups, with this study and previous observations it seems that CKs and CDX-2 staining may be helpful for identifying BE and distinguishing BE from its surrounding epithelia. For CDX-2 staining, one have to consider that only few cells might be positive in the superficial villus region and for specific the CK7/20 staining pattern one have to bear in mind that this pattern might be found in subsets of gastric cardia as well.

Acknowledgements

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Chapter 8

Summary

Samenvatting

Dankwoord

Curriculum Vitae

Summary

Barrett's esophagus (BE) is the metaplastic change of the normal lined squamous epithelium of the distal esophagus to a columnar type of epithelium as a result of chronic longstanding gastro-esophageal reflux disease (GERD). Patients with BE have a significantly increased risk of developing an esophageal adenocarcinoma (EA). Over the last 3 decades, the incidence of BE and its associated adenocarcinoma has increased in Western countries at a rate that exceeds that of any other malignancy. Despite all the research performed on BE there is still an inadequate understanding of the biological basis of this mucosal transformation.

The first part of this thesis describes the results of a gene expression profile analysis (using SAGE) comparing BE with normal squamous epithelium and gastric cardia (**chapter 2**). Genes are described that are involved in the metaplastic transformation of normal squamous epithelium into columnar epithelium. Of interest was that comparing BE with normal squamous epithelium or BE with gastric cardia mucosa more or less the same amount of tags were significantly differentially expressed. In contrast, comparing normal squamous epithelium with gastric cardia the amount of tags that were significantly differentially expressed was twice as high. This indicates that BE shows similarities to both investigated epithelia. In addition, BE proves to be an incompletely differentiated type of epithelium. In addition, several genes that were more specifically expressed in BE were found, these included TFF1, TFF2, TFF3, Annexin A10 and Galectin 4. Of interest is the unique Cytokeratin (CK) expression profile for each epithelium that is described in this chapter. One of the genes, BMP4 that was found to be uniquely expressed in BE, was used for further research to investigate whether BMP4 and the BMP pathway plays a role in the metaplastic transformation of normal squamous epithelium into BE (**chapter 3**). To this aim, tissues from patients and an esophagitis-BE rat model were investigated. Results indicated BMP-pathway activation in esophagitis and BE, furthermore cultured keratinocytes treated with BMP4 showed BMP-pathway activation by up-regulation of ID2 and phosphorylation of Smad 1/5/8. This effect could be blocked by pre-treatment with Noggin, a BMP antagonist. In addition the CK expression patterns and gene expression profiles were examined. Upon BMP4 treatment results indicated a shift of the CK expression pattern to a specific BE pattern. On the gene expression level a shift of 26% was seen to a Barrett gene expression profile. These results indicate that BMP4 is involved in the development of BE.

Despite all the research on gene expression profiles, the signal transduction events that occur in BE are inadequately understood. Therefore a comprehensive description of cellular kinase activity in BE was made and compared with the kinase activity profiles of normal squamous esophagus and gastric cardia (**chapter 4**). The activity of the MAP kinase signalling cascade was significantly diminished in BE compared to normal squamous esophagus and gastric cardia. Furthermore the EGF receptor is significantly more activated in normal squamous esophagus compared to BE; additionally there is more activity in BE compared to gastric cardia. An interesting finding was the increased glycolytic activity in BE compared to both normal squamous esophagus and gastric cardia.

In order to specifically investigate the genes involved in the epithelial cell layer of BE, SAGE was performed on primary cell cultures of BE and normal squamous epithelium (**chapter 5**). Gene expression profiles were compared between the 2 cell types with full biopsies containing epithelial cells, stromal and inflammatory cells. To obtain the epithelial cell layer, epithelial cells out of biopsies of BE and normal squamous epithelium were cultured using a Barrett specific culturing medium. Genes specifically expressed by the Barrett epithelial cells were Lipocalin 2, SOX 4, Cytokeratin 7, Galectin 7 and Cyclin D1, whereas Annexin A10, TFF1 and TFF2 were specifically expressed in the BE biopsies. This study demonstrates certain genes that were specifically expressed by the epithelial cells, whereas others were only found in the biopsies and assumingly were expressed by the stromal non epithelial tissue in the biopsy specimens.

Despite the research that is performed on esophageal cancer, the molecular basis remains uncertain. Transcriptome analysis was performed on esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC) and compared with transcriptomes of BE and normal squamous epithelium, respectively (**chapter 6**). Transcriptome analysis of EA and ESCC indicated that the molecular basis of these cancers is significantly different; both epithelia have their own specific characteristics. Genes that were significantly up-regulated in EA compared to BE were SOX 4 and Lipocalin 2, while the down-regulated genes in EA were Trefoil factors and Annexin A10. The most up-regulated genes in ESCC compared to normal squamous epithelium were BMP4, Cyclin D1, E-Cadherin and TFF3. In order to get a better overview of biological events occurring in EA and ESCC in comparison to respectively BE and normal squamous epithelium, genes corresponding to tags significantly differentially expressed between the two SAGE libraries were clustered into groups of biological processes. Interestingly, in EA an abundance of genes situated in the clusters nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, cell division, cell growth, response to stimulus and signal transduction were found, indicating that these processes play a major role in EA. Of interest is that in ESCC compared to normal squamous epithelium, the cluster analysis indicates that cell-cell signalling is five fold decreased. In contrast, the clusters cell cycle, nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, metabolism, cell division and cell communication were all more than 10 fold increased in ESCC. In more detail, 13 main clusters of biological processes were more than 5 fold increased in ESCC in contrast to 1 cluster in normal squamous epithelium. This indicates

that ESCC is a highly active epithelium in several types of processes, like cell adhesion, signal transduction, cell death, immune response and cell growth.

For diagnostic purposes it is important to find biomarkers that can specifically identify BE, for instance to differentiate Barrett epithelium from gastric cardia. In order to determine the specificity of CDX-2 and a set of CKs as specific markers for BE as compared to normal squamous esophageal and gastric cardia tissue, immunohistochemistry was performed on frozen tissue samples of BE, normal squamous esophagus and gastric cardia (**chapter 7**). Results showed that CK7, CK8, CK18 and CK20 are expressed in all BE samples. CDX-2 was seen in 87% of the BE cases, but in 26% of these cases CDX-2 expression was only visible in more than 10% of the cells. To distinguish between BE and normal squamous esophagus CDX-2 and a set of CKs can be used, between BE and gastric cardia a combination of CDX-2 and CK7 is most informative.

General Discussion

In the experimental studies described in this thesis, we tried to increase the knowledge of genes and signal transduction pathways that are involved in the metaplastic development of BE and esophageal cancer. Genetic changes that occur in BE are often specific to a particular stage of dysplasia and cause disruptions within signal transduction pathways that result in uncontrolled cell growth, differentiation and proliferation. High throughput techniques, like SAGE, microarray and pep-chip were used in this thesis in an attempt to gain insight into these pathways. The complex cross-talk between the different receptor-mediated signal transduction pathways with its numerous regulatory checkpoints, inputs and outputs can look intimidating and overwhelming. Even in a simple scenario of a single receptor's signal transduction pathway more than a million different combinations can result from just a few dozen inputs. The reality is messier, with outputs from one signalling pathway directly affecting the action of other receptors. Using the high through-put techniques a large amount of data was achieved and we tried to create an overview of the processes involved in BE. Both at transcriptome and kinome level a blueprint of BE was made, because knowledge of the fundamental mechanisms involved in the metaplastic

and dysplastic processes occurring in the esophagus is essential before new treatment, diagnostic and preventive strategies avoiding BE related adenocarcinoma can be developed.
Several studies in this thesis focussed on the metaplastic transformation of normal esophageal epithelium into BE. We showed on transcriptome and kinome level, that BE has strong correlation with normal squamous and gastric cardia epithelium, however BE is also an epithelium with its own specific characteristics. One of the BE specific genes, BMP4, was investigated in more detail and results showed that BMP4 plays an important role in the transdifferentiation of normal squamous epithelium to BE. It is known that inflammatory processes as occurring in the esophagus due to GERD, can up-regulate BMP4 expression. With the present observations we assume that BMP4 together with other environmental factors, induces phenotypical cellular changes of the normal squamous mucosa that finally results in a columnar type of mucosa.
SAGE analysis was as well performed to investigate which processes are involved in the two main types of cancers that occur in the esophagus. Phenotypically EA and ESCC are poles apart. Analysing their transcriptomes showed us important dissimilarities of both types of esophageal cancer at gene expression level. Future studies should aim at further improving our insight in the tumorigenesis of these cancers using tools a transcriptome and kinome analysis for investigating the different stages of dysplasia in the development of EA and ESCC. Eventually these transcriptomes have to be connected to the kinomes in order to make clear which actual processes are involved in the different stages of metaplasia and dysplasia.

High through-put techniques widen the scope of Barrett's esophagus

Gradually, the molecular basis necessary to develop BE and malignant transformation are becoming better understood. Biological and molecular staging of the different stages of BE and its associated EA will become increasingly important, also markers that predict the outcome of certain therapies and the likelihood of metastases or recurrences. In addition, the molecular pathogenesis of BE and EA is the basis for potential chemotherapeutic strategies. Using high through-put techniques for characterizing BE will certainly help to understand the molecular basis of BE. However since these techniques are expensive, time consuming and not practical for

standard lab tests the ultimate goal will be to find certain or a subset of markers and/or predictors that overcome this problem and can easily characterize the stage of the disease. On the other hand, we should keep in mind that over time one of the great promises of the high-through-put-techniques approach is that the ability to carry out comprehensive genomic analyses easily, accurately, rapidly and inexpensively with high sensitivity should also help to create a new generation of routine diagnostic and prognostic tools.

Samenvatting

Barrett oesofagus is de metaplastische verandering van normale plaveisel epitheel van de distale slokdarm in een cilindrisch type epitheel als gevolg van chronische langdurende reflux van de zure en gallige maaginhoud in de slokdarm. Patienten met een Barrett oesofagus hebben een significant verhoogde kans om slokdarm kanker te ontwikkelen. De laatste 3 decennia is de incidentie van Barrett oesofagus en de daarmee gerelateerde kanker het sterkst gestegen van alle kankers in de Westerse wereld. Ondanks al het onderzoek op het gebied van Barrett oesofagus is de biologische basis van deze mucosale transformatie in de slokdarm niet goed begrepen.

Het eerste gedeelte van dit proefschrift beschrijft de resultaten van gen expressie profiel analyses (gebruik makende van de techniek SAGE) waarin we Barrett epitheel vergelijken met normaal plaveisel epitheel en cardia epitheel van de maag (**hoofdstuk 2**). Er worden verschillende genen beschreven, die een rol kunnen spelen in de metaplastische transformatie van normaal plaveisel epitheel in een cilindrisch type epitheel. Interessant was dat als we Barrett oesofagus met normaal slokdarm weefsel of cardia epitheel van de maag vergeleken, bij beide vergelijkingen een vergelijkbare hoeveelheid genen verschillend tot expressie kwamen. Echter, als we normaal slokdarm weefsel met cardia van de maag vergeleken, vonden we twee keer zoveel verschillende genen. Dit laat zien dat Barrett epitheel overeenkomsten heeft met beide onderzochte epithelia en dat dit epitheel incompleet gedifferentieerd is. Daarnaast zijn er verschillende genen geïdentificeerd, zoals BMP4, TFF1, TFF2, TFF3, Annexin A10 en Galectin 4 die alleen tot expressie komen in Barrett oesofagus. In deze studie beschrijven we verder een uniek Cytokeratine expressie profiel voor ieder epitheel.

Eén van de genen die specifiek tot expressie komt in Barrett oesofagus, BMP4, is gebruikt voor verder onderzoek naar de rol van BMP4 en de BMP signaal transductie route in de metaplastische transformatie van normaal slokdarm epitheel naar een Barrett oesofagus (**hoofdstuk 3**). Hiervoor zijn weefsels van patiënten en een esophagitis-Barrett rattenmodel gebruikt. De resultaten laten zien dat in esophagitis en Barrett epitheel de BMP route is geactiveerd. Verder laten gekweekte plaveisel cellen geïncubeerd met BMP4 zien dat de BMP route door middel van upregulatie van ID2 en phosphorylering van Smad 1/5/8 is geactiveerd. Dit effect kan geblokkeerd worden door pre-incubatie met Noggin, een BMP4 antagonist. Vervolgens zijn de veranderingen in Cytokeratine patroon en gen expressie profielen onderzocht. De resultaten hiervan laten zien dat BMP4 incubatie een verschuiving in Cytokertine expressie patroon van een plaveisel specifiek patroon naar een Barrett specifiek patroon veroorzaakt. Op gen expressie profiel niveau was een verandering van 26% naar een Barrett gen expressie patroon zichtbaar. Deze resulaten bewijzen dat BMP4 een rol speelt in de ontwikkeling van een Barrett slokdarm.

Ondanks al het onderzoek met gen expressie profielen, zijn de signaal transductie routes die belangrijk zijn in een Barrett oesofagus niet goed begrepen. Daarom hebben we de cellulaire kinase activeit in Barrett slokdarm bestudeerd en deze vergeleken met de kinase activiteit profielen van normaal slokdarm epitheel en cardia epitheel van de maag (**hoofdstuk 4**). De activiteit van de MAP kinase signalerings route blijkt significant verlaagd te zijn in Barrett

epitheel in vergelijking met normaal slokdarm weefsel en cardia epitheel van de maag. Verder zien we dat de EGF receptor significant meer actief is in normaal slokdarm epitheel in vergelijking met Barrett epitheel, maar de EGF receptor is meer actief in Barrett epitheel dan in cardia epitheel van de maag. Een opvallende bevinding was de verhoogde glycolytische activiteit in Barrett in vergelijking met zowel normaal slokdarm en cardia weefsel van de maag.

Om specifiek die genen die belangrijk zijn in de epithiale cel laag van een Barrett oesofagus te onderzoeken, hebben we SAGE gebruikt om de gen expressie profielen van primaire celkweken van Barrett en normaal slokdarm epitheel te analyseren (**hoofdstuk 5**). Om de epitheliale cel laag te krijgen, zijn epitheel cellen van biopten van een Barrett en normaal slokdarm weefsel gekweekt met een speciek Barrett kweekmedium. De gen expressie profielen zijn vergeleken met elkaar en met de gen expressie profielen van een volledig Barrett en normaal slokdarm biopt dat niet alleen epitheel cellen bevat, maar ook stroma en ontstekingscellen. Genen die specifiek tot expressie kwamen in de Barrett epitheel cellen waren Lipocalin 2, SOX4, Cytokeratin 7, Galectin 7 en Cyclin D1. Annexin A10, TFF1 en TFF2 kwamen echter specifiek tot expressie in de Barrett biopten. Deze studie laat zien dat bepaalde genen specifiek tot expressie komen in de epitheel cellen, terwijl andere alleen in de biopten worden gevonden en waarschijnlijk door de stromale, niet epitheliale weefsel cellen, in het biopt tot expressie komen.

Ondanks al het onderzoek op slokdarm kanker gebied is de moleculaire basis die ten grondslag ligt van deze vorm van kanker onduidelijk. Daarom zijn gen expressie profielen van het slokdarm adenocarcinoom en plaveisel carcinoom ontwikkeld en vergeleken met de gen expressie profielen van respectievelijk Barrett oesofagus en normaal slokdarm weefsel (**hoofdstuk 6**). De transcriptoom analyse laat zien dat de moleculaire basis van deze kankers totaal verschillend is en dat beide weefsels hun eigen specifieke karakteristieken hebben. Genen die significant hoger tot expressie komen in het slokdarm adenocarcinoom dan in Barrett oesofagus, zijn SOX 4 en Lipocalin 2, terwijl de Trefoil factoren en Annexin A10 significant lager tot expressie komen in het slokdarm adenocarcinoom. De genen die significant hoger tot expressie komende in het plaveisel carcinoom in vergelijking met normaal slokdarm weefsel zijn BMP4, Cyclin D1, E-Cadherin en TFF3. Om een duidelijker beeld te krijgen van welke biologische gebeurtenissen een rol spelen in de ontwikkeling van een slokdarm adenocarcinoom en plaveiselcel carcinoom in vergelijking tot respectievelijk Barrett oesofagus en normaal slokdarm weefsel, hebben we de

genen die corresponderen tot de tags die significant verschillend zijn tussen de 2 weefsels geklusterd in groepen van biologische processen.

Interessant is dat in het slokdarm adenocarcinoom veel genen in de groepen van nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, cel deling, cel groei, reactie tot stimulus en signaal transductie vallen. Dit laat zien dat deze processen een grote rol spelen in het slokdarm adenocarcinoom. De kluster analyse van het plaveiselcel carcinoom in vergelijking met normaal slokdarm epitheel laat zien dat de groep cel-cel signalering 5 keer verlaagd is. Echter, de groepen cel cyclus, nucleobase/ nucleoside/ nucleotide & nucleic acid metabolism, metabolisme, cel deling en cel communicatie zijn allemaal meer dan 10 keer verhoogd in het plaveiselcel carcinoom. In meer detail, 13 belangrijke klusters van biologische processen waren meer dan 5 keer verhoogd in het plaveiselcel carcinoom, terwijl er maar 1 kluster verhoogd was in normaal slokdarm epitheel. Dit geeft aan dat het plaveiselcel carcinoom sterk actief is in verschillende processen, zoals cel adhesie, signaal transductie, cel dood, immuun reactie en cel groei.

Voor diagnostische doeleinden is het belangrijk om een goede biomarker te vinden die specifiek Barrett oesofagus kan identificeren, om bijvoorbeeld Barrett oesofagus van cardia epitheel van de maag te onderscheiden. Om de specificiteit van CDX-2 en een set van verschillende CKs als specifieke marker voor Barrett oesofagus te onderzoeken, hebben we gebruik gemaakt van immunohistochemie op weefsel secties van Barrett oesofagus, normaal plaveisel slokdarm en cardia epitheel van de maag (**hoofdstuk 7**). De resultaten laten zien dat CK7, CK8, CK18 en CK20 allemaal tot expressie komen in alle Barrett biopten, terwijl CDX-2 expressie maar in 87% van de Barrett slokdarm gevallen zichtbaar is. Hiervan is in 26% van de gevallen, CDX-2 expressie zichtbaar in meer dan 10% van de cellen. Hieruit blijkt dat CDX-2 en een set van CKs gebruikt kan worden om Barrett oesofagus van normaal slokdarm epitheel te onderscheiden. Om Barrett oesofagus van cardia epitheel van de maag te onderscheiden, is gebruik van CDX-2 en CK7 het beste.

Algemene discussie

In de experimentele studie beschreven in dit proefschrift hebben we geprobeerd de kennis van genen en signaal transductie routes die een rol spelen in de metaplastische ontwikkeling van Barrett oesofagus en slokdarm kanker te verhogen. Genetische veranderingen die zich voordoen in een Barrett oesofagus kunnen veranderingen in de signaal transductie routes teweeg brengen die kunnen leiden tot ongecontroleerde cel groei, differentiatie en proliferatie. In dit proefschrift is gebruik gemaakt van 'high through-put' technieken zoals SAGE, microarray en pep-chip. Het complexe netwerk van interacties tussen de verschillende receptor-gemedieerde signaal transductie routes met hun verschillende reguliere controlepunten, ingangen en uitgangen kunnen intimiderend en overweldigend overkomen. Zelfs een simpel voorbeeld van een signaal transductie route met één receptor met een paar dozijn verschillende ingangen, kan meer dan 1 miljoen verschillende combinaties opleveren. De werkelijkheid is complexer want de uitkomsten van één signaal transductie route kunnen een direct effect hebben op weer andere receptoren. Gebruik makend van de 'high through-put' technieken wordt een grote hoeveelheid data gegenereerd en hebben we geprobeerd een schema te maken van de verschillende processen die een rol spelen in een Barrett oesofagus. Zowel op transcriptoom als kinoom niveau hebben we een blauwdruk van de Barrett oesofagus gemaakt. Dit omdat de kennis van fundamentele mechanismes die belangrijk zijn in metaplastische en dysplastiche processen die voorkomen in de slokdarm, essentieel is voor de ontwikkeling van nieuwe therapeutische, diagnostische en preventieve strategiën om Barrett en de gerelateerde kanker tegen te kunnen gaan.

In dit proefschrift worden verschillende studies die gericht zijn op de transformatie van normaal slokdarm epitheel naar Barrett epitheel beschreven. We laten op transcriptoom en kinoom niveau zien dat Barrett oesofagus een epitheel is met specifieke eigenschappen. Er is echter ook een correlatie tussen Barrett oesofagus en normaal slokdarm epitheel en cardia epitheel van de maag. Eén van de gevonden Barrett specifieke genen, BMP4, is in meer detail onderzocht en de resultaten laten zien dat BMP4 een belangrijke rol speelt in de transdifferentiatie van normaal slokdarm epitheel naar Barrett. Door de ontsteking die ontstaat in de slokdarm als gevolg van reflux, kan BMP4 worden geproduceerd. BMP4 zorgt waarschijnlijk samen met andere factoren ervoor dat de plaveisel cellen in de slokdarm transdifferentieren naar Barrett type cellen.

Verder is, om de processen die een rol spelen in de neoplastische veranderingen in de slokdarm te onderzoeken, gebruik gemaakt van de SAGE techniek. Fenotypisch zijn het slokdarm adenocarcinoom en het plaveiselcel carcinoom zeer verschillend. Nadat we de gen expressie profielen van deze weefsels hadden geanalyseerd, zagen we dat ze op gen expressie niveau inderdaad significant verschillend zijn. Om een goed inzicht te krijgen in de tumorigenese van deze kankers, zou transcriptoom en kinoom onderzoek gedaan moeten worden naar alle verschillende stadia van dysplasie in de ontwikkeling naar het slokdarm adenocarcinoom of het plaveiselcel carcinoom. Uiteindelijk moeten de gen expressie profielen gekoppeld worden aan de kinomen om te onderzoeken welke specifieke processen een rol spelen in de verschillende stadia van metaplasie en dysplasie.

'High through-put' technieken verbreden de horizon van Barrett oesofagus

Langzaam aan wordt de biologische basis die ten grondslag ligt aan de ontwikkeling van een Barrett oesofagus en de maligne transformatie ervan duidelijk. De biologische en moleculaire factoren van de verschillende fases in de ontwikkeling van een Barrett oesofagus en het adenocarcinoom zullen belangrijker worden. Ook belangrijk zijn markers die de uitslag van bepaalde therapiën en de kans op uitzaaiingen en recidieven, voorspellen. De moleculaire bases van de Barrett oesofagus en het adenocarcinoom vormen de grondslag voor potentiële chemo-therapeutische strategiën. Het gebruik van 'high through-put' technieken om de eigenschappen van een Barrett oesofagus te omschrijven, zal in belangrijke mate bijdragen om deze moleculaire basis te ontdekken. Deze technieken zijn echter nog erg duur, tijd consumerend en niet praktisch voor standaard lab proeven. Daarom is het uiteindelijke doel om bepaalde of een groep van markers en predicatoren te vinden die kunnen helpen verschillende ziekte stadia makkelijk te kunnen onderscheiden. Aan de andere kant moeten we rekening houden met het feit dat de 'high through-put' technieken de genetisch vergelijkende analyses naar verloop van tijd makkelijker, precieser, goedkoper en met hoge sensitiviteit uitvoerbaar maken. Dit kan uiteindelijk leiden tot een nieuwe generatie van routine, diagnostische en prognostische mogelijkheden.

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De vriendengroep uit Roosendaal wil ik ook bedanken! We kennen elkaar al heel lang en zijn nu over heel (Zuid-)Nederland verspreid gaan wonen, gelukkig ook een paar in Utrecht, al was Neeltje weer snel vertrokken. Jullie hebben voor de nodige afleiding gezorgd met een spelletje Catan, een weekje skiën, een avondje stappen, een studentenhap of een avondje gourmetten. Roel & Maud, Jaimie & De Muf, Debbie & Erwin, Rick & Neeltje, Joost & Kirsa, Marianne, Vanessa, Sjoerd en Mangnus: zoals jullie zelf al zongen: 'één blik zegt genoeg voor die hechte vriendenploeg'.

In Roosendaal liggen m'n roots en stiekem hoort daar ook de familie Backx bij. Veel tijd hebben we doorgebracht aan de keukentafel al keuvelend of heftig discusiërend over van alles en nog wat. Bijvoorbeeld op zondagochtend, terwijl Kirby z'n rondje door het bos loopt en iedereen langzaam uit bed komt (of niet hè Lieke!), onder het genot van een gebakken ei met spek of een lekker Italiaans pesto-ham-kaas broodje. Bedankt voor de gezellige momenten en het tweepersoonsbed dat altijd klaar staat!

Pytrik (BP), m'n oudere broer, we deden altijd veel samen, van wedstrijdzwemmen tot en met de lego spelen, pingpongen en samen intro in Eindhoven vieren. Na de verbouwing zijn jullie inmiddels aan kant en kunnen jij en Karin van jullie stulp genieten. Ik hoop dat er nog veel gezellige momenten komen die waarschijnlijk bij jou tot hoofdpijn leiden!

Pa en ma, bedankt dat jullie mij altijd hebben gesteund en in me hebben geloofd! Toen het in 4 HAVO zó goed ging, lieten jullie aan mij de keuze om naar 5 VWO en vervolgens de UvA te gaan (al was pa niet echt blij dat z'n kleine meid naar het grote Amsterdam ging). Zonder jullie had ik hier niet gestaan en ik ben dan ook erg blij dat jullie bij de verdediging op de voorste rij zitten!

Last but not least: Jaap. Lieve Jaap, lekker ding! We zeggen altijd dat we nooit over werk praten; we houden beide van ons werk maar kunnen maar moeilijk voorstellen dat dat wat de ander doet nou echt leuk is. Ik weet zeker dat zonder jouw steun en toeverlaat deze 4 jaren anders waren geweest! Je bent mee gaan backpacken, we hebben een huis gekocht, zijn gaan samenwonen, op avontuur een jaartje in Chicago en binnenkort gaan we zelfs trouwen! Ik hou van je! Dikke kus!

Jantine

Curriculum Vitae (Nederlands)

De auteur van dit proefschrift werd geboren op 8 november 1978 te Roosendaal. In 1991 verliet ze de St. Joseph basisschool waarna ze in 1997 haar VWO diploma behaalde aan het Getrudiscollege te Roosendaal. Hierna begon ze haar studie Biomedische Wetenschappen aan de Universiteit van Amsterdam. Haar eerste wetenschappelijke stage heeft ze uitgevoerd op de afdeling Anatomie & Embryologie aan de Universiteit van Amsterdam. Onder begeleiding van Prof. Dr. W.H. Lamers heeft ze onderzoek gedaan naar de karaterisering van een arginine deficiënte muis. Haar literatuurstudie, over de rol van verschillende transcriptie factoren in het cholesterol metabolisme, heeft ze uitgevoerd op de afdeling Biochemie aan de Universiteit van Amsterdam. Haar tweede stage heeft ze uitgevoerd in het lab van het Unilever Health Instituut te Vlaardingen. Onder begeleiding van Dr. A.W. van Gorp heeft ze daar, gebruikmakende van RNA profiling, onderzoek gedaan naar de relatie tussen veroudering en hoge bloeddruk. Na het behalen van haar doctoraalexamen in 2001, is ze gaan reizen door Australië.

In maart 2002 is ze begonnen als assistent in opleiding bij de afdeling Laboratorium Experimentele Inwendige Geneeskunde aan het Academisch Medisch Centrum te Amsterdam.. Het onderzoek dat ze daar onder begeleiding van Prof. Dr. M.P. Peppelenbosch en Dr. K.K. Krishnadath heeft uitgevoerd, is in dit proefschrift beschreven. Doel van het onderzoek was het verkrijgen van meer inzicht welke genen een belangrijke rol spelen in de ontwikkeling van een Barrett oesofagus.

In juni 2006 is ze begonnen als post-doc op de Human Nutrition afdeling aan de University of Illinois at Chicago (VS). Met behulp van een fellowship van de Netherlands Genomics Initiative doet ze daar, onder begeleiding van Dr. G. Fantuzzi, onderzoek naar de rol van Adiponectin in de ontwikkeling van Crohn’s gerelateerde colon kanker gebruikmakende van gen expressie profiel analyses.

Curriculum Vitae (English)

The author of this thesis was born on November 8, 1978 in Roosendaal. In 1997, she graduated from high school at the Gertrudiscollege in Roosendaal. The same year she started studying Biomedical Sciences at the University of Amsterdam. She performed her first internship at the department of Anatomy & Embryology of the University of Amsterdam. Under supervision of Prof. Dr. W.H. Lamers she performed research on characterizing Arginine deficient mice. She wrote her Masters' Thesis with the subject 'Transcription factors in cholesterol metabolism' at the Biochemistry department of the University of Amsterdam. She performed her second internship at the Unilever Health Institute in Vlaardingen. Under supervision of Dr. A.W. van Gorp, she investigated the relationship between ageing and hypertension using RNA profiling. After finishing her Masters in 2001 she went backpacking through Australia.

In March 2002 she started as a PhD student at the Laboratory of Experimental Internal Medicine of the Academic Medical Center in Amsterdam. Under supervision of Prof. Dr. M.P. Peppelenbosch and Dr. K.K. Krishnadath she investigated the genes involved in the development of Barrett's Esophagus. The results of her research are described in this thesis.

In June 2006 she started as a post-doc at the Human Nutrition department of the University of Illinois at Chicago (USA). Under supervision of Dr. G. Fantuzzi and supported by a fellowship of the Netherlands Genomics Initiative, she performs research investigating the role of Adiponectin in the development of Crohn's related colon cancer using microarray analysis.

Notities