DIAGNOSTIC PATHOLOGY

Changes of microRNAs-192, 196a and 203 correlate with Barrett's esophagus diagnosis and its progression compared to normal healthy individuals

Pavla Luzna[1], Jan Gregar[2], Ivo Uberall[3], Lenka Radova[4], Vlastimil Prochazka[2] and Jiri Ehrmann Jr[1,3*]

Abstract

Background: Barrett's esophagus (BE) is a disease with a rising prevalence in western countries probably due to the unhealthy lifestyle. In significant number of cases it develops to esophageal adenocarcinoma. Two decades ago, important gene regulators (microRNAs) were discovered and their attendance in the process of malignant transformation was demonstrated (e.g. miR-192, 196a, 203). Our aim was to select the patients with the increased risk of malignant transformation before the cancer develops.

Methods: 71 patients with BE disease were selected, slides from FFPE blocks were prepared, the lesions were microdissected and a qPCR relative expression analysis for selected microRNAs (generally known to be connected with malignant transformation process) was carried out.

Results: We demonstrated unequivocal statistically significant upregulation of two microRNAs (miR-192, 196a) and downregulation of miR-203 and positive miR-196a correlation with progression from intestinal metaplasia to adenocarcinoma compared to normal individuals.

Conclusions: We hypothetize that there do exist changes of selected microRNAs which can undoubtedly distinguish the patients with BE from normal healthy individuals.

Keywords: microRNA, Barrett's esophagus, real-time PCR

Background

Barrett's esophagus

Barrett's esophagus (BE) is the gastrointestinal disorder termed after the physician Norman Barrett in the 1950s [1]. It is an infliction of a distal part of the esophageal mucosa. In physiological state, esophageal mucosa is lined by squamous stratified nonkeratinized epithelium. However, by the influence of several agents the squamous epithelium is being replaced by the columnar one, the phenomenon known as metaplasia [2]. Subjectively, the patient with BE can suffer from heartburn, eructation or nausea, nevertheless, sometimes BE can be asymptomatic [3,4]. Grossly, BE is classified on the basis of its length: long segment (afflicted part of the esophagus is longer than 3 cm), short segment (shorter than 3 cm), and ultra-short segment (which is not actually observed by the endoscopic examination) [5]. Microscopically, Barrett's esophagus was defined by the presence of goblet cells. Today, however, it is accepted that there are three histologic subtypes of columnar epithelium which are found near to the distal esophagus [6], whereas only one of them is characterized by goblet cells presence. The first type is the junctional type secreting mucus, the second one is the gastric type of columnar epithelia with the presence of parietal and chief cells and the third one called „specialized intestinal metaplasia" with the goblet cells presence which is the most proximal to the squamous epithelium [7].

* Correspondence: jiri.ehrmann@hotmail.com
[1]Department of Histology and Embryology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Hnevotinska 3, 775 15 Olomouc, Czech Republic
Full list of author information is available at the end of the article

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BE can progress through dysplasia stages (low grade and high grade dysplasias) and even to the esophageal adenocarcinoma (EAC) [8]. Thus, Barrett's esophagus presents a sequential model of carcinogenesis. The attention to the EAC diagnosis is outright because of its rising prevalence in western countries [9] and its extremely high mortality oscillating about 85% within 5 year period [10].

MicroRNAs

MicroRNAs form a large group of 18-22 nt short single-stranded RNAs with a high potential of gene expression regulation [11]. MicroRNAs were discovered as late as in 1993 by Victor Ambros and his colleagues in the worm *Caenorrhabditis elegans*. Successively, microRNAs were revealed across the plants and other animals including *Homo sapiens*. Nowadays, 1100 diverse microRNAs were identified in humans [12]. Due to their size and properties, they can create complementary bounds with high amount of mRNAs and therefore block the process of translation or to degrade the mRNA. It is estimated that 30% of all human genes are regulated by them.

It is important and fundamental to note, that their up and downregulation is correlated to various disorders including malignant tumors [13,14]. It is presumed that the huge group of microRNAs is the potential target for therapy of all sorts of diseases including malignant ones [15]. There are hundreds of studies monitoring levels of hundreds of microRNAs in malignant versus healthy tissues and hundreds of studies focused on the functional role of these molecules in the process of carcinogenesis [13,14,16-18].

Therefore the aim of this study was to identify a combination of selected microRNAs levels of which (compared to normal healthy individuals) would definitely characterize a patient with Barrett's esophagus diagnosis, even in spite of the fact that the histological examination is not clear. Further, we wanted to find out if a specific microRNA which would reflect the grading of the progression does exist. All the previous studies performed used usually frozen tissue samples and the number of the examined patients was low [19-22]. Nevertheless, archives of departments of pathology are offering enough biological material for the retrospective studies and it was confirmed that FFPE tissues are suitable for routine microRNA levels determination [23-27].

It is well known that the process of carcinogenesis is generally connected to major important genes involved in e.g. cell cycle, proliferation or inflammation. That's why we explored the miRBase and http://microRNA.org databases and searched for the concrete microRNAs which could impact the target genes from the selected groups. Due to our ongoing immunohistochemical studies we chose *IL-1beta* and *IL-8* genes, revised the literature and we found out that they could regulate the expression of miR-21 resp. miR-203 as well. According to the literature, miR-192 is tightly connected to the digestive system carcinomas and miR-196a was found to have a potential to influence the cell cycle inhibitor *p27*, and *p27* is mentioned as a colorectal carcinoma progression marker and pancreatic ductal adenocarcinoma biomarker, too. Simultaneously, we discussed our chosen miRNAs with present literature findings and finally decided to use four chosen microRNAs.

Methods

Tissue processing and staining

All the experimental and tissue handling approaches were approved by the Ethical Comittee of the University Hospital Olomouc. Bioptical specimens of esophageal mucosa were taken as a part of the regular endoscopic examination of the BE diagnosis by NBI (narrow band imaging) and AFI (autofluorescence). Immediatelly after the mucosa excision, the tissue was immersed into the Baker's solution and fixed for 24 hours. Consequently, after tissue processing it was embedded into paraffin. A routine H-E staining was performed and the grade of dysplasia was estimated. Consequently, a histochemical staining (PAS, Alcian blue) was performed according to the standard protocol.

Laser capture microdissection (LCM)

All the methodical accesses were held under RNAse free conditions. We examined 71 patients diagnosed for BE (12 BE diagnosed macroscopically by NBI - microscopically not confirmed, 20 BE without dysplasia, 27 with low grade dysplasia and 12 with high grade dysplasia/ adenocarcinoma). Since this is a retrospective study, the paraffin blocks originated from years 2006-2010. Paraffin blocks were cut for 5-6 μm thick slides which were mounted on the PET membrane-coated metal frame (before this the frame was exposed to 30 min of UV light, followed by poly-L-lysin coating in termostat for 30 minutes in 37°C). For better adhesion of the tissue the frames were put into the incubator for 30 minutes and 58°C. Concurrently, a routine H-E slide was prepared for better observation and orientation in the tissue architecture, because the slides used for microdissection have to be air-dried, thus cellular structures are worse recognizable.

The membrane coated slide with the mounted tissue was deparaffinized in two baths of xylene, 3 minutes each and consequently put into the 100% ethanol. Further, 95%, 75% and 50% ethanol solutions were used, 30 seconds each. Subsequently, the slides were stained by the cresyl violet solution for 1 minute, then washed by the rising concentrations of ethanol (50%, 75%, 95%

and 100%) and finally washed in xylene, air dried and stored under the silicagel. The following LCM process was performed afterwards immediately.

The PET coated metal frame was turned around and placed on the basic histological slide and put into the microdissector. The requested tissue area was marked and dissected by the UV laser. Immediately after the dissection, the microdissected tissue was collected by the adhesive cap of the special eppendorf tube. We used the *mmi CellCut*® (Molecular Machines & Industries, Zürich, Switzerland) microdissection instrument.

RNA extraction, multiplex reverse transcription, preamplification step

Immediately after LCM, the eppendorf tube with the tissue was filled by 100 μl of digestion buffer, freezed at -80°C or RNA was promptly isolated. Prior to the RNA isolation, protease digestion was performed for 3 hours in 50°C and 15 minutes for 80°C. RNA including the small microRNA fragments was extracted by the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion/Applied Biosystems/Life Technologies; Carlsbad, CA, USA) according to manufacturer's protocol. The elution volume was 60 μl and it was consequently spinned in a vacuum concentrator for 1,5 hour to reduce the final volume to approximately 20 μl. The concentration of RNA was measured by the NanoDrop spectrophotometer (Wilmington, DE, USA) and the yield was moving around 10 ng/μl. The isolated RNA was subjected to the reverse transcription and eventually stored at -80°C.

Every reverse transcription reaction contained the amount of 5 ng of total RNA isolated. The total volume of the reaction was 7,5 μl and it consisted of 3,5 μl of master mix (0,075 μl 100 mM dNTPs with s dTTP; 0,5 μl MultiScribe™ Reverse Transcriptase, 50 U/μl; 0,75 μl 10× Reverse Transcription Buffer; 0,095 μl RNAse Inhibitor, 20 U/μl; 2,08 μl DEPC water), all from TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems/Life Technologies; Carlsbad, CA, USA); 2,5 μl RNA and 1,5 μl RT stem-loop RT-primer pool (10 RT-primers, concentration 0,5× each: RNU44, RNU48, RNU66, RNU6B, U47, miR-16, miR-21, miR-192, miR-196a, miR-203) (all from Applied Biosystems/Life Technologies; Carlsbad, CA, USA). The reverse transcription reaction proceeded according to the protocol recommended by the manufacturer (30 minutes in 16°C, 30 minutes in 42°C, 5 minutes in 85°C). For the signal transduction the preamplification step was inserted according to [28].

Real time PCR assay, PCR efficiency, endogenous control selection

The total volume of the qPCR reaction mixture was 10 μl. The mixture consisted of 5 μl of the TaqMan® 2× Universal Master Mix II No AmpErase UNG, 2 μl of nuclease free H_2O, 0,5 μl of single TaqMan® microRNA assay (all from Applied Biosystems/Life Technologies; Carlsbad, CA, USA) and 2,5 μl of the preamplification product. The amplification step was designed according to the manufacturer's protocol [95°C for 10 minutes, 45 cycles (95°C for 15 seconds, 60°C for 60 seconds)]. All the qPCR reactions were performed in the *LightCycler*® 480 System (Roche Applied Science, Penzberg, Germany).

For the efficiency of the qPCR reaction the First-Choice® Stomach RNA (Ambion/Applied Biosystems/Life Technologies; Carlsbad, CA, USA) was used because BE is a glandular type of metaplasia. Normal glands are found in stomach which is simultaneously the examined area during the esophageal endoscopy. So, the efficiency of PCR reaction should be verified in the glandular tissue.

The qPCR reaction for the chosen microRNA assay was adjusted for the 5 RNA template concentrations. The preamplification step was inserted as well. All the volumes were the same as for the regular qPCR reaction. After the reaction run, the efficiency was counted by the plotting the Cp value towards the log of the RNA concentration. The efficiency was presented by the slope of the axis.

Each qPCR reaction was performed for 6 potential endogenous controls (RNU44, RNU48, RNU66, RNU6B, U47, miR-16) according to the most cited reference genes in literature [20,29]. The most suitable two endogenous controls were assessed by the free downloadable application geNorm. The latter calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of reference genes [30,31].

Statistical analysis

The results of the study were processed by the biostatistician. The Kruskal-Wallis test with the multiple comparison was used for the p value formulation. In the regression relations (grading compared to other chosen parameters) the test of the coefficient significance in the regression model was used [32].

Fold changes

The results of the experiments are presented as the x-fold change. It is the ratio of the relative gene expressions (relative expression of the examined gene in the sample and relative expression of this gene in the calibrator - normal healthy esophagus). The relative gene expression is the expression of the target gene divided by the expression of the reference (housekeeping) gene. The number of the fold expresses how many times is the relative expression of the target gene higher or

lower than the relative expression of the same gene in the calibrator. The x-fold is expressed by formula [33]:

$$\text{ratio} = \frac{(E_{Ref})^{Cp\,sample}}{(E_{target})^{Cp\,sample}} \div \frac{(E_{Ref})^{Cp\,calibrator}}{(E_{target})^{Cp\,calibrator}}$$

cp = threshold point
E = efficiency of the qPCR reaction
sample = Barrett's esophagus in any grade
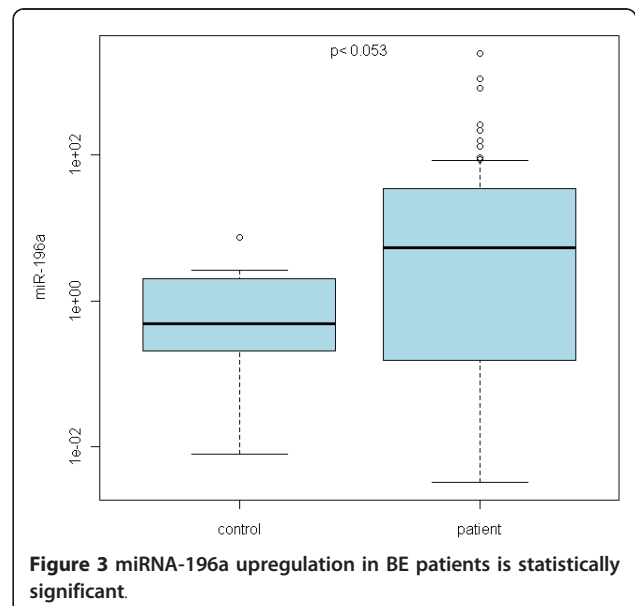calibrator = normal healthy esophageal epithelium

On the basis of geNorm, reference genes RNU44 and U47 were used and all the x-fold changes are relativized to their geometrical mean.
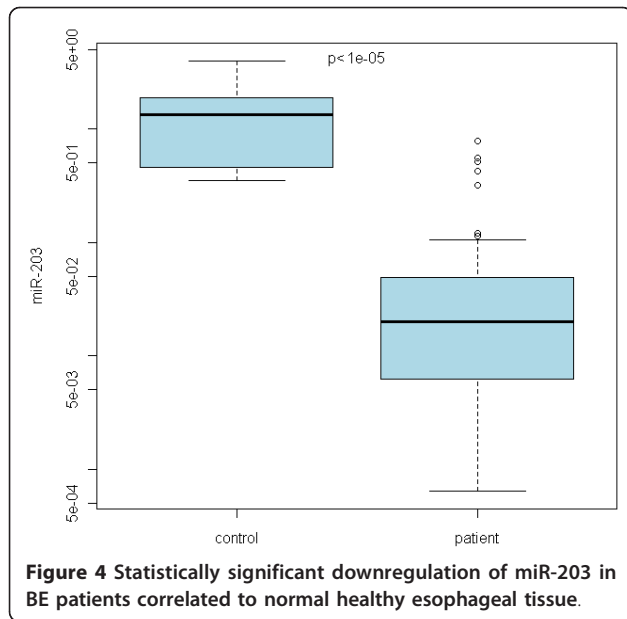
Results and discussion

In the case of miR-21, no statistically significant differences between Barrett's esophagus and normal epithelia were acquired (see Figure 1). Refering to miR-192, there is a statistically significant upregulation of miR-192 expression in any grade of the disease with the p < 0,00001 (see Figure 2). miR-196a expression shows the upregulation as well with p < 0,05 (see Figure 3). On the other hand, miR-203 is definitely downregulated in the impaired tissue with the p < 0,00001 (see Figure 4). In the case of miR-196a, there is a statistically significant (p < 0,005) trend in relative expression dependent on the disease grading (see Figure 5). The results acquired from the analysis of disease grading did not confirm the dependency on sex, hiatal hernia, obesity, smoking, alcohol abuse, family anamnesis of any cancer, each of this risk factor being estimated separately (Pearsons Chi-Quadrat Test with Monte Carlo Simulations). Kruskal-

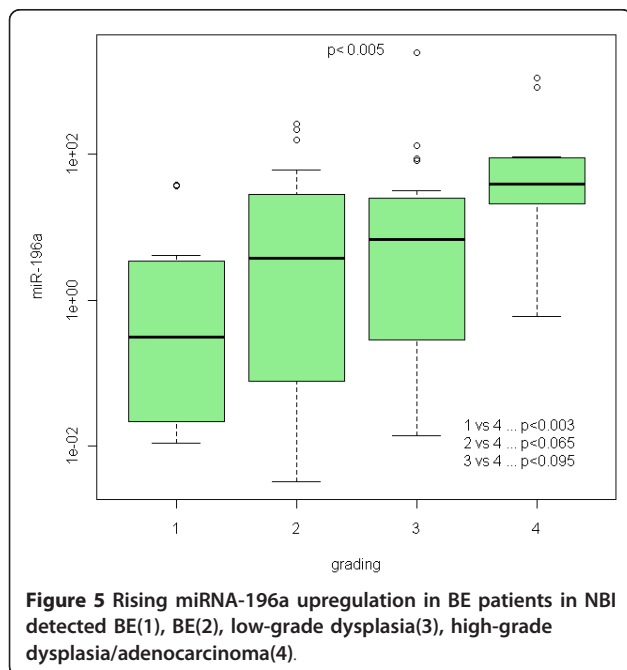**Figure 2 miRNA-192 statistically significant upregulation in BE patients**.
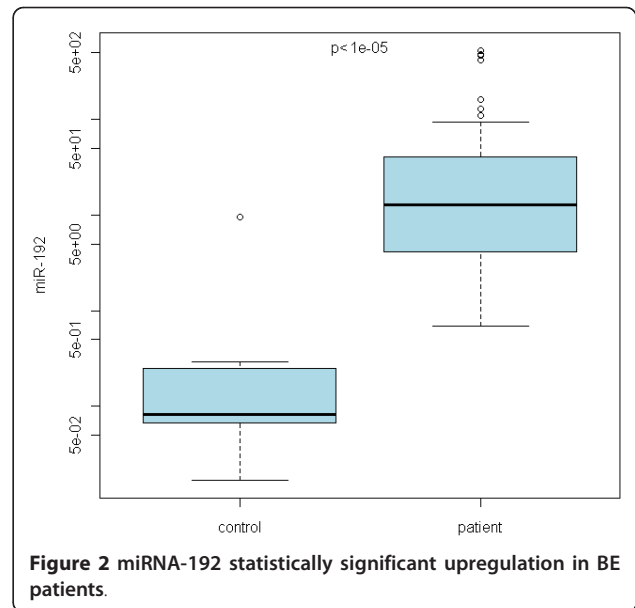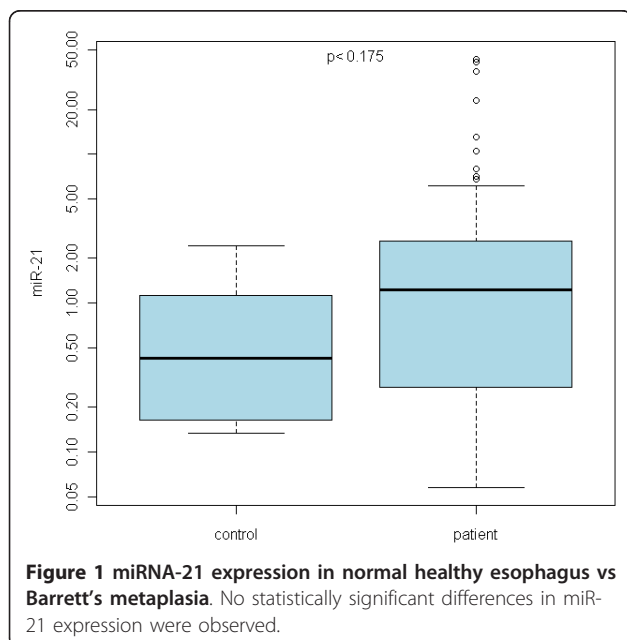
Wallis Chi-Quadrat Test did not confirm the correlation of the grading versus age of patients as well as it did not confirm the correlation of the grading versus alcohol and smoking (every risk factor considered separately). However, correlation of grading versus hiatal hernia, obesity, smoking, alcohol abuse, family anamnesis (all parameters considered together) has confirmed that there are statistically significant parameters for alcohol (p < 0,046) and smoking (p < 0,065). Further, the correlation of grading to all the previous parameters plus sex and age shows statistically significant values of the influence of sex (p < 0,008), age (p < 0,065), smoking (p < 0,02), and alcohol abuse (p < 0,0045).

Figure 1 miRNA-21 expression in normal healthy esophagus vs Barrett's metaplasia. No statistically significant differences in miR-21 expression were observed.

Figure 3 miRNA-196a upregulation in BE patients is statistically significant.

Figure 4 Statistically significant downregulation of miR-203 in BE patients correlated to normal healthy esophageal tissue.

It is supposed that Barrett's metaplasia arises on the basis of chronic inflammation [34,35] which originates as a reaction to the gastroesophageal reflux disease (GERD). GERD occurs when the acid content of the stomach returns to the distal part of the esophagus. The continuous irritation of the esophageal squamous stratified nonkeratinized epithelium induces its replacement by the columnar one which is less mechanically resistant but it resists to the low pH of the esophageal microenvironment. Connection between BE and GERD was well

Figure 5 Rising miRNA-196a upregulation in BE patients in NBI detected BE(1), BE(2), low-grade dysplasia(3), high-grade dysplasia/adenocarcinoma(4).

established in the 1970s [6,36]. However, when there is GERD in the esophagus, BE must not be necessary present. GERD is not the only risk factor for development of BE. It is the presence of hiatal hernia, obesity (visceral fat), smoking or alcohol abuse as well. Moreover, men are three times more in danger than women. O'Riordan et al. [34] demonstrated that in the inflammation - BE - EAC sequence the levels of proinflammatory cytokines IL-8 and IL-1β increase. Thus, interleukins seem to be one possible target of the therapeutic intervention in the future. Another possible target of BE therapy could be the microRNAs - short single-stranded molecules with high potential of gene regulation.

It is well known that many microRNAs are deregulated in the connection with malignant progression. Changes in microRNA-196a expression are cited in connection with both pancreatic adenocarcinoma and breast carcinoma as well as in Barrett's esophagus. In combination with another miRNA (miR-217), miR-196a level can help distinguish between malignant and benign pancreatic tissue [37]. Moreover, in long-term survival adenocarcinoma patients (24 months), the levels of miR-196a are conversely correlated with survival [38]. As well as Mathé et al. [21], we also revealed that microRNA-196a is definitely overexpressed in Barrett's esophagus of any grade compared to normal healthy esophageal tissue, even the statistical analysis shows that the trend of the rising expression of this microRNA during the BE progression does exist. Moreover, compared to [20] (11 FFPE patients), we demonstrated this fact in our study with 71 patients in 4 gradings of BE.

In the case of miR-21, it was published, that six solid tumor tissues (lung, breast, stomach, prostate, colon, pancreas) where miR-21 is the only overexpressed microRNA do exist [39]. Feber et al. [19] describes the overexpresson of miR-21 and downexpression of miR-203 in his experiments with Barrett's esophagus as well as with esophageal adenocarcinoma. In the paper from Saini et al. [40], microRNA-203 is described as a anti-metastatic small RNA with the possible therapeutic intervention. Our results suggest the unequivocal miR-203 downregulation in BE, but non-unequivocal miR-21 expression. Compared to our study, Feber performed his experiments for 35 frozen specimens without the micro-dissection step. The same conclusion (for miR-21 and miR-203) can be find in the paper from Mathé et al. [22] who compared miRNA expression profiles of eso-phageal adenocarcinoma and squamous cell carcinoma to the adjacent noncancerous tissue pairs. In addition, Mathé et al. [22] describe the elevation of levels of miR-192 in esophageal adenocarcinoma. In our set of patients, we have come to the same conclusion.

Dealing with the possibility of getting the tissue samples through all stages of BE progression in one

individuality brings controversial situation. We succeed in obtaining of tissue samples from all stages of BE progression in one patient's case, however we have to point out that the NBI technique, which we are using in the endoscopic examination helps to vizualize the architecture of the BE lesion and gives us the most precise picture of the stage of potential dysplasia and if there are any indications of proliferation to higher stage of BE dysplasia, it is therapeutically intervened. Our aim is not to let the patient to escalate to HGD/EAC stage which is very difficult to cure.

Most of the gene expression studies cited deal with relative expression data, but not all of them deal with the endogenous controls. Nonn et al. [28] use the normalization of the results to two chosen small nucleolar RNA RNU44 and RNU48, Maru et al. [21] use miRNA-16 as the reference gene. In his Application Note (Applied Biosystems/Life Technologies; Carlsbad, CA, USA) Wong et al., 2007 describe all the applicable endogenous controls suitable for microRNA data normalization, however with the necessity of verification. That's why in this paper six possible endogenous controls were chosen and the most stable couple of them was chosen for relative expression data calculation.

Conclusions

In our study, we showed that there possibly do exist such combinations of microRNA expressions of which may be able to give us an information about the patient's status and possible disease progression. Expression of microRNA-196a was unequivocally confirmed as the Barrett's esophagus molecular marker with the rising trend through the disease progression. In combination with expression of microRNA-192 and microRNA-203 the patient could be independently diagnosed and his diagnosis could be confirmed in spite the fact that the BE diagnosis is not supported by the histological examination.

List of abbreviations used

AFI: autofluorescence; BE: Barrett's esophagus; cp: threshold point; DEPC: diethylpyrocarbamate; dNTPs: deoxynucleotide triphosphates; dTTP: deoxythymidine triphosphate; E: efficiency; EAC: esophageal adenocarcinoma; FFPE: formalin fixed paraffin embedded; GERD: gastroesophageal reflux disease; H-E: hematoxylin-eosin; IL: interleukin; LCM: laser capture microdissection; miR: microRNA; mRNA: mediator ribonucleic acid; NBI: narrow band imaging; nt: nucleotide; PAS: periodic acid Schiff; PCR: polymerase chain reaction; PET: polyethylene terephthalate; qPCR: quantitative polymerase chain reaction; RNA: ribonucleic acid; RT: reverse transcription; UV: ultraviolet

Author details

[1]Department of Histology and Embryology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Hnevotinska 3, 775 15 Olomouc, Czech Republic. [2]Department of Internal Medicine II - Gastroenterology and Hepatology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, I.P. Pavlova 6, 779 00 Olomouc, Czech Republic. [3]Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Hnevotinska 3, 775 15 Olomouc, Czech Republic. [4]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Hnevotinska 3, 775 15 Olomouc, Czech Republic.

Authors' contributions

PL: participated in the design of the study, carried out the molecular studies, carried out histological stainings, JG: performed the endoscopical examination of patients, collected tissue samples and patient's history, IU: carried out the molecular studies, LR: carried out the statistical and correlation analyses, VP: conceived the study and participated in its design, collected tissue samples and patient's history, JE: conceived the study and participated in its design, evaluated endoscopical biopsies
All authors read and approved the final manuscript.

Competing interests

The autors declare that they have no competing interests.

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