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BRIEF ARTICLE

miR-200 family expression is downregulated upon neoplastic progression of Barrett's esophagus

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Abstract

AIM: To investigate miR-200 family expression in Barrett's epithelium, gastric and duodenal epithelia, and esophageal adenocarcinoma.

METHODS: Real-time reverse transcriptase-polymerase chain reaction was used to measure miR-200, *ZEB1* and *ZEB2* expression. Ingenuity Pathway Analysis of miR-200 targets was used to predict biological outcomes.

RESULTS: Barrett's epithelium expressed lower levels of miR-141 and miR-200c than did gastric and duodenal epithelia ($P < 0.001$). *In silico* analysis indicated roles for the miR-200 family in molecular pathways that distinguish Barrett's epithelium from gastric and duodenal epithelia, and which control apoptosis and proliferation. All miR-200 members were downregulated in adenocarcinoma ($P < 0.02$), and miR-200c expression was also downregulated in non-invasive epithelium adjacent to adenocarcinoma ($P < 0.02$). The expression of all miR-200 members was lower in Barrett's epithelium derived high-grade dysplastic cell lines than in a cell line derived from benign Barrett's epithelium. We observed significant inverse correlations between miR-200 family expression and *ZEB1* and *ZEB2* expression in Barrett's epithelium and esophageal adenocarcinoma ($P < 0.05$).

CONCLUSION: miR-200 expression might contribute to the anti-apoptotic and proliferative phenotype of Barrett's epithelium and regulate key neoplastic processes in this epithelium.

Key words: miRNA; Barrett's esophagus; Esophageal adenocarcinoma; miR-200; Epithelial to mesenchymal transition; Apoptosis; Proliferation; Epithelium

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INTRODUCTION

Barrett's esophagus is characterized by the replacement of the normal stratified squamous esophageal epithelium

with metaplastic columnar epithelium. Metaplasia to Barrett's esophagus is probably an adaptive response to the insult from reflux of gastric acid and duodenal bile salts into the esophagus, which occurs in chronic gastroesophageal reflux[1]. Barrett's esophagus epithelium possesses secretory and absorptive cell types, and these closely resemble those found in normal gastric and intestinal epithelia[2,3]. mRNA expression profiling studies confirm its similarity to gastric and duodenal epithelia[4-6]. However, Barrett's esophagus epithelium also expresses a specific cluster of genes, including those associated with alterations in cell cycle/proliferation, apoptosis, stress response, and cellular migration pathways, and these distinguish it from all related gastrointestinal mucosae[4]. Other studies have confirmed unique phenotypic characteristics of Barrett's epithelium that correspond with this specific gene expression cluster. For example, unlike gastric and duodenal epithelia, cellular proliferation in Barrett's esophagus continues along the upper crypt and at the luminal surface, possibly due to abnormal cell cycle entry or exit[7]. Furthermore, Barrett's esophagus epithelium expresses unusually high levels of anti-apoptotic proteins[8] and can mount a unique anti-apoptotic and proliferative response to reflux[9-12].

Barrett's esophagus is clinically important because it is the only visibly identifiable precursor to esophageal adenocarcinoma[13]. Progression to dysplastic stages involves increased abnormalities in the cell cycle and overall proliferation[13]. Neoplastic progression commonly occurs without obvious symptoms, and at the time of diagnosis, most patients with esophageal adenocarcinoma have local invasion or metastases[14]. Several lines of evidence suggest that epithelial to mesenchymal transition is required for local invasion and metastasis[15]. Epithelial to mesenchymal transition involves inhibition of E-cadherin expression and transition from epithelial to fibroblastic cell type, with associated alterations in cellular adhesion and migration[15]. Other studies have presented immunohistochemical, gene expression and cell line data that suggest a role for epithelial to mesenchymal transition in esophageal adenocarcinoma[16,17].

miRNAs downregulate target gene expression at the post-transcriptional level through the binding of their "seed" sequences with complementary sites in the 3'-untranslated region of target mRNAs[18]. The miR-200 family of miRNAs (miR-141, 200a, 200b, 200c and 429) are key regulators/inhibitors of epithelial to mesenchymal transition, and act to maintain the epithelial phenotype by targeting the expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2[19-21]. Accordingly, the number of studies reporting downregulation of miR-200 family expression in cancer is increasing[19-25]. In addition, members of the miR-200 family have recently been shown to affect other cell behaviors including proliferation, cell cycle and apoptosis[26-28].

Given the unique gene expression profile and cellular behavior in Barrett's esophagus epithelium, the phenotypic features that characterize its neoplastic progression, and the potential relevance of epithelial to mesenchymal transition to esophageal adenocarcinoma, we sought to determine the expression of miR-200 family members in gastric, duodenal and Barrett's esophagus epithelium, and to assess their expression with neoplastic progression of Barrett's esophagus. We hypothesized that Barrett's esophagus epithelium may possess a miR-200 expression profile different to gastric and duodenal epithelia, and that downregulation of miR-200 family expression may occur upon progression to esophageal adenocarcinoma.

MATERIALS AND METHODS

Tissue collection and processing

Tissues from patients diagnosed with either Barrett's esophagus (n = 17) or esophageal adenocarcinoma (n = 20) were collected at endoscopy or after surgical resection. The clinical research ethics committees of Flinders University and Erasmus Medical Centre approved the protocol for this study. Details of the collection process, information about the clinical characteristics of the patients, and RNA isolation from tissues have been published in full elsewhere[29]. In brief, endoscopic biopsy samples were obtained from the second part of the duodenum, proximal stomach, and distal esophagus. All biopsies were immediately stored in RNAlater (Ambion, Austin, TX, USA) and frozen at -20℃ until required. All biopsy samples used in this study were collected from the most distal level of endoscopically visualized Barrett's esophagus epithelium, which was confirmed by concurrent histopathology to be from columnar mucosa with intestinal metaplasia. In individuals with esophageal adenocarcinoma, a similar biopsy collection protocol was used for endoscopic biopsy. Samples were obtained from the second part of the duodenum, proximal stomach and the adenocarcinoma. Samples from surgical resection specimens were obtained from the normal upper stomach, and the tumor site, and immediately stored in RNAlater (Ambion) and frozen at -20℃ until required. If any Barrett's esophagus epithelium was present proximal to an esophageal adenocarcinoma, this was also sampled using the same protocols. Samples from patients with adenocarcinoma of the esophagus were always obtained before any neoadjuvant chemotherapy or radiotherapy was commenced, if clinically indicated.

The stored endoscopic biopsies and resection tissues were thawed in RNAlater as required. Thirty percent of each endoscopic biopsy sample, or a small portion of the resection samples, was dissected from the thawed tissue sample, fixed in formalin, embedded in paraffin, and processed for conventional histopathology. This was done to confirm that the biopsy contained only the appropriate tissue type. The remaining tissue had any remaining RNAlater removed, and was then processed in Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction. RNA was also extracted from cell lines derived from benign Barrett's esophagus (Qh) and high grade dysplastic (Ch and Gi) epithelium[30].

Quantitative reverse transcriptase-polymerase chain reaction analysis of miR-200 family, ZEB1 and ZEB2 expression

miR-200 expression was determined using commercially available TaqMan® miRNA assays specific for each member of the miR-200 family (Applied Biosystems, Foster City, CA,

Table 1 Relative miRNA expression in Barrett's esophagus, gastric and duodenal mucosal tissues

MiRNA	Duodenal (n = 10)	Barrett's esophagus (n = 17)	Gastric (n = 15)	P value (Kruskal-Wallis test)
miR-141	0.076 (0.039, 0.167)	0.026 (0.023, 0.036)	0.051 (0.042, 0.092)	0.0002[1,2]
miR-200a	0.148 (0.067, 0.340)	0.148 (0.126, 0.177)	0.247 (0.154, 0.509)	0.0314[2]
miR-200b	0.796 (0.606, 1.276)	0.833 (0.750, 0.993)	1.233 (1.089, 1.963)	0.0011[2]
miR-200c	2.700 (1.890, 3.511)	1.049 (0.929, 1.170)	2.335 (1.792, 2.773)	< 0.0001[1,2]
miR-429	0.095 (0.042, 0.070)	0.070 (0.061, 0.087)	0.078 (0.072, 0.153)	0.259

Relative expression for each epithelial tissue type. Relative expression values are median (95% CI). The group P value was the result of a Kruskal-Wallis test across the three tissue groups. Significant differences were identified by post hoc testing by the Holm-Bonferroni method for: [1]Duodenal versus Barrett's esophagus mucosa - miR-141 (P = 0.0008) and miR-200c (P < 0.0001); [2]Gastric versus Barrett's esophagus mucosa - miR-141 (P = 0.0004) miR-200a (P = 0.0078), miR-200b (P = 0.0001), and miR-200c (P < 0.0001).

USA). *ZEB1* and *ZEB2* mRNA expression was assessed using the Quantiscript® RT kit for reverse transcription and the Quantitect® SYBRGreen mastermix for polymerase chain reaction (PCR). Primer details are available upon request. miRNA expression was normalized using RNU44, and mRNA expression was normalized using 18S rRNA. Data were analyzed quantitatively using Q-Gene software[31]. Apparent differences in gene expression between the tissues were assessed for statistical significance using the Kruskal-Wallis test (significance cut-off $P < 0.05$). If significance was reached for this analysis, then the *post hoc* Holm-Bonferroni test was used for pairwise comparisons. Statistical testing was performed using Microsoft Excel. Spearman rank order correlation tests between miRNA and mRNA expression were conducted on-line (http://www.wessa.net/rankcorr.wasp). In addition to miR-200 expression, we also tested miR-215 because we have previously demonstrated downregulation of this miRNA in esophageal adenocarcinoma[29], and it was recently shown to target *ZEB2* directly[32].

miRNA target prediction and pathway analysis

Target prediction using miRecords (http://mirecords.biolead.org/)[33] and a core analysis using Ingenuity Pathway Analysis (www.ingenuity.com) were combined to elucidate possible implications of reduced miRNA expression. Predicted targets used in this analysis were required to be predicted by at least five databases in the miRecords search engine. Ingenuity Pathway Analysis parameters were set to assess a knowledge base derived from direct and indirect associations between genes in human experiments, and also in epithelial cell lines. In the Ingenuity Pathway Analysis, genes are grouped according to function and are allocated to top associated networks and cellular functions. Ingenuity Pathway Analysis uses a right-tailed Fisher's exact test to assign P values to each grouping, testing each result against a result from random groups of input predicted genes. Networks and cellular functions are ranked according to their score with the highest scoring networks representing the greatest statistical significance.

RESULTS

miR-200 family expression analysis in Barrett's esophagus, gastric and duodenal epithelia

Taqman Quantitative reverse transcriptase-PCR (qRT-PCR) revealed that expression levels of miR-141 and 200c were significantly lower in Barrett's esophagus epithelium compared with gastric and duodenal epithelia (Table 1). miR-200a and miR-200b expression was significantly lower in Barrett's esophagus epithelium than in gastric epithelium, but did not differ in expression between Barrett's esophagus and duodenal epithelia. The expression level of miR-429 was not significantly different across these three epithelial types.

Predicted implications of reduced miR-200 family expression in Barrett's esophagus

Core Ingenuity Pathway Analysis on predicted targets of miR-141 and miR-200c were performed to determine the probable biological effects of their reduced expression. The collection of target predictions from multiple algorithms, presented in miRecords, listed 272 gene targets for miR-141 and 429 targets for miR-200c. The top associated biological networks for these targets are illustrated in Figure 1, and the biological functions associated with these targets are listed in Table 2.

miR-200, ZEB1 and ZEB2 expression in Barrett's esophagus and esophageal adenocarcinoma

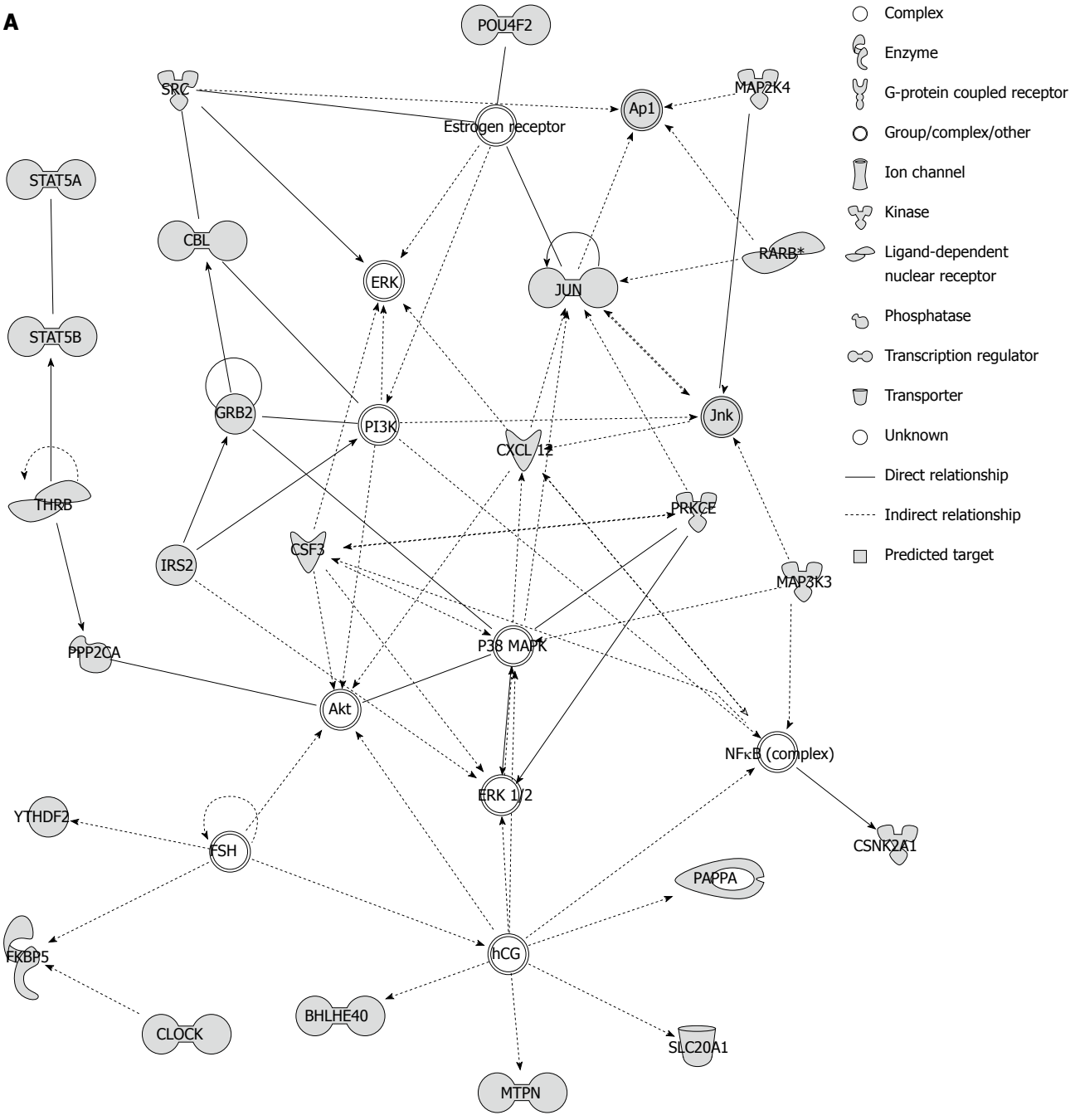
Expression of all members of the miR-200 family was significantly lower in esophageal adenocarcinoma compared with Barrett's esophagus epithelium (Table 3). The median expression of all miR-200 family members was lower in Barrett's esophagus epithelium proximal to esophageal adenocarcinoma than in Barrett's epithelium from patients without cancer or dysplasia. However, after *post hoc* analysis, only the difference in miR-200c expression was statistically significant. To determine whether this could indicate downregulation of miR-200 expression in dysplasia, prior to the development of adenocarcinoma, miR-200 family expression was further assessed in a non-dysplastic Barrett's esophagus derived cell line (Qh), and two cell lines derived from Barrett's esophagus with high-grade dysplasia (Ch, Gi). Figure 2 shows that the expression of all miR-200 members was markedly reduced in both dysplastic cell lines compared to expression in the benign cell line.

To determine whether the miRNA switch for epithelial to mesenchymal transition might be active in esophageal adenocarcinoma development, we also assessed the mRNA expression of miR-200 targets *ZEB1* and *ZEB2*.

Table 2 Top molecular and cellular functions of miR-200 gene targets

miRNA	Molecular and cellular functions	Molecules involved	*P* value
	Cell cycle	41	1.90E-07-2.82E-02
	Gene expression	53	4.94E-07-2.82E-02
miR-141	Cellular movement	39	2.45E-05-2.82E-02
	Cellular assembly and organization	61	2.88E-04-2.82E-02
	Cellular growth and proliferation	68	2.88E-04-2.28E-02
	Gene expression	84	1.37E-09-2.32E-02
	Cellular growth and proliferation	108	5.65E-05-2.32E-02
miR-200c	Cell cycle	53	9.17E-05-2.32E-02
	Cell death	95	9.62E-05-2.32E-02
	Cellular assembly and organization	55	1.19E-04-2.32E-02

The top five functions associated with the predicted targets of miR-141 and miR-200c, as determined by Ingenuity Pathway Analysis, are shown in this table. This analysis used a right-tailed Fisher's exact test to calculate the probability (P value above) that each cellular function ascribed to the predicted target gene list was due to chance alone. For each miRNA, the molecular and cellular functions are ranked according to their *P* value.



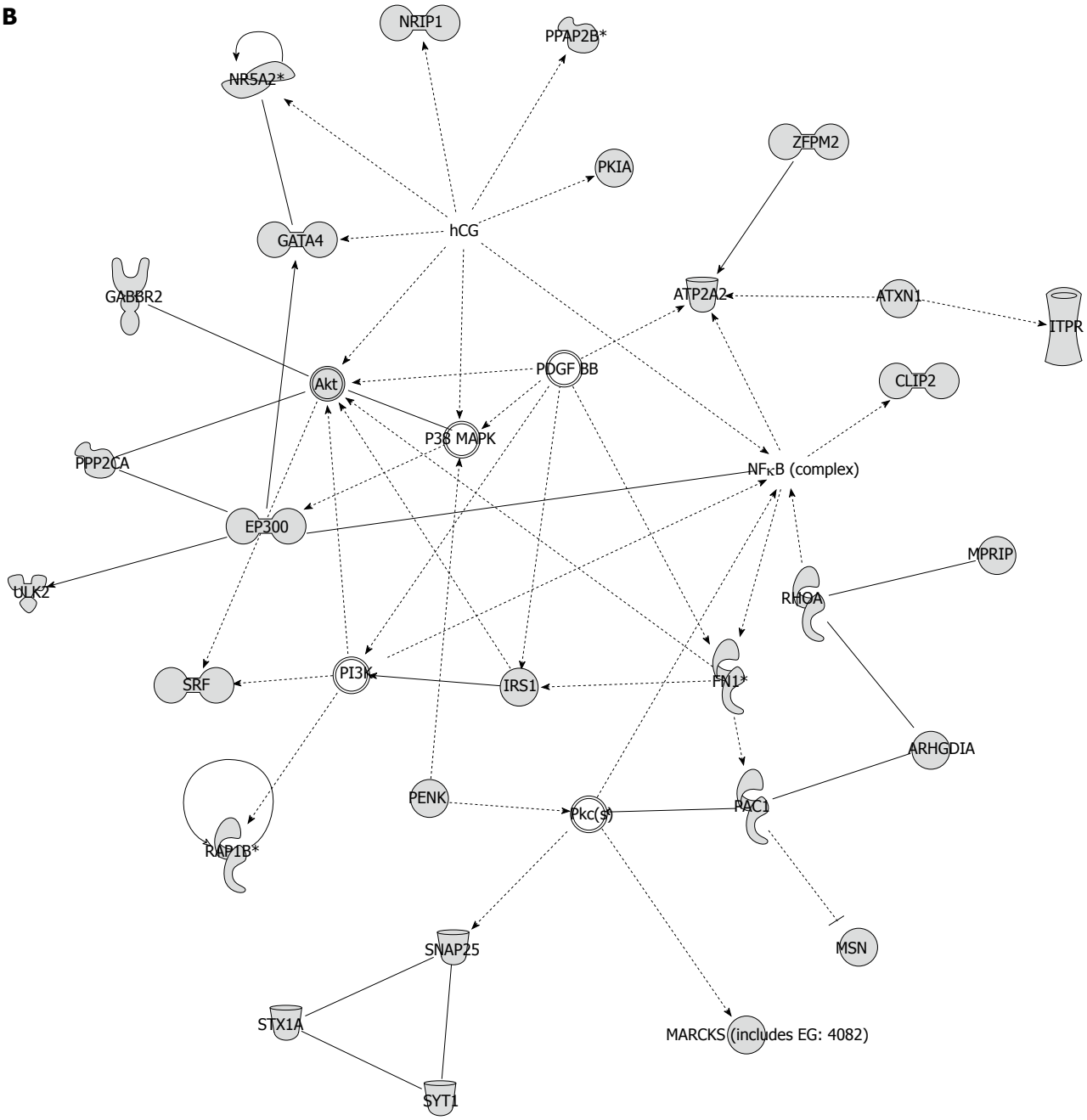


Figure 1 Top associated network functions for miR-141 and miR-200c. A: Ingenuity Pathway Analysis predicted that the top associated network functions for miR-141 were gene expression, cell death and cell cycle ($P = 1 \times 10^{-35}$). B: The top associated network functions for miR-200c were cell morphology, cellular assembly and organization, cellular function and maintenance ($P = 1 \times 10^{-38}$). Predicted targets of miR-141 or miR-200c are highlighted in grey. Uncolored entries represent molecules that are associated with the pathway but are not predicted miR-141 or miR-200c targets. The P values were derived from a right-tailed Fisher's exact test to calculate the probability that each predicted miRNA target matches the ascribed network function due to chance alone.

ZEB1 and *ZEB2* expression was significantly higher in esophageal adenocarcinoma compared to Barrett's esophagus epithelium from patients without cancer or dysplasia (Table 4). There were significant inverse correlations between the expression of *ZEB1*/*ZEB2* and the expression of some miR-200 members. miR-215 and *ZEB2* expression were inversely correlated (Table 4).

DISCUSSION

We found that miR-141 and miR-200c were expressed at lower levels in Barrett's esophagus epithelium, compared to normal gastric and duodenal epithelia. Bioinformatics analysis indicated that this might contribute to the cell cycle, stress response (proliferation, apoptosis), and cellular migration behavior, which are known to make Barrett's esophagus epithelium different to gastric and duodenal epithelia[4,7-12,34]. The reduced miR-200 levels that we observed in Barrett's esophagus epithelium adjacent to adenocarcinoma, and Barrett's esophagus with high-grade dysplasia derived cell lines suggested an association between down-regulation of miR-200 expression and neoplastic progres-

Table 3 miRNA expression in Barrett's esophagus and esophageal adenocarcinoma

miRNA	BE (n = 17)	Adeno carcinoma (n = 20)	BEC (n = 9)	P value (Kruskal-Wallis test)
miR-141	0.026 (0.023, 0.036)	0.012 (0.011, 0.025)	0.015 (0.01, 0.04)	0.03398[1]
miR-200a	0.148 (0.126, 0.177)	0.057 (0.013, 0.042)	0.08 (0.017, 0.201)	0.00079[1]
miR-200b	0.833 (0.75, 0.993)	0.387 (0.316, 0.572)	0.399 (0.258, 1.13)	0.00068[1]
miR-200c	1.05 (0.929, 1.170)	0.551 (0.461, 0.939)	0.662 (0.438, 0.965)	0.00323[1,2]
miR-429	0.07 (0.062, 0.087)	0.039 (0.029, 0.06)	0.042 (0.036, 0.09)	0.01355[1]

Relative expression for each tissue type. Relative expression values are median (95% CI). BE = Barrett's esophagus epithelium from individuals without cancer, BEC = Barrett's epithelium taken proximal to adenocarcinoma and confirmed by histology to be free of invasive cancer. The group P value was the result of a Kruskal-Wallis test across the three tissue groups. Significant differences were identified by post hoc testing by Holm-Bonferroni method for: [1]Adenocarcinoma versus Barrett's esophagus mucosa from individuals without cancer - miR-141 (P = 0.0126), miR-200a (P = 0.0001), miR-200b (P < 0.0001), and miR-200c (P = 0.0014) and miR-429 (P = 0.0031); [2]Barrett's esophagus mucosa from individuals with versus without cancer - miR-200c (P = 0.0191).

Table 4 *ZEB1* and *ZEB2* expression in Barrettt's epithelium and esophageal adenocarcinoma

	Fold ↑	miR-141	miR-200a	miR-200b	miR-200c	miR-429	miR-215
ZEB1	2.9	R = -0.2	R = -0.4	R = -0.5	R = -0.5	R = -0.3	R = -0.3
	P < 0.0001	P = 0.341	P = 0.046	P = 0.009	P = 0.006	P = 0.159	P = 0.152
ZEB2	1.5	R = -0.3	R = -0.3	R = -0.5	R = -0.5	R = -0.4	R = -0.4
	P = 0.029	P = 0.101	P = 0.085	P = 0.008	P = 0.015	P = 0.067	P = 0.044

The fold increase (↑) in median ZEB1 and ZEB2 expression in esophageal adenocarcinoma versus Barrett's esophagus mucosa from individuals without cancer, and the P value derived using the Mann-Whitney test are given. Spearman correlations (R = Spearman's rho) between ZEB1/2 expression and each miR-200 member, with associated P values are also shown.

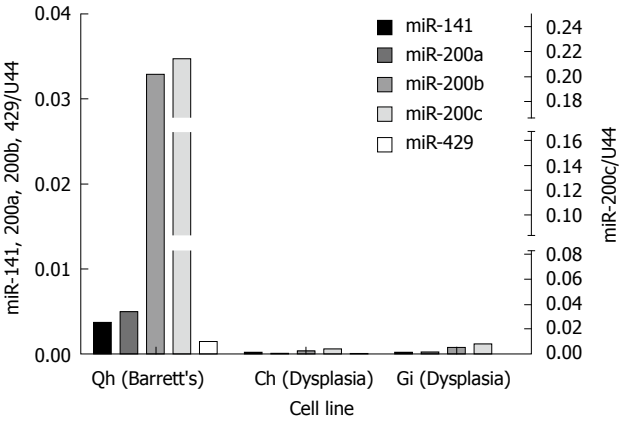


Figure 2 miR-200 family expression in benign Barrett's and dysplastic cell lines. Relative expression of all miR-141, miR-200a, miR-200b and miR-429, normalized to U44 expression is shown on the left hand y axis. Relative expression of miR-200c is shown on the right hand y axis. The pattern of relative expression of miR-200 members in the Qh (benign Barrett's) cell line closely resembled that in benign Barrett's esophagus mucosa (see relative expression values in Table 1).

sion in Barrett's esophagus. The increased expressions of *ZEB1* and *ZEB2* in esophageal adenocarcinoma, and their inverse correlations with miR-200 expression, are consistent with induction of epithelial to mesenchymal transition mediated by loss of miR-200 expression. Taken together with the known biological functions of the miR-200 family[26-28], our study provides evidence for their influence in patterning the known unique gene expression[4] and phenotypic characteristics[4,7-12,34] of benign Barrett's esophagus epithelium, as well as features that characterize its neoplastic progression[13]. Furthermore, our data indicate that the miRNA switch for turning on epithelial to mesenchymal transition[20] might be activated during the development of esophageal adenocarcinoma.

Although the miR-200 family has some redundancy in seed sequence, and therefore the genes they target, they act co-operatively to control the expression of their targets, and a change in expression of just one member is sufficient to alter target transcript levels[20]. miR-141 and miR-200c are known to modulate apoptosis, cell cycle and proliferation, and cellular migration through regulation of their target genes[26-28]. Several *in vivo* and *in vitro* studies have elucidated the molecules/complexes that are upregulated/activated in Barrett's epithelium and are responsible for its unique anti-apoptotic and proliferative responses to reflux-induced stress. These include three mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase, p38 and C-Jun N-terminal kinase[35]; protein kinase C[11]; phosphatidylinositol 3-kinases (PI3K) and downstream Akt[36]; and transcription factors nuclear factor (NF)-κB[37] and activator protein-1[36]. Our combined target prediction and Ingenuity Pathway Analysis predicted indirect (or direct in the case of Akt) targeting of all of the aforementioned molecules/complexes in the top associated network functions of miR-141 and/or miR-200c. Overall, this suggests that reduced miR-141 and miR-200c expression in Barrett's esophagus epithelium (*vs* gastric and duodenal epithelia) might activate molecular pathways that are known to promote the specific response of Barrett's esophagus epithelium to the insult of gastroesophageal reflux.

Our analysis predicted direct targeting and downregulation of fibronectin by miR-200c, and this was evident in the top associated network. A recent study has shown that fibronectin expression is reduced in cell lines in direct

response to miR-200c expression[38]. Therefore, it is reasonable to expect that fibronectin expression should be higher in Barrett's esophagus epithelium than in gastric and duodenal epithelia; due at least partly to reduced miR-200c expression in Barrett's esophagus. In agreement with this, fibronectin is in the gene expression cluster that separates Barrett's esophagus epithelium from gastric and duodenal epithelium[4]. Fibronectin has key roles in cellular adhesion and migration[39]. In the development of Barrett's esophagus, its expression is proposed to help facilitate expansion of epithelial cell populations in order to replace areas of denuded squamous epithelium with metaplastic cells[4]. Furthermore, fibronectin expression contributes to increased proliferative and anti-apoptotic behavior through NF-κB and PI3K signaling[40]. Taken together, these observations lend support to the biological validity of our bioinformatics-based approach, and further promote a likely role for reduced miR-200c expression in Barrett's esophagus.

The development of high-grade dysplasia significantly increases the risk of progression to esophageal adenocarcinoma[41,42]. Features of dysplastic epithelium that make it distinct from benign Barrett's esophagus epithelium include increased proliferative and anti-apoptotic behavior[13]. We found that miR-200c expression is downregulated in Barrett's esophagus sampled from patients with a concurrent esophageal cancer, and the entire miR-200 family was downregulated in cell lines derived from patients with high-grade dysplasia. Our pathway analysis indicates that this could result in the molecular events known to contribute to the features of dysplastic Barrett's esophagus, including increased signaling through MAPK pathways[43], increased activation and expression of NF-κB[44,45], and increased activation of Akt[43,46]. Activation of Akt is stimulated by interaction of the obesity-related hormone leptin and its receptor, and this results in increased proliferation and resistance to apoptosis in Barrett's esophagus derived cancer cell lines[46]. The leptin receptor is downregulated *in vitro* in response to miR-200c expression[38], and our miRecords searches predicted direct targeting of the leptin receptor by miR-200c. We speculate that reduced miR-200 family expression in dysplasia could be an important mechanism for leptin receptor mediated activation of Akt, and this could contribute to the established link between obesity and an increased risk of esophageal adenocarcinoma development[47]. In further support of the direct relevance of decreased miR-200 expression to the molecular features of neoplastic progression of Barrett's esophagus, we found that three gene transcripts (*EGR3*, *HS3ST1* and *RPS6KB1*), listed in miRecords to be targets of miR-200c, were present in a panel of 18 transcripts that are upregulated in Barrett's epithelium, from which cancer has arisen *versus* benign Barrett's esophagus epithelium[48].

Downregulation of E-cadherin expression *via* transcriptional repression is a central mechanism for epithelial to mesenchymal transition[15]. ZEB1 and ZEB2 are amongst a group of transcription factors that repress transcription of E-cadherin[15]. *ZEB1* and *ZEB2* are targets of the miR-200 family, and an increase in ZEB1 and ZEB2 activity caused by downregulation of miR-200 expression is sufficient to induce epithelial to mesenchymal transition[20]. Epithelial to mesenchymal transition that is promoted by downregulation of the miR-200 family has now been implicated as an important mechanism for invasion and metastasis in several cancers[20,22-25]. Previous epithelial and mesenchymal cell marker studies have provided evidence for the involvement of epithelial mesenchymal transition in the development of esophageal adenocarcinoma[16,17], but to the best of our knowledge, no studies have investigated loss of miR-200 mediated control of *ZEB1* and *ZEB2* expression as a possible mechanism for epithelial to mesenchymal transition in this disease. We found downregulation of the entire miR-200 family and upregulation of *ZEB1* and *ZEB2* transcription levels upon progression of Barrett's esophagus to adenocarcinoma. We observed significant inverse correlations between expression of miR-200 members and *ZEB1*/*ZEB2* transcripts. miR-215 expression, which we have previously reported as downregulated in neoplastic progression of Barrett's esophagus[29], was also inversely correlated with *ZEB2* expression. miR-215 has recently been shown to target *ZEB2* expression directly in kidney cells[32], and our results suggest the same in Barrett's esophagus. Together, these results suggest that the miR-200 family contributes to control of *ZEB1* and *ZEB2* expression in Barrett's esophagus, and this might be important for maintaining the epithelial phenotype. They add to the current evidence for the involvement of epithelial to mesenchymal transition in esophageal adenocarcinoma, and indicate downregulation of miR-200 expression as a potential mechanism for this.

With regard to the potential clinical relevance of our findings, we hypothesize that the miR-200 family might be useful biomarkers for identifying patients with Barrett's esophagus who are at increased risk of adenocarcinoma. They may also be helpful for assessing the potential antineoplastic effects of medical and surgical treatment of Barrett's esophagus. Further studies are required to evaluate this hypothesis. Recent advances in delivery of small RNAs in a clinical setting[49], and the demonstrated *in vitro* antineoplastic effects of endogenous miR-200 expression[38,50], suggest a possible future role for the therapeutic use of this family of miRNAs in treating early cancer in Barrett's epithelium.

Our study had some limitations. First, we used Barrett's esophagus epithelium proximal to cancer, and cell lines derived from dysplastic epithelium, to determine evidence for reduced miR-200 expression in dysplasia. We did not demonstrate miR-200 downregulation in dysplastic epithelium from patients who did not have invasive cancer, and this is an important area for investigation in future studies. Second, although our study provides evidence for effects of miR-200 expression on known gene-expression and phenotypic features of benign and dysplastic Barrett's esophagus, we did not expand this evidence using functional studies.

In summary, we showed that the miR-200 expression profile in Barrett's esophagus distinguished it from gastric and duodenal epithelia, and that downregulation of miR-200 expression was associated with dysplasia and adenocarcinoma. Further investigation is warranted to evaluate whether changes in the expression of these miRNAs can

be used to identify patients with Barrett's esophagus who are at risk of progression to esophageal adenocarcinoma.

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COMMENTS

Background

Barrett's esophagus is a precursor to esophageal adenocarcinoma and develops in response to chronic gastroesophageal reflux. Barrett's esophagus epithelium closely resembles that of the stomach and small intestine. However, some genes are expressed uniquely in Barrett's esophagus, and this is associated with pronounced anti-apoptotic and proliferative behavior in response to reflux. Esophageal adenocarcinoma development may involve an epithelial to mesenchymal transition-mediated acquisition of invasive phenotype. The miR-200 family of miRNAs can regulate cell death and proliferation, and control epithelial to mesenchymal transition through targeting of *ZEB1* and *ZEB2* gene expression.

Research frontiers

miRNAs have been implicated in almost every cellular process investigated. Recent evidence highlights a role for the miR-200 family members in numerous cellular processes including cell proliferation, cell death and epithelial to mesenchymal transition. All of these processes have been implicated in Barrett's esophagus and esophageal adenocarcinoma. We identified aberrant expression of the miR-200 family in Barrett's esophagus and esophageal adenocarcinoma and hypothesize that this differential expression contributes to aspects of neoplastic progression.

Innovations and breakthroughs

This study is the first to identify differential expression of the miR-200 family in Barrett's esophagus and esophageal adenocarcinoma. The miR-200 family expression data in Barrett's esophagus paired with functional studies in other cell types provides evidence that aberrant miR-200 family member expression may contribute to the increased proliferation and decreased cell death associated with Barrett's esophagus. The complete downregulation of the miR-200 family and subsequent upregulation of mRNA targets ZEB1 and ZEB2 in esophageal adenocarcinoma mimics what is seen in epithelial to mesenchymal transition and other invasive solid tumors, which suggests that the miR-200 family is involved in invasive esophageal adenocarcinoma development.

Applications

The authors hypothesize that the miR-200 family might be useful biomarkers for identifying patients with Barrett's esophagus who are at increased risk of adenocarcinoma. They may also be helpful for assessing the potential antineoplastic effects of medical and surgical treatment of Barrett's esophagus.

Terminology

Barrett's esophagus is a precursor to esophageal adenocarcinoma and develops in response to chronic gastroesophageal reflux. The miR-200 family of miRNAs can regulate cell death and proliferation, and control epithelial to mesenchymal transition through targeting of *ZEB1* and *ZEB2* gene expression.

Peer review

This is a well-written manuscript that describes miRNAs in Barrett's epithelium. The authors' conclusions are supported by the data.

REFERENCES

1 **Orlando RC.** Mucosal Defense in Barrett's Esophagus. In Barrett's Esophagus and Esophageal Adenocarcinoma. Sharma P, editor. Oxford, UK: Blackwell Publishing, Ltd, 2006; 60-72
2 **Zwas F**, Shields HM, Doos WG, Antonioli DA, Goldman H, Ransil BJ, Spechler SJ. Scanning electron microscopy of Barrett's epithelium and its correlation with light microscopy and mucin stains. *Gastroenterology* 1986; **90**: 1932-1941
3 **Levine DS**, Rubin CE, Reid BJ, Haggitt RC. Specialized metaplastic columnar epithelium in Barrett's esophagus. A comparative transmission electron microscopic study. *Lab Invest* 1989; **60**: 418-432
4 **Barrett MT**, Yeung KY, Ruzzo WL, Hsu L, Blount PL, Sullivan R, Zarbl H, Delrow J, Rabinovitch PS, Reid BJ. Transcriptional analyses of Barrett's metaplasia and normal upper GI mucosae. *Neoplasia* 2002; **4**: 121-128
5 **van Baal JW**, Milano F, Rygiel AM, Bergman JJ, Rosmolen WD, van Deventer SJ, Wang KK, Peppelenbosch MP, Krishnadath KK. A comparative analysis by SAGE of gene expression profiles of Barrett's esophagus, normal squamous esophagus, and gastric cardia. *Gastroenterology* 2005; **129**: 1274-1281
6 **Hao Y**, Triadafilopoulos G, Sahbaie P, Young HS, Omary MB, Lowe AW. Gene expression profiling reveals stromal genes expressed in common between Barrett's esophagus and adenocarcinoma. *Gastroenterology* 2006; **131**: 925-933
7 **Lao-Sirieix P**, Brais R, Lovat L, Coleman N, Fitzgerald RC. Cell cycle phase abnormalities do not account for disordered proliferation in Barrett's carcinogenesis. *Neoplasia* 2004; **6**: 751-760
8 **Dvorakova K**, Payne CM, Ramsey L, Holubec H, Sampliner R, Dominguez J, Dvorak B, Bernstein H, Bernstein C, Prasad A, Fass R, Cui H, Garewal H. Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. *Clin Cancer Res* 2004; **10**: 2020-2028
9 **Fitzgerald RC**, Omary MB, Triadafilopoulos G. Dynamic effects of acid on Barrett's esophagus. An ex vivo proliferation and differentiation model. *J Clin Invest* 1996; **98**: 2120-2128
10 **Kaur BS**, Ouatu-Lascar R, Omary MB, Triadafilopoulos G. Bile salts induce or blunt cell proliferation in Barrett's esophagus in an acid-dependent fashion. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G1000-G1009
11 **Kaur BS**, Triadafilopoulos G. Acid- and bile-induced PGE(2) release and hyperproliferation in Barrett's esophagus are COX-2 and PKC-epsilon dependent. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G327-G334
12 **Dvorakova K**, Payne CM, Ramsey L, Bernstein H, Holubec H, Chavarria M, Bernstein C, Sampliner RE, Riley C, Prasad A, Garewal H. Apoptosis resistance in Barrett's esophagus: ex vivo bioassay of live stressed tissues. *Am J Gastroenterol* 2005; **100**: 424-431
13 **Reid BJ**, Li X, Galipeau PC, Vaughan TL. Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. *Nat Rev Cancer* 2010; **10**: 87-101
14 **Eloubeidi MA**, Mason AC, Desmond RA, El-Serag HB. Temporal trends (1973-1997) in survival of patients with esophageal adenocarcinoma in the United States: a glimmer of hope? *Am J Gastroenterol* 2003; **98**: 1627-1633
15 **Lee JM**, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006; **172**: 973-981
16 **Rees JR**, Onwuegbusi BA, Save VE, Alderson D, Fitzgerald RC. In vivo and in vitro evidence for transforming growth factor-beta1-mediated epithelial to mesenchymal transition in esophageal adenocarcinoma. *Cancer Res* 2006; **66**: 9583-9590
17 **Jethwa P**, Naqvi M, Hardy RG, Hotchin NA, Roberts S, Spychal R, Tselepis C. Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma. *World J Gastroenterol* 2008; **14**: 1044-1052
18 **Smith CM**, Watson DI, Michael MZ, Hussey DJ. MicroRNAs, development of Barrett's esophagus, and progression to esophageal adenocarcinoma. *World J Gastroenterol* 2010; **16**: 531-537
19 **Bracken CP**, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF, Goodall GJ. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res* 2008; **68**: 7846-7854
20 **Gregory PA**, Bert AG, Paterson EL, Barry SC, Tsykin A,

Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008; **10**: 593-601

21 **Korpal M**, Kang Y. The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis. *RNA Biol* 2008; **5**: 115-119

22 **Dykxhoorn DM**, Wu Y, Xie H, Yu F, Lal A, Petrocca F, Martinvalet D, Song E, Lim B, Lieberman J. miR-200 enhances mouse breast cancer cell colonization to form distant metastases. *PLoS One* 2009; **4**: e7181

23 **Adam L**, Zhong M, Choi W, Qi W, Nicoloso M, Arora A, Calin G, Wang H, Siefker-Radtke A, McConkey D, Bar-Eli M, Dinney C. miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. *Clin Cancer Res* 2009; **15**: 5060-5072

24 **Li Y**, VandenBoom TG 2nd, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* 2009; **69**: 6704-6712

25 **Kong D**, Li Y, Wang Z, Banerjee S, Ahmad A, Kim HR, Sarkar FH. miR-200 regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells. *Stem Cells* 2009; **27**: 1712-1721

26 **Du Y**, Xu Y, Ding L, Yao H, Yu H, Zhou T, Si J. Down-regulation of miR-141 in gastric cancer and its involvement in cell growth. *J Gastroenterol* 2009; **44**: 556-561

27 **Cochrane DR**, Howe EN, Spoelstra NS, Richer JK. Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers. *J Oncol* 2010; 2010: 821717

28 **Uhlmann S**, Zhang JD, Schwäger A, Mannsperger H, Riazalhosseini Y, Burmester S, Ward A, Korf U, Wiemann S, Sahin O. miR-200bc/429 cluster targets PLCgamma1 and differentially regulates proliferation and EGF-driven invasion than miR-200a/141 in breast cancer. *Oncogene* 2010; **29**: 4297-4306

29 **Wijnhoven BP**, Hussey DJ, Watson DI, Tsykin A, Smith CM, Michael MZ. MicroRNA profiling of Barrett's oesophagus and oesophageal adenocarcinoma. *Br J Surg* 2010; **97**: 853-861

30 **Palanca-Wessels MC**, Klingelhutz A, Reid BJ, Norwood TH, Opheim KE, Paulson TG, Feng Z, Rabinovitch PS. Extended lifespan of Barrett's esophagus epithelium transduced with the human telomerase catalytic subunit: a useful in vitro model. *Carcinogenesis* 2003; **24**: 1183-1190

31 **Simon P**. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 2003; **19**: 1439-1440

32 **Wang B**, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, Saleem M, Goodall GJ, Twigg SM, Cooper ME, Kantharidis P. E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-beta. *Diabetes* 2010; **59**: 1794-1802

33 **Xiao F**, Zuo Z, Cai G, Kang S, Gao X, Li T. miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Res* 2009; **37**: D105-D110

34 **Galipeau PC**, Prevo LJ, Sanchez CA, Longton GM, Reid BJ. Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. *J Natl Cancer Inst* 1999; **91**: 2087-2095

35 **Jaiswal K**, Lopez-Guzman C, Souza RF, Spechler SJ, Sarosi GA Jr. Bile salt exposure increases proliferation through p38 and ERK MAPK pathways in a non-neoplastic Barrett's cell line. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G335-G342

36 **Song S**, Guha S, Liu K, Buttar NS, Bresalier RS. COX-2 induction by unconjugated bile acids involves reactive oxygen species-mediated signalling pathways in Barrett's oesophagus and oesophageal adenocarcinoma. *Gut* 2007; **56**: 1512-1521

37 **Hormi-Carver K**, Zhang X, Zhang HY, Whitehead RH, Terada LS, Spechler SJ, Souza RF. Unlike esophageal squamous cells, Barrett's epithelial cells resist apoptosis by activating the nuclear factor-kappaB pathway. *Cancer Res* 2009; **69**: 672-677

38 **Cochrane DR**, Spoelstra NS, Howe EN, Nordeen SK, Richer JK. MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol Cancer Ther* 2009; Epub ahead of print

39 **Pankov R**, Yamada KM. Fibronectin at a glance. *J Cell Sci* 2002; **115**: 3861-3863

40 **Han SW**, Roman J. Fibronectin induces cell proliferation and inhibits apoptosis in human bronchial epithelial cells: pro-oncogenic effects mediated by PI3-kinase and NF-kappa B. *Oncogene* 2006; **25**: 4341-4349

41 **Pera M**, Trastek VF, Carpenter HA, Allen MS, Deschamps C, Pairolero PC. Barrett's esophagus with high-grade dysplasia: an indication for esophagectomy? *Ann Thorac Surg* 1992; **54**: 199-204

42 **Wong T**, Tian J, Nagar AB. Barrett's surveillance identifies patients with early esophageal adenocarcinoma. *Am J Med* 2010; **123**: 462-467

43 **Ogunwobi O**, Mutungi G, Beales IL. Leptin stimulates proliferation and inhibits apoptosis in Barrett's esophageal adenocarcinoma cells by cyclooxygenase-2-dependent, prostaglandin-E2-mediated transactivation of the epidermal growth factor receptor and c-Jun NH2-terminal kinase activation. *Endocrinology* 2006; **147**: 4505-4516

44 **O'Riordan JM**, Abdel-latif MM, Ravi N, McNamara D, Byrne PJ, McDonald GS, Keeling PW, Kelleher D, Reynolds JV. Pro-inflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Gastroenterol* 2005; **100**: 1257-1264

45 **Konturek PC**, Nikiforuk A, Kania J, Raithel M, Hahn EG, Mühldorfer S. Activation of NFkappaB represents the central event in the neoplastic progression associated with Barrett' s esophagus: a possible link to the inflammation and overexpression of COX-2, PPARgamma and growth factors. *Dig Dis Sci* 2004; **49**: 1075-1083

46 **Beales IL**, Ogunwobi O, Cameron E, El-Amin K, Mutungi G, Wilkinson M. Activation of Akt is increased in the dysplasia-carcinoma sequence in Barrett's oesophagus and contributes to increased proliferation and inhibition of apoptosis: a histopathological and functional study. *BMC Cancer* 2007; **7**: 97

47 **Whiteman DC**, Sadeghi S, Pandeya N, Smithers BM, Gotley DC, Bain CJ, Webb PM, Green AC. Combined effects of obesity, acid reflux and smoking on the risk of adenocarcinomas of the oesophagus. *Gut* 2008; **57**: 173-180

48 **Helm J**, Enkemann SA, Coppola D, Barthel JS, Kelley ST, Yeatman TJ. Dedifferentiation precedes invasion in the progression from Barrett's metaplasia to esophageal adenocarcinoma. *Clin Cancer Res* 2005; **11**: 2478-2485

49 **Ashihara E**, Kawata E, Maekawa T. Future prospect of RNA interference for cancer therapies. *Curr Drug Targets* 2010; **11**: 345-360

50 **Hurteau GJ**, Carlson JA, Roos E, Brock GJ. Stable expression of miR-200c alone is sufficient to regulate TCF8 (ZEB1) and restore E-cadherin expression. *Cell Cycle* 2009; **8**: 2064-2069

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