MicroRNAs and esophageal cancer - implications for pathogenesis and therapy

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ABSTRACT

There are several microRNAs that have been consistently reported to be differentially expressed in esophageal squamous cell carcinoma vs. normal squamous tissue, with prognostic associations for miR-21 (invasion, positive nodes, decreased survival), miR-143 (disease recurrence, invasion depth), and miR-375 (inversely correlated with advanced stage, distant metastasis, poor overall survival, and disease-free survival). There is also evidence that miR-375 regulates gene expression associated with resistance to chemotherapy. Hence, microRNA expression assays have the potential to provide clinically relevant information about prognosis and potential response to chemotherapy in patients with esophageal squamous cell carcinoma. Results are inconsistent, however, for microRNAs across different studies for esophageal adenocarcinoma (EAC) vs. its precursor lesion Barrett's esophagus. These inconsistencies may partly result from pathological and/or molecular heterogeneity in both Barrett's esophagus and EAC, but may also result from differences in study designs or different choices of comparator tissues. Despite these inconsistencies, however, several mRNA/protein targets have been identified, the cancer related biology of some of these targets is well understood, and there are clinico-pathological associations for some of these mRNA targets. MicroRNAs also have potential for use in therapy for esophageal cancers. The development of new delivery methods, such as minicells and autologous microvesicles, and molecular modifications such as the addition of aromatic benzene pyridine analogs, have facilitated the exploration of the effects of therapeutic microRNAs in vivo. These approaches are producing encouraging results, with one technology in a phase I/IIa clinical trial.

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KEY WORDS
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TEXT

Esophageal cancer continues to be a lethal disease, with the majority of patients presenting at an advanced stage relative to other cancers such as colon, melanoma and breast [1]. Late stage esophageal cancers are aggressive, and despite advances in both medical and surgical therapies, the five year survival rate is at best around 15-20% [1]. Furthermore, over the last 30 years the incidence of esophageal adenocarcinoma has increased more than that of any other cancer in the Western world [2], to become the fifth leading cause of cancer related death in men in the US [1]. The two main histological types of esophageal cancer, adenocarcinoma (EAC) and squamous cell carcinoma (ESCC), have different causes and patterns of incidence, very different tumor biology, are more common in different parts of the esophagus, and differ somewhat in their clinical management [3].

Esophageal adenocarcinoma

The principal factor driving the development of EAC appears to be gastro-esophageal reflux, which is very common in the Western World. In some people, chronic reflux induces an adaptive response in the epithelium of the distal esophagus, whereby the squamous epithelium is replaced by metaplastic columnar epithelium, a condition known as Barrett's esophagus (BE) [4]. Approximately 10% of patients with chronic reflux have Barrett's esophagus [5]. Barrett's esophagus is the only identifiable precursor lesion for EAC, and progression to EAC occurs in 0.2 to 2.1% of patients per year [6]. Although ESCC still represents 90% of esophageal cancer cases in many Eastern countries, the incidence of EAC has now surpassed that of ESCC in most Western countries [7]. Furthermore, the incidence of EAC in Europe [8], North America [9], and Australia [10] is increasing at a rate exceeding that of any other cancer, with a near 6 fold increase over the last 3 to 4 decades, predominantly in men. The prognosis for this cancer is poor and approximately 90% of sufferers will die from this disease. This is because potentially curative treatment, usually esophagectomy, is only feasible in approximately 25-30% of individuals, and surgical treatment is associated with significant morbidity and mortality. The two main risk factors for EAC are gastro-esophageal reflux and obesity [11].

Esophageal squamous cell carcinoma (ESCC)

Although EAC is increasingly common in Western countries, ESCC is still dominant in East Asia [12]. ESCC develops from the original squamous epithelium of the oesophagus, and this is strongly influenced by lifestyle factors. The two main risk factors for ESCC of the esophagus are tobacco smoking and high alcohol consumption, particularly in combination. Esophagitis and atrophy of

squamous epithelium may be clinically relevant precursor lesions [13, 14], while strong evidence suggests that varying grades of dysplasia and carcinoma *in situ* are relevant precursors to development of ESCC [13, 15]. ESCC is mostly diagnosed at late stages, and the prognoses of affected patients are poor despite advances in therapeutic options such as surgery, chemotherapy and radiotherapy. With the exception of a small number of patients with early cancers confined to the mucosa, attempted curative therapies for ESCC involve either neoadjuvant treatment (chemotherapy or chemoradiotherapy, CRT) followed by surgical resection [16], or CRT alone [17, 18].

Need for Biomarkers in Esophageal Cancer

Although the tumor-node-metastasis (TNM) system provides classification of tumor stage, it provides little therapeutic and biological information, such as the metastatic potential, or the sensitivity or resistance of the tumor to radiotherapy and chemotherapy. There is therefore a need for better prognostic indicators to distinguish high-risk patients from other patients. Increasing evidence suggests that biomarkers such as microRNA's might be able to be meet this requirement.

microRNAs

Aberrant expression and/or functions of miRNAs (microRNAs) are implicated in tumorigenesis [19]. MiRNA-expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment. In addition, profiling has been exploited to identify miRNA genes that might be downstream targets of activated oncogenic pathways, or that target protein-coding genes involved in cancer [19].

Methods

We performed a PubMed search with the following search phrase, "(microRNA OR micro-RNA OR microRNA OR mir-*) AND (esophag* OR oesophag*)", on the 4th Jan 2012. This returned 99 publications. From those articles relevant publications dealing directly with micro-RNAs and esophageal cancer were obtained by screening the abstracts or, if necessary, the entire article. Further articles were extracted by screening the references within these papers. In case of non-availability of the whole article the abstract was taken into consideration despite the limited data provided. This search was repeated weekly until the 29th May 2012 to capture recent publications.

Identification of miRNAs with Diagnostic or Prognostic Relevance in Esophageal Cancer

Several studies have been published investigating the differential expression of miRNAs in ESCC and EAC. The first investigation of miRNA expression profiles in ESCC and EAC was reported by

Feber et al (2008) and found differences between the two diseases. MiR-194, miR-192 and miR200c were observed to be up-regulated in EAC compared with normal squamous epithelium, but not in ESCC, whereas miR-342 was up-regulated in ESCC [20]. Mathe et al (2009) found that miR-194 and miR-375 were elevated in adenocarcinoma compared with ESCC [12]. Subsequent studies have reported associations between specific miRNAs and disease classification, clinical outcomes, and various target genes known to be involved in the development of cancer.

MiRNAs in Esophageal Squamous Cell Carcinoma (ESCC)

Pathology and Differentiation

Guo et al (2008) were the first to report the expression of miRNA in esophageal cancer tissues using miRNA microarray techniques. They found that three miRNAs (miR-25, miR-424, and miR-151) were up-regulated and four miRNAs (miR-100, miR-99a, miR-29c, and mmu-miR-140*) were reduced in ESCC versus normal squamous tissue. They also found that five miRNAs (miR-335, miR-181d, miR-25, miR-7 and miR-495) correlated with gross pathologic classification (fungating *vs* medullary), and two miRNAs (miR-25 and miR-130b) correlated with histopathological differentiation (well *vs* moderate *vs* poor) [21].

Diagnosis, and Survival

Guo et al (2008) reported that expression of miR-103/107 in ESCC correlated with poor survival [21]. Ogawa et al (2009) reported that high expression levels of 6 miRNAs correlated with significantly lower survival rates in patients with ESCC. Of these, the over-expression of miR-129 was identified as a significant and independent prognostic factor (HR = 18.1; P = 0.031) in 49 patients with surgically treated ESCC [22].

Hiyoshi et al (2009) observed that miR-21 levels were higher in ESCC tissues compared with normal squamous tissues [23], and Zhu et al (2011), using laser capture micro-dissection of normal basal, normal differentiated, and tumor tissues in 5 ESCC cases, found that miR-21, miR-25 and miR-106b were up-regulated in ESCC tumors compared with matched normal tissues [24]. Mathe et al (2009) found that elevated miR-21 expression in non-cancerous tissue from patients with ESCC was associated with worse prognosis, and that this association was independent of tumor stage, lymph node status, and chemoradiation therapy [12].

Kurashige et al (2012) found that miR-223 expression was significantly higher in cancerous ESCC tissues than in the corresponding normal tissues, and that patients with high miR-223 expression had a significantly poorer prognosis than those with low expression [25]. In contrast, Li et al (2011)

reported that over-expression of miR-223 in an ESCC derived cell line decreased cell migration and invasion. These authors also reported that miR-223 targets ARTN in ESCC cells, a known tumor metastasis-related gene [26].

Lin et al (2012) reported that high miR-142-3p expression was associated with a poor prognosis in 91 patients with ESCC, but more importantly, stratified analysis indicated that high miR-142-3p expression was correlated with a poor prognosis in a subset of patients within generally good-prognosis groups; i.e. patients with a small ESCC, no lymph node metastases, and early stage [27]. Kong et al (2012) reported that miR-375 levels were inversely correlated with advanced stage, distant metastasis, poor overall survival, and disease-free survival in ESCC. Li et al (2011) reported that miR-375 is down-regulated by hyper-methylation of its promoter in ESCC [28], and Kong et al (2012) found that the miR-375 promoter was methylated in 58% of patients with ESCC [29]. Isozaki found that miR-375 was strongly up-regulated by treatment with a histone deacetylase inhibitor (HDAC) in an ESCC cell line [30].

Serum and plasma

Zhang et al (2011) reported that miR-31 was up-regulated in ESCC tissue and in serum. Patients with high serum levels of miR-31 had a poorer prognosis, with early tumor recurrence and a higher risk of tumor-related death. MiR-31 has also been reported to be up-regulated in other squamous cell cancers such as lung [31], oral [32], and head and neck [33]. This is in contrast to EAC, in which miR-31 has been reported to be down-regulated relative to Barrett's esophagus [34]. However, Lynam-Lennon et al (2012) reported that miR-31 was down-regulated in radioresistant ESCC cells, both basally and in response to radiation, in a an established isogenic model of radioresistance in oesophageal adenocarcinoma [35].

Komatsu et al (2011) reported that miR-21 levels were higher in patients with ESCC, that they reflected tumor levels, and correlated with disease recurrence. Plasma levels of miR-21 were also reduced in postoperative samples. MiR-375 plasma levels were observed to be lower in patients with ESCC [36].

Zhang et al (2010), using solexa sequencing, observed upregulation of 25 serum miRNAs in ESCC patients compared with controls. Using real-time PCR and a panel of 7 serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a and miR-127-3p) in 290 patients with ESCC and 140 age- and sex-matched controls, they were able to achieve an approximate 70% sensitivity at 100% specificity in diagnosing patients with histologically confirmed ESCC [37].

Invasion

Ma et al (2009) reported that patients with ESCC invasion deep into the esophageal wall showed significantly higher expression of miR-21, and that protein expression of the miR-21 target PTEN (a tumor suppressor) was significantly lower in tumor, compared with normal tissues [38]. Mori et al (2009) reported that miR-21 expression levels were significantly higher in T3 or T4 tumors than in T1 or T2 tumors, and also that miR-21 expression was significantly higher in patients with more invasive infiltrative growth pattern tumors than in patients with less invasive tumors [39].

Lymph node involvement/metastases

Chen et al (2011) found that up-regulation of miR-92a was significantly correlated with lymph node status, metastasis, and TNM stage in 107 patients with ESCC. Up-regulation of miR-92a was also associated with poorer survival of patients with ESCC, and the authors suggested that this miRNA had potential for use as an independent prognostic factor [40]. Akagi et al (2011) reported that expression levels of miR-21 were higher in ESCC tissues compared to normal epithelium, and that miR-21 and miR-205 levels were higher in ESCC patients who were node-positive [41]. Cai et al (2012) reported that miR-21 levels were associated with the presence of metastases in ESCC patients, but not with TNM staging [42].

Disease recurrence, and response to therapy

Akagi et al (2011) reported that miR-143, miR-145 and miR-205 levels were higher in ESCC tissues from patients who had recurrent disease after surgery [41]. Komatsu et al (2011) found that patients with a high plasma level of miR-21 tended to have greater vascular invasion, and that plasma miR-21 correlated strongly with tumor recurrence. Importantly, the plasma level of miR-21 was significantly reduced in postoperative samples [36]. Kurashige et al (2012) also observed a significant reduction in serum miR-21 levels postoperatively versus preoperatively, and found that miR-21 levels were significantly reduced in patients with ESCC who responded well to chemotherapy [43].

Down-regulation of miR-27a in 2 ESCC cell lines via transfection with antagomirs reduced the IC_{50} for effect on cell viability of both the drug effluxer P-glycoprotein-related (Vincristine, adriamycin) and P-glycoprotein-non-related (cisplatin, 5-flurouracil) drugs by an average of 68% [44]. However, in contrast to the observations in ESCC, down-regulation of miR-27a in leukaemic cell lines was associated with chemotherapy resistance, and miR-27a was inversely correlated with the expression of P-glycoprotein [45].

Chemotherapy-sensitivity

Hamano et al (2011) found that miR-200c expression was increased in ESCC tissues compared with matched normal squamous tissues, and that miR-200c was inversely correlated with response to chemotherapy. These authors also determined that miR-200c expression was increased in a cisplatin-resistant cell line compared with the parent cell line. In an anti-miR-200c-transfected ESCC-derived cell line, chemosensitivity to cisplatin was found to increase [46]. Sugimura et al (2012) reported that low expression of let-7b and let-7c in before-treatment biopsies from patients with ESCC correlated significantly with poor response to chemotherapy. Low expression of let-7c also correlated with poor prognosis. In cultured cells transfection of let-7c restored sensitivity to cisplatin and increased the rate of apoptosis after exposure to cisplatin [47].

Esophageal Adenocarcinoma (EAC)

Survival

Feber et al (2011) explored whether global miRNA expression in resected EAC tissues could predict patient survival and lymph node involvement, and found that a combined expression signature of increased expression of five miRNAs (miR-100, miR-143, miR-145, miR-199a_3p and miR-199a_5p) was associated with patient survival independent of lymph node involvement and overall stage (HR = 3.6, p = 0.005) [48]. Hamano et al (2011) also observed increased survival of patients with esophageal adenocarcinoma in those that had higher levels of miR-145 [46]. However, Feber et al (2011) observed reduced levels of miR-143 and miR-145 in EAC compared with normal tissue [48]. Wijnhoven et al (2010) also reported reduced levels of miR-143 and miR-145 in EAC compared with Barrett's epithelium [49]. The incongruent observations for miR-145 are supported by studies with cell lines. Ectopic expression of miR-145 in a metastatic colon carcinoma cell line to levels similar to normal colonic epithelium resulted in increased proliferation and anchorage independent growth [50], whereas several studies have demonstrated that over-expression of miR-145 has tumor suppressor effects in non-metastatic colorectal cancer cell lines [51]. These combined observations suggest that loss of expression may be required for neoplastic transformation, but either re-expression of miR-145, or at least higher levels, may be needed for the development of a more aggressive phenotype.

Hu et al (2011) investigated the prognostic significance of 10 selected miRNAs in 99 patients with EAC. In univariate analysis miR30e, miR-195p and miR-200a were associated with shorter overall

survival in patients with EAC. In multivariate analysis only miR-195p was associated with shorter overall survival and disease free survival [52].

Hamano et al (2011) found that over-expression of miR-200c correlated significantly with shortened overall duration of survival of patients with esophageal cancer who had received preoperative chemotherapy followed by surgery [46]. Yang et al (2009) reported that miR-200a and miR-200b levels, measured by hybridization microarray, were higher in some patients with adenocarcinoma compared with normal squamous tissue, and that miR-200a* was up-regulated 13-fold in high grade dysplasia compared with low grade dysplasia [53]. However, members of the miR-200 miRNA family are reported to be down-regulated in many human cancer cells, and appear to play a critical role in the suppression of epithelial-to-mesenchymal transition (EMT), tumor cell adhesion, migration, invasion and metastasis, by targeting and repressing the expression of key mRNAs that are involved in EMT [54]. In agreement with this, Smith et al (2011) reported that miR-200a, miR0-200b and miR-200c levels, measured using qRT-PCR, were lower in adenocarcinoma compared with non-dysplastic Barrett's esophagus tissues, with corresponding higher mRNA levels of ZEB1 and ZEB2 , observations which are consistent with the induction of an epithelial to mesenchymal transition [55].

Leidner et al (2012) reported that miR-31 expression is decreased in some patients with high grade dysplasia compared with Barrett's esophagus, and that miR-375 expression is decreased in some patients with EAC compared with high grade dysplasia and Barrett's esophagus [34]. Patients with EAC with decreased expression of both miR-31 and miR-375 had decreased survival (2.8 years vs. 3.5 years).

Lymph node involvement

Feber et al (2011) reported that the expression of three miRNAs in EAC (miR-99b, miR-199a-3p and miR-199a-5p) was associated with the presence of lymph node metastasis, with a receiver operator characteristic (ROC) curve derived optimal sensitivity of 78% and specificity of 78% [48]. This suggests that a panel of miRNAs could potentially be effective predictors of lymph node metastases. This might be applicable where, for example, endoscopic therapy is being considered for use as the definitive treatment [56].

Disease progression

Fassan et al (2011) investigated miRNA expression in tissues from 14 patients who had undergone esophagectomy for EAC. Microarray results were validated by quantitative real-time PCR and in

situ hybridization. This study identified increased expression of 6 miRNAs (miR-215, miR-560, miR-615-3p, miR-192, miR-326 and miR-147) and reduced expression of 7 miRNAs (miR-100, miR-23a, miR-605, miR-99a, miR-205, let-7c and miR-203) that correlated with cancer progression from normal squamous mucosa to adenocarcinoma. However, there were no indications that the expression of any of these miRNAs changed during the progression from Barrett's esophagus to dysplasia to adenocarcinoma [57], so it is therefore possible that these miRNAs are only associated with differences between squamous epithelium and Barrett's esophagus, and may not be directly associated with the development of adenocarcinoma. Other miRNAs identified in the hybridization micro-arrays in this study (miR-23a, miR-99a, miR-100, miR-605, miR-147, & miR-560) appear to be differentially expressed between Barrett's esophagus and EAC, but are yet to be validated by real time PCR [57].

Maru et al (2009) reported that miR-196a is a potential marker of progression from Barrett's metaplasia to dysplasia and invasive adenocarcinoma in the esophagus. Higher levels of miR-196a were observed in Barrett's esophagus, dysplastic mucosa and EAC, compared with normal squamous mucosa, and in high grade dysplasia and EAC compared with Barrett's esophagus and low grade dysplasia, but only in some patients [58]. Luzna et al (2011) used micro-dissection in combination with quantitative real–time PCR to investigate changes in miRNA expression in patients who progressed from Barrett's esophagus to adenocarcinoma. These authors also observed up-regulation of miR-196a, and also of miR-192 and, down-regulation of miR-203. They also found a positive correlation between miR-196a and progression from Barrett's esophagus to adenocarcinoma, although there is considerable overlap in the expression levels of miR-196a between these tissues [59]. However, Fassan et al (2011) did not find that miR-196a levels were increased in dysplastic and EAC tissues compared with Barrett's esophagus from surgical resection specimens [57]. Furthermore, Bansal et al (2011) reported that miR-196a was not differentially expressed in biopsies containing Barrett's esophagus and biopsies with adenocarcinoma or high grade dysplasia [60], but did observe that miR-15b, miR-21, and miR- 203 were up-regulated in biopsies containing adenocarcinoma compared with non-dysplastic intestinal metaplasia, and that miR-486-5p and let-7 were down-regulated [60].

Leidner et al (2012), using next generation sequencing as a discovery platform, followed by quantitative real-time PCR validation of micro-dissected tissues, and by adopting a stringent methodology to determine the timing of miRNA alterations in esophageal neoplasia, observed that miR-375 expression, relative to matched normal squamous tissue, is decreased in 60% of patients with EAC, compared with patients with high grade dysplasia or Barrett's esophagus. These authors

also observed that miR-31 expression is decreased in 36% of patients with high grade dysplasia, and 45% of patients with EAC, compared with Barrett's esophagus [34]. This is a low level of sensitivity, and indicates that there may be several different pathways to the development of these lesions, or that down-regulation of these miRNAs may not be required for the development of these lesions.

Chemotherapy-sensitivity

Hummel et al (2011) reported that miR-148a expression levels were inversely associated with EAC cancer cell differentiation [61]. In esophageal cancer cell lines, transient transfection with a miR-148a mimic increased the sensitivity of chemotherapy-sensitive cells to cisplatin, but did not have this effect in chemotherapy-resistant cell lines [62].

Summary tables of diagnostic and prognostic miRNAs

The published reports of observed differential miRNA expression, clinical associations, and mRNA/protein targets in ESCC and EAC are summarised in Table 1, in which the miRNAs are listed in ascending order. For the major studies, the study size and the main methods are summarised in Table 2. MiRNAs that have been reported in multiple publications are summarised in for ESCC in Table 3, and for EAC in Table 4. For some of the miRNAs the reports have given qualitatively inconsistent results, and these are indicated in Tables 3 and 4, along with the number of studies reporting each type of result.

Non-expression based miRNA markers

Single nucleotide polymorphisms (SNPs)

Ye et al (2008) investigated 41 SNPs within 26 miRNA-related genes in a case-control study of 346 Caucasian patients with esophageal cancer versus 346 frequency-matched (age, gender, and ethnicity) controls. Seven SNPs were significantly associated with esophageal cancer risk. The most notable finding was that a SNP located in the pre-miR-423 region was associated with a per-allele odds ratio of 0.64. In a combined unfavorable genotype analysis, the high-risk group had a 3.14-fold (95% CI, 2.03 - 4.85) increased risk of cancer [63].

Umar et al (2012) investigated 4 common SNPs within pre-miRNAs (mir-196a-2C>T, mir-146aG>C, mir-499T>C, and mir-423C>A) in 239 ESCCs and 309 control patients. Patients with 2-4 pre-miRNA polymorphisms had 1.4-fold higher risk of ESCC compared to patients with pre-miRNA polymorphisms. Patients with 2-4 pre-miRNA polymorphisms also had significantly lower median survival (11.60 vs. 30.2 months) [64]. Wang et al (2012) genotyped SNPs in the 3' UTRs of

seven genes in 537 ESCC cases and 608 normal controls and found that SNP rs6573 in the 3' UTR of RAP1A was significantly associated with the risk of developing ESCC, and was also associated with pathology stage. This SNP lies within the binding site for miR-196a, and in vitro assays confirmed that this SNP interfered with the binding of miR-196a. Importantly, these authors observed that observed that RAP1A was overexpressed in the majority of esophageal squmous cell carcinoma tissues, and that the RAP1A genotype correlated with lymph node metastasis [65].

Methylation of miRNAs

Chen et al (2011) used bisulfite sequencing and methylation specific PCR to investigate previously reported dysregulated miRNAs (miR-34a, miR-203, miR-34b/c, miR-424 and miR-129-2) that are embedded in CpG islands. These authors reported that miR-34a, miR-34b/c and miR-129-2 had significantly higher levels of methylation than corresponding non-tumor tissues in 67%, 41%, and 96% respectively of patients with ESCC [66]. Li et al (2011), using methylation specific-PCR, observed an association between promoter hyper-methylation of miR-375 and its down-regulation in esophageal cancer tissues compared with adjacent non-cancerous tissue [28]. Kong et al (2012) used bisulfite sequencing and methylation specific PCR, and reported that methylation of the promoter of miR-375 was detected in 58% of ESCC specimens [29].

MiRNA biogenesis defects

RNASEN (ribonuclease 3) is a double-stranded RNA–specific endonuclease that converts pre-cursor forms of miRNA into mature forms. Expression levels of RNASEN measured by immunohistochemistry in 73 patients with ESCC were found to be strongly associated with overall ($P = 0.0003$) and disease free ($P = 0.0005$) patient survival. Multivariate Cox regression analysis showed that this prognostic effect of RNASEN may be independent of disease stage. Knockdown of RNASEN in esophageal cancer cell lines resulted in a 46% to 85% reduction in cell number [67].

Yoo et al (2010) investigated the expression of RISC proteins in tissues from patients with ESCC and found that neither Ago2 (Argonaute2, an endonuclease responsible for the cleavage of targeted mRNA) nor TNRC6A (which silences the expression of bound mRNAs; recruited to miRNA targets through an interaction with an Argonaute protein) were expressed in normal squamous cells, while Ago2 and TNRC6A were expressed in 59% and 62% of ESCC patients, respectively [68].

Biological roles of identified miRNAs

Preliminary work has been done investigating how miRNAs may be involved in the development of esophageal cancers, and in the differential therapeutic responses of esophageal cancers. A wide

range of potential messenger RNA (mRNA) and protein targets of miRNAs have been identified in esophageal cancers, and some of these targets have been validated in vitro. These are listed in Table 1. Furthermore, cellular phenotype changes characteristic of neoplasia have also been associated with some miRNAs and their targets in vitro. Here we provide details of investigations into the targets of miR-21 and miR-375, as they have been consistently reported to be diagnostic for esophageal cancers, and have also been reported to be associated with prognostic features.

Hiyoshi et al (2009) found that 18 of 20 micro-dissected cancer tissues over-expressed miR-21, in comparison with the normal esophageal epithelium. Furthermore, patients with lymph node metastasis or venous invasion showed significantly higher expression of miR-21. These authors also investigated the relationship between miR-21 and a previously identified target gene, the tumor suppressor programmed cell death 4 (PDCD4), in a panel of ESCC-derived cell lines, and observed an inverse correlation between PDCD4 protein levels and miR-21 expression. They also observed that anti-miR-21-transfected cells had increased PDCD4 protein expression, and increased luciferase-reporter activity from a construct containing the PDCD4-3' untranslated region, which suggests a direct effect of miR-21 on PDCD4 expression. These same anti-miR-21-transfected ESCC cell lines showed a reduction in cellular proliferation, and reduced invasion into matrigel [23]. Zhu et al (2011) subsequently reported that miR-21 was increased [24], and that PDCD4 mRNA was down-regulated [69], in ESCC cancer tissues compared with matched normal squamous tissue, and Fassan et al (2011) reported that nuclear PDCD4 protein, measured by immuno-histochemistry, was decreased in ESCC resection tissues compared with unmatched normal squamous tissues [70].

Increased miR-21 has also been reported to be associated with a decrease in the mRNA [24] and protein [38] of the metastasis-suppressor PTEN (phosphatase and tensin homologue) in ESCC. Loss of function of PTEN contributes to the development of many cancers [71]. The loss of function of PTEN has also been associated with miR-21 over-expression in several cancers. Furthermore, miR-21 has been shown to directly regulate PTEN [72], and miR-21 expression has been observed to be inversely correlated with PTEN protein, but not mRNA, in an ESCC cell line transfected with either miR-21 mimics or antagonists [38]. PTEN has also been reported to be associated with responsiveness to chemotherapy [73], radiotherapy [74], and hormone therapy [75]. The only study investigating PTEN in EAC (Kulke et al (2001)), did not find significant mutations or LOH of PTEN in 80 patients with EAC, and suggested that PTEN may be inactivated through other mechanisms [76]. However, evaluations of miR-21 expression in EAC have been inconsistent, and so far no associations with clinical outcomes have been reported.

Mathe et al (2009) reported that reduced levels of miR-375 in cancerous tissue of patients with EAC were associated with worse prognosis [12]. Leidner et al (2012) observed reduced miR-375 levels in EAC, but not in high grade dysplasia or Barrett's esophagus, suggesting that this miRNA may be associated with progression to invasive adenocarcinoma [34]. In patients with ESCC, plasma levels of miR-375 have been reported to be lower than in healthy patients [36]. miR-375 has also been reported to be correlated with advanced stage, distant metastasis, poor overall survival, and poor disease-free survival in ESCC [29]. Li et al (2011) observed hyper-methylation of the miR-375 promoter in 58% of ESCC patients [28]. Promoter hyper-methylation usually results in silencing or reduced transcription, and could therefore account for the reduced levels of miR-375 observed in ESCCs.

Kong et al (2012) reported that miR-375 levels in ESCC tissues were negatively correlated with insulin like growth factor 1 receptor (IGF1R) expression [29]. IGF1R is frequently over-expressed in many malignancies, and plays a crucial role in promoting cell proliferation, survival, tumorigenesis, metastasis, and resistance to existing forms of cancer therapy. The observation that miR-375 may regulate IGF1R in ESCC is important as Phase III clinical trial results targeting IGFR1 with monoclonal antibodies in unselected patients have been disappointing [77].

Potential of miRNAs as therapeutic targets

The inhibitory effects induced by miRNAs on a specific target may be mild, and may only lead to a subtle reduction of protein expression. However, the simultaneous down-regulation of a broad range of mRNA targets can determine the cellular phenotype. MiRNAs that regulate protein levels across multiple pathways can potentially cause a switch from one program of cellular behavior to a different program of behavior [78]. This implies that only small changes in miRNA expression may be required for therapeutic effects. For cancer therapy miRNA replacement aims at restoring the expression of tumor suppressive miRNAs. MiRNAs may also be involved in the self-renewal of tumor-initiating cancer cells (cancer stem cells) [79, 80]. This suggests that miRNAs might be able to be used to target cancer cells that are associated with chemotherapy-resistance, metastasis, and recurrence.

To date few tumor suppressor miRNAs have been discovered that have been shown to be workable in animal models of cancer. The best characterized tumor suppressor miRNA is let-7, which was originally identified as a switch gene required for proper development in *Caenorhabditis elegans*. let-7 is now know to comprise a family of miRNAs that were the first group of oncomirs shown to

regulate the expression of an oncogene, specifically the Ras genes. Ras proteins are membrane-associated GTPase signaling proteins that regulate cellular growth and differentiation, and activating mutations result in the increased expression of Ras and cause cellular transformation [81]. Studies using cultured lung cancer cells, as well as mouse models of lung cancer, have shown that the reintroduction of let-7, via the in vitro transfection of cell lines, blocks the proliferation of cancer cells and reduces the growth of both murine and human lung tumors in mice [82, 83]. Conversely, inhibition of let-7 augmented tumorigenesis in a KRAS-induced mouse model, further indicating that let-7 functions as a tumor suppressor. In the same study exogenous delivery of let-7 to established tumors in a mouse model of non-small-cell lung cancer (NSCLC) significantly reduced the tumor burden [84], suggesting that therapeutic miRNA delivery may be possible in humans.

Challenges that have become apparent from reported studies

Molecular heterogeneity

High throughput array techniques have been used to screen for potential miRNAs that are diagnostic or prognostic for ESCC and EAC. However, the various studies that have used hybridisation micro-arrays have not produced comparable results for associations of individual miRNAs, with the exception of miR-21 in ESCC. There has even been a report of qualitatively different results from different cohorts of patients within the one study [12]. Furthermore, within each study, irrespective of the method used to measure miRNA levels, there is often considerable overlap in the expression levels of each miRNA between normal healthy vs. diseased patients, thus making it difficult to obtain sufficient specificity and sensitivity. Both of these issues may be a consequence of the molecular heterogeneity which is apparent in lesions throughout the gastro-intestinal tract [85-88].

A potential solution to the problem of molecular heterogeneity is to find a panel of several miRNAs that are altered in different pathways of disease development, and in this way the sensitivity could potentially be increased. However, in studies where panels of miRNAs have been tested, disease classification overlap has still been observed [20, 53]. Feber et al (2008), using micro-arrays containing 328 human miRNA probes, and unsupervised hierarchical clustering of the data, generated four groups corresponding to normal squamous, ESCC, Barrett's esophagus, and EAC. The first branch contained 7 samples of normal squamous mucosa and 1 ESCC, the second branch contained 7 ESCC and 1 normal squamous mucosa , the third branch contained 4 Barrett's esophagus and 1 ESCC, and the fourth branch contained 10 EAC, 1 Barrett's esophagus, 1 normal

squamous mucosa, and 1 ESCC [20]. While it is possible that the patient with Barrett's esophagus who was classified into the EAC group may have been at risk of developing EAC (and therefore appeared EAC-like), this seems unlikely for the patient with normal esophageal squamous mucosa. It is encouraging that this approach was able to correctly classify all of the EAC patients. This may not be possible for patients with dysplastic lesions, however, as Yang et al (2009), who also used unsupervised hierarchical clustering of miRNA micro-array data (470 human miRNA probes), misclassified 3 of 16 dysplastic or adenocarcinoma tissues as normal squamous mucosa, and misclassified 3 of 16 normal squamous tissues [53].

For EAC, miR-100 is the only miRNA that has been consistently reported to be down-regulated in EAC compared with normal squamous tissue [20, 53, 57], and to have potential prognostic significance. However, the prognostic association of miR-100 is the inverse of the diagnostic relationship, in that decreased levels of miR-100 in cancerous tissues are associated with better survival [48], and with cancer progression [57]. This is in contrast to observations in epithelial ovarian cancer [89], and potentially also in prostate cancer [90]. MiR-199a_3p has also been reported to be down-regulated in EAC [91], as well as hepatocellular carcinoma [92], and epithelial ovarian cancer [93]. As for miR-100, the prognostic association with survival is the inverse of the diagnostic relationship. Low expression levels of miR-199a_3p in cancerous tissues have been reported to be associated with dramatically increased survival in EAC [48]. Similar observations for diagnostic and prognostic associations have been made for miR-143 and miR-145 [48]. It is possible that these incongruent observations are the result of increased stromal involvement in tumor formation (as cells such as myofibroblasts express high levels of these miRNAs) [94], and/or are the result of transition of epithelial to mesenchymal cells in the tumors [95]. The latter explanation is supported by the association of the epithelial-mesenchymal-transition (EMT) with metastasis.

Issues with blood based measurements

While there has not been any work reported investigating the diagnostic or prognostic potential of miRNAs in serum or plasma for EAC, several groups have reported associations in ESCC. There are, however, potential problems with using serum or plasma for these types of studies. Red blood cells contain miRNAs, and hemolysis is difficult to prevent during blood collection. This can effect the levels of miR-16, which is commonly used as a normalization reference in these studies [96]. More recently it has been observed that plasma levels of miRNA biomarkers expressed by myeloid (e.g., miR-223, miR-197, miR-574-3p, and let-7a) and lymphoid (e.g., miR-150) blood cells were tightly correlated with corresponding white blood cell counts [97]. High levels of miRNAs of blood

cell origin could potentially mask changes in miRNAs secreted by cancer or associated stromal cells.

Stability and delivery of miRNAs

MiRNAs are relatively unstable and have anionic charge, and these characteristics present challenges to their application in gene silencing, particularly with respect to their effective delivery to target tissues . MiRNA therapy also faces several further potential challenges including lack of tissue specificity, poor cellular uptake, and risk of systemic toxicity. However, mouse studies that have evaluated the therapeutic delivery of tumor suppressor miRNAs have not observed problems associated with the miRNAs, and suggest that delivery of miRNA to normal tissues is well tolerated [98], [99]. Therapeutic miRNA mimics may be better tolerated by normal cells compared with cancer cells because the pathways regulated by the miRNA mimic are already regulated by endogenous miRNA.

Transient expression systems that use viral or liposomal delivery have been trialed for administering large quantities of miRNAs. Although the use of similar methods to that used for siRNAs (small interfering RNAs) for cancer gene therapies has shown that the immune response can limit the effectiveness of RNA delivery [100, 101], Ibrahim et al (2011) recently reported the effective use of polyethylenimine (PEI)-mediated delivery of unmodified miRNAs in a mouse model of colon carcinoma. Low molecular weight PEI/miRNA complexes were delivered systemically or by local application into mouse xenograft tumors, where they caused profound antitumor effects and repression of oncogene expression [102]. Wu et al (2011) reported on the use of an optimized cationic lipid based miRNA delivery method. In this study mice treated with pre-miR-133b containing lipoplexes had mature miR-133b expression in their lungs that were 52-fold higher than in untreated mice, and 50-fold higher than mice treated using an non-optimized commercial transfection reagent [103]. Kitade et al (2010) investigated improving the clinical delivery of miR-143 by adding aromatic benzene-pyridine (BP-type) analogs to the 3'-overhang region of the RNA-strand, and by changing the sequences of the passenger strand in the miR-143 duplex (miR-143BPs), leading to greater activity and increased resistance to nuclease activity. The modified miR-143 showed a significant tumor-suppressive effect on xenografted tumors of human colorectal cancer cells in mice [104]. The self-assembly of MS2 bacteriophage capsids has been used to develop virus-like particles (VLPs) for RNA and drug delivery. Pan et al (2012) reported that MS2 VLPs conjugated with HIV-1 Tat peptide effectively transferred packaged pre-miR146a RNA into various cells and tissues, and suppressed expression of a target gene by 80% [105].

The Australian company EnGeneIC (Lane Cove, New South Wales, Australia) have recently adapted bacterially derived 400 nm particles, called minicells, for encapsulation and cancer cell targeting of various chemotherapeutics including miRNAs [106]. The size of the minicells (~400 nm) ensures retention within the reticuloendothelial system and prevents them from penetrating into normal tissues, which is a problem for drug-conjugated monoclonal antibody therapeutics. The minicells have been targeted to tumor cells via bi-specific antibodies: antibodies to LPS on the bacterial minicells, and antibodies to receptors on cancer cell membranes. The antibodies are conjugated together at their Fc regions with protein A/G. This reduces complement-mediated in vivo toxicity, since the Fc part of each monoclonal antibody is blocked by protein A/G. Post-intravenous administration, the bi-specific-antibody-targeted RNA-packaged minicells rapidly transfer out of the vascular circulation and into the tumor microenvironment, possibly due to the leaky vasculature associated with solid tumors. Xenograft studies in mice revealed that therapeutically significant concentrations of shRNA (small-hairpin-RNA) were expressed from plasmids in tumor xenografts, and this resulted in tumor stabilization and regression. In drug studies significant anti-tumor effects were observed with over 1000-fold lower concentrations delivered to xenografts via minicells compared with the respective free drugs [107]. This approach has also been used to reduce the expression of drug resistance proteins with shRNA encoding plasmids. This approach resulted in increased sensitivity to subsequent chemotherapeutic drug treatment, and survival of mice with otherwise drug resistant xenografts. These authors have generated data that suggests that the potent anti-tumor effects observed in mouse xenografts are unlikely to be due to interferon or inflammatory cytokine responses [108]. A phase I/ IIa multi-center clinical trial of this technology is currently in progress in cancer patients.

Microvesicles and exosomes, which are shed from cells and appear to be involved in cell-to-cell communication, are also possible vehicles for targeting miRNA molecules to body tissues. Exosomes are formed in endosomes containing multivesicular bodies, which have been functionally linked to miRNA effector complexes [109,]. This indicates potential mechanisms for miRNA targeting to exosomes. Akao et al (2012) recently reported that miRNA molecules transfected into in vitro differentiated human macrophages were shed from these cells as contents in microvesicles during incubation in serum-free medium. The transfected cells also secreted microvesicles containing miRNAs when injected into xenografted nude mice [110]. These results suggest an approach similar to that being investigated for cancer immunotherapy using autologous dendritic cells as tumor-specific antigen presenting cells, where peripheral blood monocytes could be differentiated in vitro into macrophages and then transfected with therapeutic miRNA. The cells, or secreted microvesicles, could then be transferred back into the patient.

Summary

There are 6 miRNAs (miR-21, miR-29c, ,miR-99a, miR-143, miR-203 & miR-375) that have been consistently reported to be differentially expressed in ESCC vs. normal squamous tissue, with prognostic associations for miR-21 (invasion, positive nodes, decreased survival) and miR-143 (disease recurrence, invasion depth). Results are inconsistent for reported miRNAs across different studies for Barrett's esophagus vs. EAC. Several studies have reported differences in miRNA expression between EAC and normal squamous esophageal mucosa, but in most cases it is not possible to determine whether the differential miRNA expression is just reflecting differences between Barrett's esophagus and normal squamous mucosa. The most comprehensive EAC study is from Leidner et al (2012) as they used a careful study design combined with laser capture micro-dissection to determine which miRNAs were differentially expressed between Barrett's esophagus and EAC [34]. However, the results from this group have yet to be replicated.

The inconsistencies in miRNA expression in EAC may be due to differences in study design involving the use of comparator tissues, for instance the use of apparently unaffected Barrett's esophagus mucosa from cancer patients rather than the use of Barrett's mucosa from cancer free patients. The inconsistencies could also be the result of pathological and/or molecular heterogeneity in both Barrett's esophagus and EAC [86, 88, 111], and might therefore be an intrinsic aspect of this disease. However, in at least one clinical aspect (chemotherapy resistance), the more important and informative tissues are likely to be metastases [112]. Although there may also be within patient molecular heterogeneity in metastases [113], there is some evidence that metastases are homogenous for allelic losses of tumor suppressor loci in a proportion (58%) of patients [114]. Irrespective of these issues, intra- and/or inter-lesion biopsy sampling errors due to heterogeneity might be averted by assaying miRNA levels in serum, plasma, or secreted microvesicles in peripheral blood. This approach might improve the ability to detect miRNAs associated with chemotherapy response in EAC, and has the added advantage of being less invasive.

Several mRNA targets have been identified, and the cancer related biology of some of these targets is well understood (e.g. PDCD4, PTEN, CDH1). Furthermore, there are clinico-pathological associations for some of these mRNA targets, which means that they may prove useful as biomarkers in combination with prognostic miRNAs. Identified target mRNAs may also be regulated by other miRNAs that have not yet been extensively investigated, thus providing an alternative to high throughput methods for identifying potentially differentially expressed miRNAs.

In assessment of the literature to date, it seems that miRNAs have the potential to become part of the clinical assessment of patients with esophageal cancer, to help determine prognosis, and to predict the likely response to therapy. Furthermore, the development of new delivery methods, such as minicells and autologous microvesicles, and molecular modifications such as the addition of aromatic benzene pyridine analogs, have facilitated the exploration of the effects of therapeutic miRNAs in vivo. These approaches are producing encouraging results, especially the work using minicells with mouse xenografts, and suggest that miRNA based treatments are possible.

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Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	ESCC vs NS ↑↓	source	EAC vs NS ↑↓	EAC vs BE ↑↓	source	Association with clinical feature, mRNA, or protein.
miR-1-1	↓	[12]				
miR-1-2	↓	[12]				
miR-7	↑	[12]				
miR-7-2	↑	[12]				
Let-7	↓	[115]				Correlation between low expression of let-7 and lymph node metastasis in ESCC [115]
Let-7a				↓	[60]	
Let-7b						Correlation with poor response to chemotherapy [47]
Let-7c	↓		↓	↑	[12, 57]	Correlation with poor response to chemotherapy and poor prognosis [47]
miR-10a	↓	[116]				
miR-10b	↑	[117]				
miR-15b				↑	[60]	
miR-16-1	↑	[12]				
miR-16-2	↑	[12]				Associated with lymph node metastasis, & shorter overall and disease-free survival in EAC [52]
miR-19a	↑	[118]				TNF-alpha confirmed target in ESCC [118]
miR-19b						
miR-21	↑	[12, 20, 21, 23, 24, 38, 41, 119, 120]	↑ - ↑ ↑	↑ - - -	[12, 20, 70] [53] [60] [49]	Decreased survival in ESCC HR=4.7 [12], Association with deep invasion into esophageal serosa [38], Association with positive nodes [41], Decreased PTEN, PDCD4, SPRY1, IAM1, &LRRFIP1 mRNA [24], decreased PTEN protein [38], decreased PDCD4 protein [23], higher in ESCC if distant lymph node metastases were present [61], high **plasma** miR-21 levels reflected ESCC tumor levels, and high correlation with recurrence [36], high **serum** levels in patients with ESCC reduced in patients who responded to chemotherapy [43] Associated with the presence of metastases in ESCC patients, but not with TNM staging [42]. No association with survival in EAC [12]
miR-23a			↓	↓	[57]	
miR-25	↑	[21, 24]	↑		[53]	Decreased KLR4 mRNA [24]
miR-27b	↓	[20]	↓	↓	[20, 53]	
miR-29			↑		[53]	
miR-29c	↓	[12, 21, 121]	↑		[53]	Suppresses cyclin E expression [121]

Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	ESCC vs NS ↑↓	source	EAC vs NS ↑↓	EAC vs BE ↑↓	source	Association with clinical feature, mRNA, or protein.
miR-30a_5p			↑		[53]	
miR-30b	↓	[12]				
miR-30c-1	↓	[12]				
miR-30c-2	↓	[12]				
miR-30e						Associated with shorter overall and disease-free survival in EAC [52]
miR-31	↑	[122]		↓	[34]	**Serum** miR-31 associated with poorer prognosis of ESCC. Validated targets EMP1, KSR2, RGS4 [122] miR-31 down-regulated in radioresistant ESCC cells, both basally and in response to radiation[35].
miR-31 & miR-375				↓	[34]	Decreased survival in EAC
miR-92a	↑	[40]				Correlated with lymph node status, metastasis, TNM stage, and poor survival. Repressed CDH1 expression.
miR-93	↑	[20]	↑		[20]	
miR-99a	↓	[12, 21]	↓	↓	[53, 57]	
miR-99b						Associated with node status in EAC [48]
miR-100	↓	[21]	↓	↓	[20, 53, 57]	Inversely associated with survival in EAC [48], inversely correlated with cancer progression [57]
miR-103/107						High expression of hsa-miR-103/107 correlated with poor survival [21]
miR-103-1				↑	[12]	
miR-106a						Lower in patients with SCC who developed recurrent disease or who died from tumor [61]
miR-106b	↑	[24]				
miR-107				↑	[12]	
miR-122a	↑	[12]				
miR-125a	↑	[12]				
miR-125b			↓	↓	[20]	
miR-125b-1	↓	[12]				
miR-125b-2	↓	[12]				
miR-126	↓	[12]				Associated with tumor cell dedifferentiation and lymph node metastasis in EAC[52]
miR-129	↑	[22]				Significant and independent prognostic factor in surgically treated ESCC patients [22]

Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	ESCC vs NS ↑↓	source	EAC vs NS ↑↓	EAC vs BE ↑↓	source	Association with clinical feature, mRNA, or protein.
miR-133a						Inhibits cell proliferation and cell invasion in ESCC cells [123]
miR-133a-1	↓	[12]				
miR-133a-2	↓	[12]				
miR-133b						Inhibits cell proliferation and cell invasion in ESCC cells [123]
miR-140				↑	[53]	
Mmu-miR-140*	↓	[21]				
miR-141				↓	[55]	Ectopic expression reduced sensitivity of ESCC cell lines to cisplatin [124]
miR-142-3p						Correlated with poor prognosis in ESCC [27]
miR-143	↓ - [125] [126]	[12, 41]	↓↑	↓	[48, 49, 53]	Disease recurrence in ESCC [41], correlated with tumor invasion depth in ESCC [126], decreased level associated with survival in EAC [48]
miR-145	↓-↑	[12, 24, 41, 125, 126]	↓↑	↓	[48, 49, 53]	Disease recurrence in ESCC [41], correlated with tumor invasion depth in ESCC [126], decreased level associated with survival in EAC [48], inhibits cell proliferation and cell invasion in ESCC cells [123]
miR-146a	↑	[12]	↑		[12, 53]	Correlation between rs2910164 C/G variant and TNM stage in ESCC[127]
miR-146b	↑	[12]	↑		[12]	
miR-147			↑	↑	[57]	
miR-148a						Inversely associated with cancer differentiation in EAC, lower in patients with ESCC who developed recurrent disease or had a tumor-related death [61]
miR-149			↓		[53]	
miR-151	↑	[21]				
miR-155	↑	[12]				
miR-181-1	↑	[12]				
miR-181a			↑		[53]	
miR-181a-1			↑		[12]	
miR-181a-2			↑		[12]	
miR-181b			↑		[53]	
miR-181b-1	↑	[12]				

Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	ESCC vs NS ↑↓	source	EAC vs NS ↑↓	EAC vs BE ↑↓	source	Association with clinical feature, mRNA, or protein.
miR-181c	↑	[12]				
miR-181d	↑	[12]				
miR-181-2	↑	[12]				
miR-192			↑ ↑	↑	[20] [12, 57, 59]	
miR-194			↑	↑	[12, 20]	
miR-195			↑		[53]	
miR-195p						Associated with higher pathologic disease stages in patients with EAC [52]
miR-196a				↑-	[57-60, 128]	Inverse correlation with protein levels of KRT5, SPRR2C, and S100A9 [58]. Homozygous SNP in pre-miRNA-196a associated with increased risk of ESCC [129]. SNP in miR-196a target RAP1A associated with risk of ESCC, and pathology stage [65].
miR-199			↑		[53]	
miR-199*			↑		[53]	
miR-199a_3p						Associated with survival in EAC [48], Associated with node status [48]
miR-199a_5p						Associated with survival in EAC [48], Associated with node status [48]
niR-199b			↑		[53]	
miR-200a				↓	[55]	Associated with approx. 10% less overall and disease-free survival at 6 months in EAC [52] (Note: not inversely correlated as expected)
miR-200a*				↑	[53]	
miR-200b	↓	[12]		↓	[55]	
miR-200c	↑	[46]	↑	↑↓	[20, 55]	Associated with poor response to preoperative chemotherapy in ESCC [46]
miR-202	↓	[12]				
miR-203	↓	[12, 20, 24]	↓	↓↑	[12, 20, 57] [53, 60]	inhibits cell proliferation by targeting DeltaNp63 in ESCC cell lines [130]
miR-205	↓-↑	[20] [41, 116]	↓	↓	[12, 20, 57]	Associated with positive nodes in ESCC [41], and with cellular migration in ESCC cells [116]

Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	ESCC vs NS ↑↓	source	EAC vs NS ↑↓	EAC vs BE ↑↓	source	Association with clinical feature, mRNA, or protein.
miR-210	↓	[131]	↓	↓	[12] [53]	Inverse association with poorly differentiated carcinomas. FGFRL1 is a target in ESCC [131].
miR-215			↑	↓	[49, 57]	
miR-220	↓	[12]				
miR-221				↓	[53]	
miR-223	↑	[25]				Inverse relationship with the expression levels of FBXW7 protein. Poorer prognosis in ESCC [25], over-expression of miR-223 in ESCC cells decreased cell migration and invasion [26]
miR-224	↑	[12]				
miR-223	↑	[12]	↑		[12]	
miR-296	↑	[132]				Inversely associated with survival in ESCC [132]
miR-320	↓	[12]				
miR-326			↑	↑	[57]	
miR-342	↑	[20]				
miR-373	↑	[133]				Inversely correlated with LATS2 protein expression [133]
miR-375	↓	[12, 29]		↓	[34]	Associated with worse prognosis of EAC [12], and with progression to invasive [34]. **Plasma** level lower in ESCC [36], correlated with advanced stage, distant metastasis, poor overall survival, and disease-free survival in ESCC. Negatively correlated with IGF1R expression in ESCC [29], down-regulated by hyper-methylation in ESCC [28].
miR-378	↓	[12]				
miR-424	↑	[21]	↑		[53]	
miR-429				↓	[55]	
miR-486-5p				↓	[60]	
miR-494			↓		[53]	
miR-499	↓	[12]				
miR-513			↓		[53]	
miR-560			↑	↑	[57]	
miR-605			↓	↓	[57]	
miR-615-3p			↑		[57]	
miR-617			↓		[53]	

Table 1. Summary of changes in microRNAs that have been reported between ESCC and normal squamous tissue, between EAC and normal squamous tissue, and between EAC and Barrett's esophagus. legend: "↑" = increased level, "↓" = decreased level, "-" = no change

miRNA	**ESCC vs NS ↑↓**	**source**	**EAC vs NS ↑↓**	**EAC vs BE ↑↓**	**source**	**Association with clinical feature, mRNA, or protein.**
miR-1322	↑	[134]				Diagnosis of ESCC, also higher in **serum** of ESCC patients [134]

Table 2. The number of patients, and methods, in each major study.

Study	Number of patients	Methods used
[41]	55 ESCC	rt-PCR of precursor and mature microRNAs, and rt-PCR of microRNA-processing elements mRNA
[20]	10 ESCC, 10 EAC	Hybridization microRNA micro-array
[21]	55 ESCC	Hybridization microRNA micro-array rt-PCR validation
[12]	100 EAC, 70 ESCC	Hybridization microRNA micro-array, validation by rt-PCR
[116]	cell lines	Hybridization microRNA micro-array, validation by rt-PCR ESCC patient biopsies – number not reported
[53]	91 EAC	Hybridization microRNA micro-array, validation by rt-PCR
[24]	5 ESCC	Laser capture micro-dissection followed by rt-PCR of microRNA and hybridization micro-array of mRNA
[57]	14 EAC	Hybridization microRNA micro-array, validation by rt-PCR and by in situ hybridization
[55]	20 EAC	Quantitative real-time PCR
[12]	68 ESCC 100 EAC	Quantitative real-time PCR

Table 3. microRNAs that have been identified in several studies in ESCC and may therefore be good candidates as diagnostic and/or prognostic biomarkers

miRNA	Number of studies	Implications
miR-21	9	*Diagnostic:* high in ESCC, and in **plasma** and **serum** of patients with ESCC *Prognostic*: invasion, node positive, decreased survival
miR-29c	3	*Diagnostic*: decreased in ESCC *Prognostic*: no associations to date
miR-99a	2	*Diagnostic*: decreased in ESCC *Prognostic*: no associations to date
miR-143	3 1	*Diagnostic*: decreased in ESCC Unchanged in ESCC *Prognostic*: recurrence, invasion depth
miR-145	5	*Diagnostic*: conflicting evidence *Prognostic*: recurrence, invasion depth
miR-203	3	*Diagnostic*: decreased in ESCC *Prognostic*: no associations to date
miR-205	3	*Diagnostic*: conflicting evidence *Prognostic*: node positive
miR-375	2	*Diagnostic*: decreased in ESCC and **plasma** *Prognostic*: correlated with advanced stage, distant metastasis, poor overall survival, and disease-free survival in ESCC

Table 4. microRNAs that have been identified in several studies in EAC and may therefore be good candidates as diagnostic and/or prognostic biomarkers

miRNA	Number of studies	Implications
miR-21		*Diagnostic*:
	2	increased in EAC vs. Barrett's esophagus,
	3	No change in EAC vs. Barrett's esophagus
	3	Increased in EAC vs. normal squamous
		Prognostic: No association with survival in EAC, no associations to date
miR-100	3	*Diagnostic*: decreased in EAC vs. normal squamous
	1	Decreased by 20% in EAC vs. Barrett's esophagus
		Prognostic: survival , inversely correlated with cancer progression
miR-143	4	*Diagnostic*: conflicting evidence, although potentially increased in EAC vs. normal squamous as Feber et al data inconsistent [20, 48]
		Prognostic: survival
miR-145	4	*Diagnostic*: Conflicting evidence, although potentially increased in EAC vs. normal squamous as Feber et al data inconsistent [20, 48]
		Prognostic: survival
miR-192	3	*Diagnostic*: increased in EAC vs. normal squamous
	1	Increased EAC vs. Barrett's esophagus
		Prognostic: no associations to date
miR-196a	4	*Diagnostic*: conflicting evidence
		Prognostic: no associations to date
miR-200c	2	*Diagnostic*: conflicting evidence
		Prognostic: response to chemotherapy
miR-203	4	*Diagnostic*: decreased in EAC vs. normal squamous
	3	EAC vs. Barrett's esophagus, conflicting evidence,
		Prognostic: no associations to date
miR-375	2	*Diagnostic*: decreased in EAC vs. Barrett's esophagus (1 study)
		Prognostic: worse prognosis, suggested association with progression to invasive carcinoma (1 study).