MiRNAs Predicting Response to Oesophageal Cancer Treatment

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Oesophageal cancer (OC) is the ninth most common cancer worldwide. Patients receive neoadjuvant therapy (NAT) as standard of care, but less than 20% of patients with oesophageal adenocarcinoma (OAC) or a third of oesophageal squamous cell carcinoma (OSCC) patients, obtain a clinically meaningful response. Developing a method of determining a patient's response to NAT before treatment will allow rational treatment decisions to be made, thus improving patient outcome and quality of life. MicroRNAs are valuable biomarkers of response to NAT in OC. Research is needed to understand the effects different types of chemotherapy and chemoradiotherapy have on the predictive value of microRNAs; studies also require greater standardization in how response is defined.

oesophageal adenocarcinoma | oesophageal squamous cell carcinoma | predicting response | chemotherapy | chemoradiotherapy | neoadjuvant therapy | microRNAs

1. Introduction

1.1. Oesophageal Cancer Epidemiology

Oesophageal cancer (OC) is the ninth most common cancer diagnosed globally, yet the sixth most common cause of cancer related death [1], resulting in an estimated 436,000 patient deaths in 2017 [2][3]. Five-year survival for OC is 17% [4]. The prognosis is bleak when compared to other cancers such as colorectal cancer, of which the 5-year survival is almost 3 times greater than that of OC [5]. These poor outcomes can at least in part be attributed to late presentation, with 47.9% of patients diagnosed at stage 4 [6]. OC is more common in men who are 3 to 4 times more likely than women to develop OC, and the median age of diagnosis is 68 years [1]. There are two major OC subtypes, oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC), for which OSCC estimated to contribute for over 70% of all OC diagnoses globally [7][8]. OSCC, shows greater prevalence across Asia within an "oesophageal cancer belt" that stretches from north-east Iran to north-west China [9]. This is likely due to the higher prevalence of tobacco use, along with genetic differences in alcohol metabolism which leads to acetaldehyde accumulation, a known carcinogen [7][10]. OAC is, however, predominant in the Western world, probably due to the high incidence of obesity and gastro-oesophageal reflux disease [11] which are the primary risk factors for OAC development. The UK has the highest incidence of OC in Europe with approximately 10,000 new cases per year [12]. The UK's 5-year survival for OC is 12% in line with average across Europe; however this varies largely from 9% in Latvia to 25% in Belgium [4].

1.2. Pathophysiology of OAC and OSCC

OAC is most often found in the lower part of the oesophagus and at the gastro-oesophageal junction, where it frequently develops from its precursor Barrett's oesophagus [13]. Persistent exposure to acid and bile reflux, results in mucus-secreting glandular metaplasia [14]. Increased genetic mutations and loss of heterozygosity are seen during epithelial proliferation. Notably in the progression to OAC two defining genetic mutations are present that can be utilised to differentiate between non-dysplastic Barrett's oesophagus, high grade dysplasia and OAC tissues, these are *TP53* and *SMAD4*. Mutations in the tumour suppressor gene *TP53* occur in 50–71% of all OAC cases [7] but have been shown to be recurrently mutated in high grade dysplasia (HGD) and OAC samples. *SMAD4* mutation is shown to be exclusive to OAC tissue and thus it can be concluded it lies at the boundary between progression from HGD to OAC [15]. The loss of these tumour suppressor genes (TSG) or mutation of proto-oncogenes leads to dysplastic Barrett's due to a greatly increased rate of uncontrolled cellular proliferation [7]. Further mutagenic changes and chromosomal instability over time result in the formation of OAC and without intervention, infiltration of the basement membrane and subsequent metastasis.

OSCC pathology is subtly different; it more commonly presents in the middle and upper parts of the oesophagus [16]. Persistent physical insults, primarily chronic alcohol and tobacco use, lead to squamous cell hyperplasia [17]. Over time should exposure be unchanged, genetic mutations will accumulate due to greatly increased rates of cellular proliferation. *TP53* mutation in OSCC is almost universally found in all patients, approximately 92% [7][18]. However, *NOTCH1* and *NOTCH3* mutations have been shown to be significantly more frequent in OSCC than OAC, at 33% versus 25%, respectively [18]. These mutagenic accumulations eventually result in a loss of negative feedback mechanism due to malfunction of tumour suppressors. The result is uncontrolled rapid rates of cellular proliferation producing a carcinoma [7].

1.3. Current Pathways of Screening

Currently there is no national screening programme for OC in the UK [19], in contrast to Asian countries such as Japan and Korea where a higher disease incidence is seen. These have been shown to significantly improve outcomes [20][21], likely due to earlier detection and thus earlier intervention. Screening programmes consist of fibreoptic upper gastrointestinal endoscopy or Barium Upper Gastrointestinal Series for those over 40 years, recommended every 2 years thereafter [20][21]. With these interventions approximately two thirds of upper GI cancers are detected at an early stage, compared to the 70% of late-stage diagnoses across Europe [1][20][21]. The largest issue with screening for OC is the invasiveness of this procedure and therefore the risk it poses to the patients [19], particularly should these results be negative and therefore futile. Mass screening would therefore place 1 in 200 to 1 in 10,000 patients at risk of adverse events such as infection, perforation and bleeding [22]. The American and British Societies of Gastroenterology suggest screening for OAC should be provided to patients with reflux >5 years, Caucasian males and family history of Barrett's oesophagus or OAC [7]. Despite recent evidence showing one off endoscopic screening in China led to a reduction in incidence and mortality, no current OSCC screening is in place [23] possibly due to its lower population prevalence in the UK. Traditionally, there have been no minimally invasive procedures with high enough sensitivities to consider their use in widespread screening programmes. However, new research into Cytosponge™ technology for detection of Barrett's oesophagus and early dysplasia suggests a specificity and sensitivity of 79.9% and 92.4% respectively, which is comparable to current screening programmes for colorectal cancer in the UK with a false positive rate of 2–9% [24], as well as improved detection rates of Barrett's oesophagus in the primary care setting [25]. Utilisation of this minimally invasive sampling method, alongside modern genetic testing could prove to be a highly sensitive and specific way of detecting and tailoring treatment regimens to patients.

1.4. Treatment of Oesophageal Cancer

Where OC is potentially curable at presentation, it is locally advanced in the majority of cases. Standard of care treatments in this setting for both OSCC and OAC are usually extensive and invasive, requiring neoadjuvant chemotherapy or chemo-radiotherapy (NAT) followed by surgical resection as recommended by NICE guidelines [26]. Guidance on the treatment pathway of OSCC and OAC based upon staging and functional assessment of the patient, as recommended by the European Society of Medical Oncology are outlined in **Figure 1** [27]. A variety of studies have investigated the benefits of NAT. The MRC MAGIC trial showed patients who receive a neoadjuvant regimen of epirubicin, cisplatin and 5-fluorouracil (5-FU) (ECF) therapy had a higher rate of overall survival (5-year survival, 36% vs. 23%) and progression free survival (0.53 to 0.81, $p<$ 0.001) in comparison to patients undergoing surgery alone [28]. Similarly, the CROSS trial demonstrated that neoadjuvant chemoradiotherapy improved median overall survival from 24 to 49.4 months vs. surgery alone [29]. However, only 25% to 30% of patients achieve a partial or complete pathological response [30][31], and it carries a 0.5 to 2% mortality rate [32]. Early identification of patients that respond well could improve outcomes by preventing the administration of treatment regimens that are unlikely to be effective and facilitating treatment modulation [33]. Response to therapy is usually assessed via assignment of the Mandard Tumour Regression Grade (TRG) ranging 1 to 5 [34]. Responders are usually defined as TRG1 (complete regression with no viable tumour cells evident) and TRG2 (presence of residual cancer cells), at least for patients receiving chemotherapy. By administering NAT in patients who do not respond well, surgery is delayed, which if carried out earlier may have proven more effective. The main benefits of NAT are the increased chance of complete resectability of the primary tumour, as reduced tumour mass induced by NAT decreases the area of resection required, as well as improved prognostic outcome due to the decreased incidence of nodal micrometasteses [32][35][36]. On the contrary, tumour progression during therapy can occur in those patients who do not respond well to NAT or conversely overtreatment of tumours with a favourable prognosis that are unlikely to respond to NAT. Therefore, identifying biomarkers that allow successful identification of who will or will not respond to therapy are desperately needed to allow rational treatment decisions to be made.

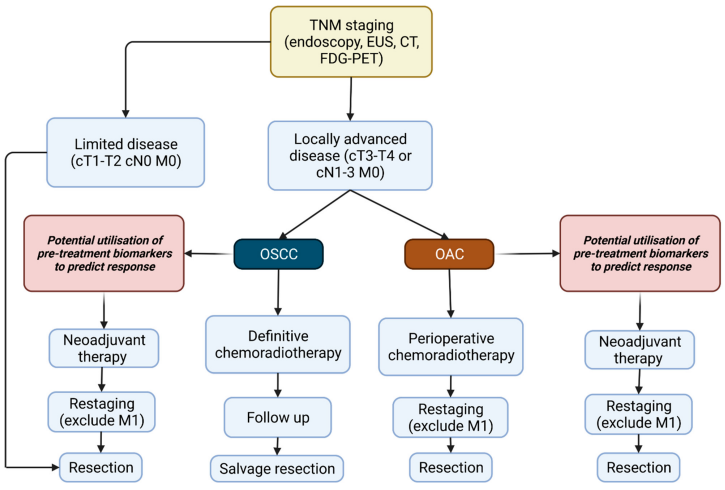

Figure 1. Current treatment strategies for oesophageal cancer as outlined by the European Society of Medical Oncology. TNM staging: T describes tumour size and any cancer spread into adjacent tissue; N describes cancer spread to adjacent lymph nodes; M describes metastasis (Adapted from "Oesophageal Cancer: ESMO clinical Practice Guidelines" (2016) [27]. Created with BioRender.com, accessed on 17 February 2022).

1.5. Function of miRNAs and Their Role in Cancer

MicroRNAs (miRNAs) are single stranded noncoding RNA molecules approximately 22 nucleotides long, regulating gene expression at the transcriptional or post-transcriptional level [36][37]. They do this by binding in a specific sequence to the complementary region in the 3′ untranslated mRNA region, which then regulates the translation of mRNAs to proteins [38]. miRNAs can bind to a complementary mRNA region resulting either in blockage of translation or degradation of a section of miRNAs via the RNA-induced Silencing Complex (RISC) complex, both leading to inactivation of a gene (**Figure 2**) [37][39]. Common molecules regulated are signalling proteins as well as transcription factors to RNA binding proteins [40]. miRNAs play an important role in biological pathways and their expression is dysregulated in multiple pathological mechanisms [41]. Aberrant miRNAs expression patterns are involved in the initiation and progression of oncogenesis due to their role as TSG and oncogenes. Oncogenic miRNAs target and prevent the expression of endogenous TSG, which activate pathways associated with OC, such as the reduced expression of miR-27a leading to permanent activation of the *KRAS* pathway [42]. TSG are often downregulated, dysfunctional or completely lost in OC such as miR-30b-5p [43] or miR-34a [44] in OSCC whereas those associated with proto-oncogenes are upregulated [45].

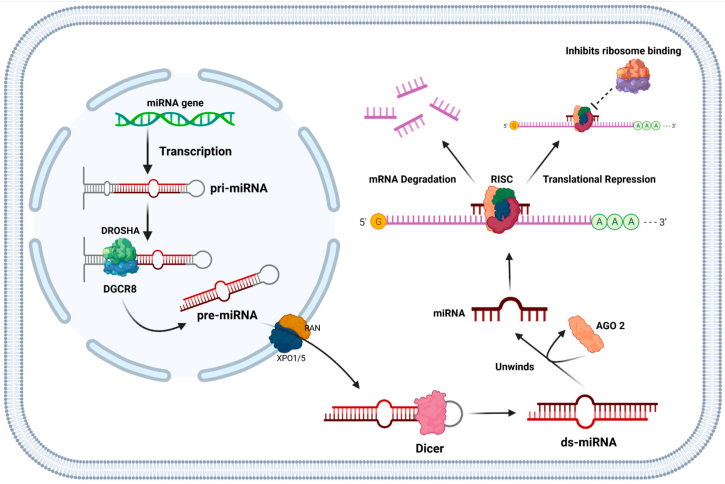

Figure 2. Synthesis and action of miRNA in post-transcriptional gene regulation. Transcription of miRNA gene via RNA Polymerase II forms pri-miRNA, DROSHA (class 2 ribonuclease III enzyme) and DGCR8 cleave the terminal end of the miRNA hairpin to form pre-miRNA. This is exported via RAN and XPO1/5. The miRNA hairpin is then cleaved by Dicer. AGO-2 binds to the double stranded miRNA, unwinds and dissociates the strands then forms a complex with RISC. This leads to either miRNA degradation or inhibition of ribosome binding. Abbreviations: pri-miRNA: primary-miRNA, pre-miRNA: precursor-miRNA, DGCR8: DiGeorge syndrome critical region gene 8, XPO 1/5: Exportin 1/5, RISC: RNA-induced silencing complex. (Created with BioRender.com, accessed on 16 January2022).

Recent research into the effects of NAT on miRNAs expression in other cancers such as breast cancer and rectal cancer have shown promising results. Over the course of NAT Lindholm et al. (2019) [46] showed that tumour suppressor miRNAs expression, such as miR-100-5p and miR-125b, were upregulated following treatment [46] which may reflect a role in the regulation of chemosensitivity. Kheirelseid et al. (2013) [47] identified expression signatures of miR-16, miR-590-3p and miR-561 that were predictive of complete versus incomplete response to neoadjuvant chemoradiotherapy in pre-treatment samples of rectal cancer [47]. A recent study showed that several miRNAs can predict poorer overall survival in both OSCC and OAC [48] such as the upregulation of miR-21 and downregulation of miR-133a. Hence, using miRNAs as predictors of pre-treatment response as well as other factors such as survival, seems to be a viable non-invasive potential solution to improving the accuracy of patient allocation to treatment.

2. Current Insights

The ability to predict OC in patients that will respond to NAT is vital to improve clinical outcomes whilst reducing treatment associated morbidity. This entry demonstrates the potential utility of miRNAs as biomarkers of response (**Figure 3**), with miR-193b, miR-21 and miR-200c showing the most promising results. However, the utilization of singular miRNAs to predict response to NAT is unlikely to be as sensitive or specific as looking at the miRNA expression profiles of multiple miRNAs. The

findings of Wen et al. (2016) [49] and the utilization of machine learning models such as SVM–RBF are likely to prove most beneficial, with the latest research showing excellent ability for machine learning techniques to predict events such as recurrence of OC after surgery [50] when looking at postoperative histopathological characteristics. This same model could be utilized preoperatively, looking at miRNA expression profiles to discern whether patients are likely to respond to NAT.

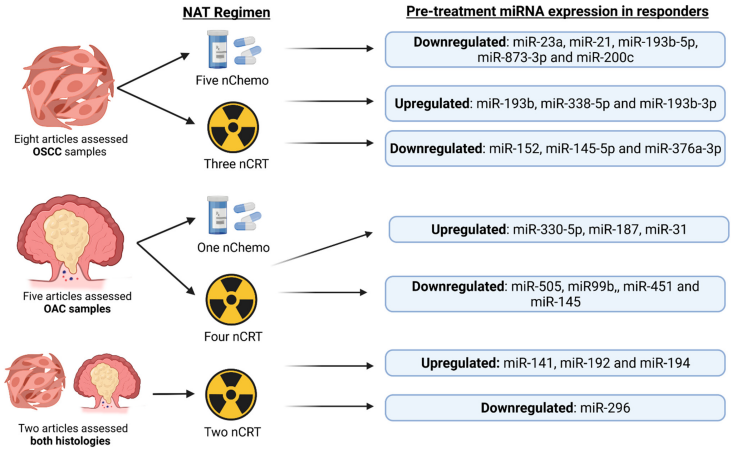

Figure 3. Overview of results produced by all 15 discussed articles. The NAT regimen utilised and how this relates to pre-treatment expression profiles in responders to nChemo or nCRT is shown. Abbreviations: OSCC; Oesophageal Squamous Cell Carcinoma, OAC; Oesophageal Adenocarcinoma, nChemo; Neopadjuvant chemotherapy, nCRT; Neoadjuvant Chemoradiotherapy. (Created with BioRender.com, accessed on 17 February 2022).

In clinical practice, there are currently no means of stratifying patients into responders and non-responders to NAT in OC. The implementation of miRNA screening prior to the initiation of NAT would allow for patients and the healthcare professionals supporting them to make a more informed decision as to whether treatment is likely to prove beneficial. This is important due to the risks of NAT and the effect it has on the limited length of time and quality of life patients may have. By utilizing a method of predicting response to NAT, such as miRNA screening, in conjunction with new non-invasive diagnostics such as Cytosponge™ technology [25][51] a minimally invasive widespread screening programmed in those at high risk of OC could be formulated. However, research would be needed to understand the ability to predict response utilizing miRNAs from pretherapeutic Cytosponge™ samples.

Despite the number of biomarkers discovered and studied, less than 0.1% are utilized in clinical practice [52]. Most often, this is a result of restricted study design and insufficient sample size or representation. In practice, utilization of miRNAs could surpass these clinical hurdles via the use of multiple different models for miRNA expression in patients with OAC versus OSCC and those receiving nChemo versus nCRT. Establishing the clear differences in miRNA expression between treatment types and doses, and linking these with OC histology, is a key step in establishing miRNAs as clinically viable biomarkers. For each set of treatments, a well-designed study with a large sample size and accurate measurements of predictability, such as AUROC, would prove robust enough for potential implementation into practice. Despite this, there are considerable obstacles for the application of pre-treatment miRNA testing within clinical practice. For example, standardisation in the extraction of miRNAs from either tissues or body fluids. Studies have shown miRNA concentrations in samples can not only differ between tissues and bodily fluids but also be directly affected by the method of extraction itself [53][54]. Further to this, when assessing circulating fluid samples miRNA concentration differs between subfractions (e.g., whole blood, peripheral blood mononuclear cells and plasma), thus it is important to standardise the method of extraction and the subfraction from which miRNAs are to be studied [55]. Studies identifying circulating miRNAs that can predict response to NAT are much more likely to be valuable in clinical practice due their greater accessibility and obtaining these samples is less invasive compared to tissue biopsies. Articles reviewing intratumoral miRNA concentrations are more useful in determining the functional mechanisms by which miRNA expression links to how patients respond to nChemo/nCRT. In addition to this, assessing miRNA expression *in vitro* often leads to differential results between samples due to the interplay between miRNA expression and the intratumoural microenvironment [56][57]. Based on this, future translational research must focus on the standardisation of miRNA sampling and extraction in circulating fluids, in order to become robust enough biomarkers to use in clinical practice.

Current Use of miR-21, miR-193b and miR-200c in Cancers and Their Functional Roles

This entry has provided evidence suggesting that miRNAs may be robust biomarkers for predicting response to NAT by differentiating between responders and non-responders. These studies suggested miR-505, miR-99b, miR-45 (for OAC patients) and miR-193b (for OSCC patients) are accurate biomarkers for predicting response to NAT. In the literature presented in this entry the three miRNAs that have consistently appeared significant in both histological OC subtypes, namely

OAC and OSCC, are miR-21, miR-193b and miR-200c thus their overall function in OAC and OSCC and other common cancers is discussed.

MiR-21 is a commonly dysregulated in a wide variety of cancers such as renal carcinoma, non-small cell lung cancer, gastric cancer, colon cancer and breast cancer [58]. In oesophageal cancer, high miR-21 has been associated with increased stromal fibroblast activity and increased cell migration [59]; therefore, it is thought to act as an oncogene during the neoplastic life cycle of OSCC with its function being less clear in OAC [58][60]. Studies suggest miR-21 is a useful biomarker in the prediction of response to other cancers such as *HER2* positive breast cancer and colorectal cancers [61][62].

The absolute role of miR-193b in oesophageal cancer is not fully understood, despite the miRNA being known to act as a TSG in various types of gastric and colon cancer [63][64]. Various studies have shown miR-193b initiates apoptosis via the Akt pathway such as in gastric cancers or promotes autophagy and non-apoptotic cell death thereby sensitising cells to chemotherapy [64]. In oesophageal cancer, miR-193b directly targets the *KRAS* pathway and thus, as discussed previously for Hummel et al. (2014), its upregulation in the state of cancer would be expected as it exerts negative transcriptional control to halt cellular proliferation [65]. A 2013 study suggested that dosages of ionizing radiation can manipulate the expression profile for miR-193b in some cancers [66], as supported by the results of Chan et al. (2017) [67] and Wen et al. (2016) [49] whereby miR-193b's expression profile differed with the addition of radiotherapy. Therefore, this could have affected the patient's expression profiles should their radiation dosages have differed between patients.

Despite the conclusions made by Tanaka et al. (2013) [68] and Mahawongkajit et al. (2020) [69] there is little conclusive evidence as to the exact function of miR-200c. The miR-200 family have been shown to be tumour suppressor genes in ovarian cancers [70] in addition to their downregulation upon neoplastic progression of Barrett's oesophagus [71]. There is additional evidence that miR-200c overexpression may play a role in chemoresistance of oesophageal cancers via also interacting with the Akt pathway [72].

Despite this, research is still needed to elucidate the absolute role of these miRNAs in a therapeutic response, and crucially, how the timings of therapeutic administration may affect miRNA expression and thus their value in predicting response. The efforts of future research need to focus on understanding the effects NAT regimes have on the predictive value of each miRNA individually, yet links must be formed to produce a multi-miRNA model for accurate prediction of NAT response with clinical utility to allow optimal patient benefit. Given the low response rate to standard of care chemotherapy agents and significantly high mortality and morbidity of OAC and OSCC, robust and predictive biomarkers of NAT response are urgently needed in the clinic and must become a research priority.

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