EDITORIAL

From blood to breath: New horizons for esophageal cancer biomarkers

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Abstract

Esophageal cancer is a lethal cancer encompassing adenocarcinoma and squamous cell carcinoma sub-types. The global incidence of esophageal cancer is increasing world-wide, associated with the increased prevalence of associated risk factors. The asymptomatic nature of disease often leads to late diagnosis and five-year survival rates of less than 15%. Current diagnostic tools are restricted to invasive and costly endoscopy and biopsy for histopathology. Minimally and non-invasive biomarkers of esophageal cancer are needed to facilitate earlier detection and better clinical management of patients. This paper summarises recent insights into the development and clinical validation of esophageal cancer biomarkers, focussing on circulating markers in the blood, and the emerging area of breath and odorant biomarkers.

Key words: Breath analysis; Cancer; MicroRNA; Non-invasive; Esophageal cancer; Biomarker

Core tip: The current "gold standard" test for detection of esophageal cancer is endoscopic imaging and confirmation by biopsy. There are several barriers to endoscopy as a clinical tool to monitor patients at high risk of esophageal cancer, including high capital and personnel costs and the invasive nature of the procedure. This paper highlight new insights into the development and clinical validation of circulating and breath biomarkers of esophageal cancer.

INTRODUCTION

Esophageal cancer is the 6th leading cause of cancer related mortality death worldwide[1,2]. Histologically it is classified into two sub-types, squamous cell carcinoma

and adenocarcinoma, each with a specific cellular origin, pathogenesis and epidemiology[3,4].

The current gold standard techniques for the detection and diagnosis of esophageal cancer, endoscopy and biopsy, are invasive. Furthermore, due to the lack of symptoms at earlier disease stages, presentation and diagnosis usually occurs late, leading to poor prognosis and 5 year survival rates as low as 15%[4,5]. Early diagnosis is associated with much higher 5 year survival rates[4], and when confined to the mucosa disease specific survival rates of up to 98% are reported[6]. Better diagnostic methods are needed to shift the majority of diagnoses to the earliest stages, and expanded access to conventional endoscopy and wider use in a screening context is not cost effective. Minimally and non-invasive biomarkers, primarily in the blood and breath, represent the most likely candidates to facilitate early detection of esophageal cancer.

Several candidate biomarkers for esophageal cancer have been proposed. However, their translation into clinical use has been slow. Biomarkers can be broadly defined as quantifiable parameters that assist in distinguishing normal from pathological processes[7,8] with applications for diagnosis, prognosis and tailoring of patient treatment[9]. In this paper we summarise recent insights into the development and clinical validation of esophageal cancer biomarkers. Whilst we recognise that there is a significant body of research which has been undertaken evaluating tissue based biomarkers in esophageal cancer, this review has deliberately focussed on minimally invasive and non-invasive methods for detection of esophageal cancer, principally circulating markers in the blood, and the emerging area of breath and odorant biomarkers. The development of robust, minimally invasive, cost effective biomarkers for early cancer will change current diagnostic, prognostic and surveillance paradigms, and could open the possibility of population screening.

SQUAMOUS CELL CARCINOMA

Esophageal squamous cell carcinoma is the most frequently diagnosed subtype of esophageal cancer worldwide[10] and typically arises in the mid and lower thirds of the esophagus[10]. The highest prevalence of squamous cell carcinoma is found within regions of Eastern Asia[11], largely attributed to the prevalence of risk factors such as tobacco smoking and the consumption of herbal tea maté and pickled vegetables[12,13]. Conversely, in Westernised societies such as the United States and Australia, incidence rates of squamous cell carcinoma have been in decline since 1998, largely due to a decline in cigarette smoking[4,12,14].

The pathogenesis of squamous cell carcinoma is highly complex, involving an accumulation of genetic modifications within the esophageal mucosa, causing progressive changes that result in invasive carcinoma[15,16]. Environmental factors, diet, smoking and alcohol consumption have been strongly implicated in the molecular mechanisms for squamous cell carcinoma; however, evidence of a causal relationship is lacking[15,17]. Genetic mutations within cyclin D1 and the tumour suppressor gene, *TP53,* are among the most frequently isolated genetic abnormalities from esophageal squamous cell carcinomas[15].

TP53 is a tumour suppressor gene with roles in DNA repair and cell cycle arrest, and is the most common mutation found in cancers, including esophageal squamous cell carcinoma[15,17,18]. *TP53* mutations have been reported in as little as 10% and up to 80% of esophageal squamous cell carcinoma[19]. Additionally, mutations to the *TP53* gene are also found in dysplastic lesions[15,16], indicating *TP53* mutations may be an event in the early stages of esophageal squamous cell carcinoma carcinogenesis. *TP53* mutations produce abnormal TP53 protein that accumulates in the nuclei of cells which may be identified by immunohistochemistry[15,19,20]. Positive *p53* staining has been demonstrated in the non-cancerous cells adjacent to tumors[19] and in cells lacking the commonly identified *TP53* mutations[20]; indicating poor specificity and sensitivity of the technique and potentially additional mutations within the gene accounting for the positive staining[20].

ESOPHAGEAL ADENOCARCINOMA

Esophageal adenocarcinoma is a highly lethal tumour usually developing in the lower third of the esophagus, or at the gastro-esophageal junction[21]. Incidence rates have risen gradually in developed countries since 1984, with a 4% increase in the incidence of adenocarcinoma in Australia between 1988 and 2005[22]. Adenocarcinoma is most prevalent in males, the elderly and the obese. However, the most significant risk factor identified for adenocarcinoma is Barrett's esophagus[12,23,24].

Barrett's esophagus is a metaplastic condition of the esophageal epithelium, affecting up to 2% of the adult population[6]. It is defined histologically by the replacement of the normal stratified squamous epithelium with a columnar epithelium with intestinal metaplasia defined by the presence of goblet cells, as a result of chronic gastro-esophageal reflux disease[24]. Gastro-esophageal reflux disease is characterised by increased acid and bile exposure to the esophageal mucosa, a consequence of extended relaxation of the lower esophageal sphincter, which may lead to esophagitis and progress to Barrett's esophagus[25]. The development of Barrett's epithelium is considered a protective mechanism, as columnar epithelium is more resistant to the harmful effects of acid and bile than the normal stratified squamous epithelium of the esophagus[26]. However, Barrett's esophagus, is also a hyper-proliferative condition, susceptible to malignant progression in some individuals[27]. Dysplasia is one of

the initial changes identified with malignant progression, characterised by cellular distortion and changes in the nuclei such as crowding and hyperchromatism[28].

Patients with Barrett's esophagus have varied risk of progression to adenocarcinoma[29], with some studies suggesting 40%-75% of cases of esophageal adenocarcinoma lack evidence of Barrett's eso-phagus[30,31]. It has been suggested the absence of any evidence of Barrett's esophagus could suggest an alternate, yet to be identified, pathogenic pathways[31], and that Barrett's esophagus is simply a strong risk factor in a subset of the population, but not a nece-ssary carcinogenic step in the development of eso-phageal adenocarcinoma[32]. However, the absence of useful tools for the early identification and ongoing assessment of Barrett's esophagus and progression to adenocarcinoma has made assessment of this relationship challenging.

CURRENT DIAGNOSTIC METHODS OF ESOPHAGEAL CANCER

The diagnosis of esophageal cancer and its prema-lignant lesions is currently limited to endoscopy and subsequent biopsy analysis[11]. Endoscopy is a highly invasive and costly diagnostic procedure[33,34] and is the current gold-standard diagnostic technique for esophageal cancer and its precursor lesions[4]. Standard white light endoscopy is limited in its scope, restricted to the identification of macroscopic abnormalities that may indicate cancer, such as nodules and ulcers, consequently failing to identify early lesions that appear macroscopically normal[4]. Whilst Barrett's esophagus is visible endoscopically, dysplasia within the Barrett's segment is more difficult to identify as lesions are often flat and difficult to distinguish from surrounding non-dysplastic columnar epithelium[33].

Classification of dysplasia is subjective and studies have shown differentiation between grades of dysplasia is highly variable amongst pathologists, leading to incorrect diagnosis and un-necessary procedures[5,35]. Likewise, random biopsy protocols is prone to sampling error[36], furthering the potential for misdiagnosis.

Surveillance using endoscopy and biopsy is generally recommended for patients with Barrett's esophagus, in order to diagnose esophageal cancer at its earliest stage[37]. As a result of the low progression rate of early lesions, such as Barrett's esophagus to adenocarcinoma and the costs of endoscopy surveillance, it may not be cost effective to employ the current diagnostic procedures in surveillance programs for esophageal cancer, and screening programs have never been considered feasible[35]. Thus, there is an acute need for the development of more selective and less invasive diagnostic techniques for individuals at risk of eso-phageal cancer.

EMERGING BIOMARKERS OF ESOPHAGEAL CANCER

Blood biomarkers

Autoantibodies have drawn appeal as serology markers for esophageal cancer, owing to their stability and persistence in serum samples. With improvements in antibody detection technologies improving the detection limits, there is a growing interest in the utility of autoantibodies as diagnostic and prognostic biomarkers for esophageal cancer. Perhaps the most comprehensively investigated has been the tumour suppressor gene, *TP53*. The protein product of *TP53* is a nuclear phosphoprotein and in normal human plasma, the TP53 protein and anti-p53 antibodies are absent[38]. *p53* mutations can cause accumulation of non-functional protein that has increased stability and a longer half-life than the native protein[38]. The subsequent production of anti-p53 has been detected in tissue, blood and other body fluids of several cancer types, including esophageal cancer. A meta-analysis by Zhang *et al*[38] summarizing the diagnostic value of anti-p53 for esophageal cancer found that patients with esophageal cancer were seven times more likely to be positive for plasma anti-p53 compared to non-cancer controls. However, despite the high specificity, the authors reported low sensitivity, suggesting limited clinical application.

More recently, a systematic review investigated the diagnostic utility of 35 different circulating autoantibodies, both alone and in combination, as biomarkers for the early detection of esophageal cancer[39]. Although the study did not distinguish between the two esophageal cancer sub-types, the majority of the studies included in the review were esophageal squamous cell carcinoma, a greater world-wide burden of this variant compared to the adenocarcinoma sub-type[39]. Although the vast majority of studies reviewed reported positive associations between their candidate biomarker, and esophageal cancer, with high specificity reported, the sensitivity values were generally too low to be of any clinical significance[39]. However, combinations of autoantibodies did slightly improve the median sensitivity. The authors also conducted a meta-analysis on the diagnostic value of anti-p53, reporting a significant association of serum anti-p53 with esophageal cancer, with sensitivity of 91.4% and specificity of 65%[39], contrasting their previous findings for meta-analysis of anti-p53[38].

Six serum biochemical markers that included anti-p53, carcinoembryonic antigen, squamous cell cancer antigen, cytokeratin 21-1 fragment (CYFRA21-1), vascular endothelial growth factor-C and microRNA (miRNA) were reviewed in a meta-analysis by Zhang *et al*[40]. Although each biomarker candidate was associated with a positive odds ratio for esophageal cancer, and high specificity values by receiver operating characteristic curves, the sensitivity for each test was

again low, with high variability between studies[40]. Although the authors suggest that combinations of the serum markers are likely to yield better sensitivity and specificity, it is more likely that the better designed, more robust, prospective, multi-centre studies are needed to better optimise and validate candidate serum biochemical markers.

Circulating tumor cells (CTCs) originate from the primary tumor, and are released into the circulation, where they may form micro-metastases. Various assays have been developed and used to assess the diagnostic and prognostic potential of CTCs in several cancer types, including breast, colorectal, gastric and esopahgeal cancer[41]. A recent meta-analysis by Qiao *et al*[42] aimed to determine the association between CTCs and clinicopathological characteristics and prognosis (tumor stage, lymph node metastasis, distant metastasis and patient survival) in esophageal cancer. The presence of CTCs was found to correlate strongly with poor overall patient survival, and predicted poor progression free survival in Asian populations with esophageal squamous cell carcinoma[42]. CTCs also correlated with venous invasion and metastasis to local lymph nodes (N-staging). New methodologies to quantify circulating tumor DNA might also offer new diagnostic potential, but more work is needed to evaluate this possibility.

Blood biomarkers for esophageal cancer represent new tools for the early detection and prognosis. However, despite the large number of candidate markers that have been published, there remains a paucity of large, well-designed, prospective multi-centre validation studies for both esophageal squamous cell carcinoma and esophageal adenocarcinoma.

CIRCULATING miRNA

miRNA's are single stranded, non-coding RNA's that can regulate gene and protein expression[43,44]. miRNAs are abundantly expressed in a stable form, with highly consistent levels amongst individuals in a range of extracellular fluids including blood serum and plasma, and have drawn attention as biomarkers for cancer and disease[43,44]. Recent studies have reported plasma/serum circulating miRNAs to be potential diagnostic and prognostic markers in some gastrointestinal cancers - esophageal squamous, esophageal adeno-carcinoma, gastric and colorectal[43,44]. Although still an emerging area of research, recent meta-analyses have highlighted the potential of circulating miRNAs for the detection of esophageal cancer.

A review by Wang *et al*[45] of eight manuscripts investigated a total of 16 different types of miRNAs in serum and saliva of Asian esophageal squamous cell carcinoma patients. The authors reported relatively high sensitivity and specificity values for combination and single miRNA markers, suggesting some diagnostic application[45]. The prognostic utility of miRNAs have also been reviewed, with Fu *et al*[46] reporting on 39 potentially prognostic miRNAs in 25 individual studies. miR-21 and miR-375 were found to be potentially prognostic of overall survival[46]. However, the small number of manuscripts that could be included in the study, and the lack of validation studies performed using the miRNA markers limits the conclusions that can be drawn for translational application. A more comprehensive meta-analysis by Fu *et al*[47] found that although increased expression of miR-21 and decreased expression of miR-375 were significantly associated with poor overall survival in esophageal cancer, both miR-21 and miR-275 were associated with low hazard ratios.

Circulating miRNAs have also been investigated as biomarkers of esophageal adenocarcinoma and the pre-cursor condition, Barrett's esophagus. In a retrospective study of bio-banked serum samples from esophageal cancer patients, Chiam *et al*[43] identified five miRNA ratios, derived from ten unique miRNAs that were discriminatory for esophageal adenocarcinoma over non-dysplastic Barrett's eso-phagus and healthy controls. The predictive accuracy of the miRNA ratios was enhanced with stepwise addition of each miRNA ratio to an analysis of the cancer patient's blood sample[43], highlighting the potential for biomarker combination approaches to enhance test specificity and sensitivity.

BREATH BIOMARKERS

Breath analysis represents an attractive modality for the early detection of cancer, as it is completely non-invasive, relatively cheap compared to conventional methods, and provides a rapid result following sample collection. Breath volatile organic compounds (VOCs) as biomarkers of disease have been recognised since the time of Hippocrates in Ancient Greece, who described *fetor hepaticus* and *fetor oris* in his treatise on breath aroma and disease[48]. It is now known that a single human breath is a complex gas mixture of more than 2000 unique VOCs, representing a reservoir of potential cancer biomarkers[49]. Breath VOCs have already shown clinical utility as possible biomarkers for lung[50,51], breast[52,53], prostate[54], colorectal[55], gastric[56] and recently, esophageal cancer.

Many studies have associated breath alkanes with cancer, presumably as a bi-product of oxidative stress pathways[57]. Breath ethane has previously been investigated in late stage esophageal squamous cell carcinoma and adenocarcinoma, with no differences compared to healthy controls[58]. More advanced technologies have since been used to characterise VOCs associated with esophageal cancer. Headspace analysis of urine[59] and gastric contents[60] from esophageal cancer patients by selected ion flow tube-mass spectrometry identified several VOCs that were differentially regulated compared to healthy controls. However, there was no predominant group of VOCs in the cancer group.

The first breath analysis study to define breath VOCs in esophageal cancer identified a phenols dominant expression pattern, with phenol, methyl phenol, ethyl phenol and hexanoic acid significantly increased in esophageal cancer compared to healthy controls[61]. In the most comprehensive study to date, Kumar *et al*[62] investigated breath VOCs in esophageal squamous cell carcinoma, esophageal adenocarcinoma, Barrett's esophagus, benign conditions and gastric adenocarcinoma, compared to healthy controls. A total of 12 VOCs, comprised of phenols, aldehydes and fatty acids were identified as being discriminatory for esophageal cancer and gastric cancer compared to normal upper gastrointestinal (GI) tract[62]. Additionally, the authors found the VOC profile distinguished esophageal cancer from Barrett's metaplasia and from benign conditions of the upper GI tract (which included esophagitis, esophageal stricture, and esophageal candidiasis). Developing a risk prediction model, the authors reported eight significant predictors for adenocarcinoma: decanal, nonanal, phenol, ethyl phenol, methyl phenol, hexanoic acid, heptanal, and butyric acid, with sensitivity and specificity of 98% and 91.7% respectively when compared to normal upper GI tract[62]. Furthermore, the model accurately discriminated esophageal adenocarcinoma from non-cancer controls (benign conditions, Barrett's meta-plasia and normal upper GI tract), with sensitivity and specificity of 87.5% and 82.9% respectively[62]. Interestingly, no differences in VOCs were detected between early and late stage cancers, or between tumour size and concentrations of VOCs.

Proton Transfer Reaction-Mass Spectrometry has recently been used to identify breath VOCs in a small study of Chinese esophageal cancer patients[63]. Although the study did not differentiate between esophageal squamous cell carcinoma and esophageal adeno-carcinoma, the authors reported 20 ion peaks in the full mass spectra that were significantly different in cancer patients compared to healthy controls[63]. Using stepwise discriminant analysis, the authors identified seven ions that were highly discriminatory for esophageal cancer[63]. In contrast to the study by Kumar *et al*[62] the authors also suggested that their predictive model discriminated for early and late stage cancer. However, these interpretations should be carefully balanced against the small participant numbers used in the study.

CONCLUSION

Current diagnostic and surveillance procedures for esophageal cancer are invasive, expensive and ill-adapted for early detection. Recent advances have been made in the development and validation of new minimally and non-invasive biomarkers for esophageal cancer. Although several novel serology markers have been investigated, these have not translated to validated clinical tools. Circulating anti-p53 and

CTCs have shown the most promise as diagnostic and prognostic markers of esophageal cancer, with recent meta-analyses supporting their use. However, the absence of well-designed, robust clinical validation trials in large patient cohorts largely limits the power of these meta-analyses. This is highlighted by the lack of differentiation between esophageal squamous cell carcinoma and esophageal adenocarcinoma, despite distinct pathologies and molecular profiles.

Circulating miRNAs have emerged as promising new biomarkers of esophageal cancers. Despite their promise, several studies have limited their focus to esophageal squamous cell carcinoma, and small clinical cohorts, with many focussing on single miRNAs rather than combined approaches. Advances in bioinformatics have facilitated analysis of large, complex miRNA microarray datasets, and future studies are likely to employ combined approaches for miRNA analysis.

The emerging field of breath and gas analysis for cancer detection represents a completely non-invasive approach to early detection, and ongoing screening of at risk individuals. With improvements in the sensitivity of VOC detection technologies, it is likely that the pool of possible breath and odorant biomarkers will significantly increase. Despite relatively few studies having investigated breath biomarkers in esophageal cancer, the initial predictive models have shown some promise. Moving forward in this rapidly developing field, it is critical that standardised approaches to the collection of breath samples are employed, to minimise study heterogeneity. Furthermore, with little evidence to support the biological origins of VOCs, mechanistic studies to better understand how VOCs are produced in cancer cells will help to improve the sensitivity and specificity of future tests.

As the incidence of esophageal cancer continues to grow world-wide, new diagnostic and prognostic tools are needed to improve survival and direct clinical management. The advances being made in new minimally and non-invasive biomarkers represents a suite of ancillary tests that could stratify patients for endoscopic and other imaging modalities, ultimately leading to improved patient care. While it is unlikely that there will ever be a single "silver bullet" biomarker, the most likely scenario will be derived from predictive algorithms based on multiple biomarkers, which could also include combinations of blood and breath analysis.

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