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Blood-based biomarkers for early detection of esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a common malignant tumor of the digestive system worldwide, especially in China. Due to the lack of effective early detection methods, ESCC patients often present at an advanced stage at the time of diagnosis, which seriously affects the prognosis of patients. At present, early detection of ESCC mainly depends on invasive and expensive endoscopy and histopathological biopsy. Therefore, there is an unmet need for a non-invasive method to detect ESCC in the early stages. With the emergence of a large class of non-invasive diagnostic tools, serum tumor markers have attracted much attention because of their potential for detection of early tumors. Therefore, the identification of serum tumor markers for early detection of ESCC is undoubtedly one of the most effective ways to achieve early diagnosis and treatment of ESCC. This article reviews the recent advances in the discovery of blood-based ESCC biomarkers, and discusses the origins, clinical applications, and technical challenges of clinical validation of various types of biomarkers.

Key words: Esophageal squamous cell carcinoma; Biomarker; Diagnosis; Blood-based; Autoantibodies; MicroRNA

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Core tip: At present, the early detection of esophageal squamous cell carcinoma (ESCC) mainly depends on endoscopy and histopathological biopsy. However, the high cost and invasiveness of endoscopy have limited its use as a tool for screening the general population. Blood tests provide a non-invasive method for early detection of ESCC. Therefore, this article reviews the recent advances in the discovery of blood-based

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biomarkers in the early detection of ESCC.

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INTRODUCTION

Esophageal cancer (EC) is one of the most aggressive carcinomas of the digestive tract. The incidence of EC ranks seventh among all malignant tumors, and the mortality rate ranks sixth among cancer-related deaths. Globally, there were an estimated 572034 new cases of and 508585 deaths due to EC in 2018^[1]. There are two main histological forms of EC: Esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC)^[2,3]. These two major histological subtypes differ in etiology and geographic incidence, with esophageal adenocarcinoma being more common in Western populations^[3-5] and ESCC being more common in the Eastern populations^[3,6,7]. China has one of the highest incidences of ESCC, with more than 90% of EC patients in China suffering from ESCC^[1,6,7]. In recent years, although the incidence of EC in China has declined, the absolute incidence of EC remains high because of the large population^[8].

At present, the five-year survival of EC is 15%-25%^[9,10], but survival can be as high as 80% if EC is caught in the early stages^[11-13]. The reality is that most EC patients are diagnosed and treated in the advanced stages, which is the primary cause of the poor prognosis of EC. Although endoscopy has been proven to be effective in detecting early-stage EC and can reduce the mortality^[14], the high cost and invasiveness limit its use as a wide-ranging screening tool for early-stage EC^[15,16] (Table 1). Finding a non-invasive method for early detection of EC will undoubtedly be an effective way to improve the early diagnosis rate and prognosis of EC^[16-18].

Blood-based biomarker tests provide simpler, less invasive alternatives^[17]. Early detection of susceptible populations by detecting nucleic acid or protein molecular markers in the blood has become an area of intense investigation in current tumor diagnosis research. In recent years, studies on ESCC serum biomarkers have revealed a variety of cancer-related molecules, including autoantibodies against various tumor-associated antigens (TAAs), microRNAs (miRNAs), various non-coding RNAs, cytokines, proteins, circulating tumor cells (CTC), and circulating tumor DNA (ctDNA). Each type of biomarker provides different information on disease status, with different advantages and disadvantages and different clinical applications. Detection of these biomarkers may provide a new effective means for screening, diagnosis, monitoring, and prognosis of tumors. Here, we provide an overview of the most promising blood-based biomarkers for future screening of ESCC, and extract basic performance characteristics [e.g., sensitivity, specificity, and area under the receiver operating characteristic curve (AUC)] for each study of serum tumor biomarkers.

ORIGIN OF AND SCREENING CRITERIA FOR TUMOR MARKERS

A tumor marker (TM) is defined here as a substance secreted by or released from tumor cells, or a host reaction to the tumor tissue, and present within body fluid and tissue. TMs can reflect the occurrence, development, and detection of tumor response to treatment, and include a wide range of molecules, such as protein, miRNA, RNA, DNA, methylated DNA, metabolites, carbohydrates, autoantibody, lipids, and circulating tumor cells themselves^[19]. Since Henry Bence Jones discovered Bence Jones protein in 1846, providing the first TM for clinical diagnosis, in this case for multiple myeloma^[20], TMs have been studied for more than 100 years. However, it was not until Abelev *et al*^[21] discovered alpha-fetoprotein in 1963, and Gold and Freeman^[22] discovered carcinoembryonic antigen in 1965 that TM assays became widely used clinically.

The advantage of TM detection is that it might be able to demonstrate the existence

Table 1 Technologies for detection of esophageal squamous cell carcinoma

Technology	Quantitative	Qualitative	Advantages	Disadvantage	Ref.
Endoscopy	-	+	(1) Obvious observation of esophageal mucosal changes, lesion changes, and lesion size and morphology; (2) Low false negative rate and false positive rate	(1) Invasiveness; (2) Easy to cause complications such as sore throat after examination; (3) High cost	[15,16]
Blood-based biomarker	+	+	(1) Non-invasive; (2) Easy to operate; (3) Low cost; (4) Suitable as a screening tool; (5) Identification of asymptomatic patients at risk	High false Negative rate and false positive rate	[16-18]

of a malignant tumor before observable imaging changes, and assist in diagnosis and analysis of the patient condition, leading to the early diagnosis and treatment of cancer. The ideal TM needs to have the following characteristics: (1) High sensitivity and expression in early-stage tumors^[23]; (2) Good specificity, only being positive in tumor patients, for differential diagnosis between benign and malignant tumors^[23]; (3) The ability to locate the tumor and possess organ specificity (although so far, no markers with complete organ and tumor specificity have been found. At present, alpha-fetoprotein is the only marker that can be used for early diagnosis and screening of primary liver cancer^[24], whereas pathological diagnosis is still the main way to diagnose other malignant tumors); (4) Be related to the severity of the disease, tumor size, or stage; (5) Can predict the prognosis of tumor treatment (such as response to postoperative radiotherapy or chemotherapy), and the corresponding TM can be quickly reduced to normal levels and maintained, indicating a good prognosis; for example, prostate-specific antigen (PSA) is a typical biomarker for recurrence after non-surgical treatment of prostate cancer^[25]; and (6) Appears in body fluids, especially blood, and is easy to detect.

The research and application of TMs have opened up a new field for tumor diagnosis and clinical treatment. When the amount of tumor-related substances in the body fluid of tumor patients changes, it can indicate the existence of certain tumors. Identifying cancer specific molecules that help to distinguish between normal and cancerous conditions may develop more effective ESCC diagnostic tools.

CANCER-ASSOCIATED AUTOANTIBODIES

The theory of cancer autoantibody production is complex and not fully understood^[26]. Studies have shown that the human immune system can sense TAAs with abnormal structure, distribution, and function of certain cellular components involved in tumorigenesis^[27], and induce an autoantibody response. In addition, circulating anti-TAA antibodies can be detected several years before clinical diagnosis and might serve as new screening markers^[28-30]. Research on autoantibodies has been carried out for more than 100 years^[31]. At the beginning, researchers only found that autoantibodies were closely related to autoimmune diseases^[32]. However, a large body of epidemiological studies in recent years have shown that patients with autoimmune diseases have a significantly increased or decreased risk for certain cancers, suggesting that autoantibodies may promote or inhibit cancer progression^[32,33]. Antigen changes in cancer cells, which have been shown to be closely related to tumor proliferation and grade, induce the production of autoantibodies by the immune system^[34]. Therefore, autoantibodies targeting TAAs have been extensively studied in different types of cancer as novel tumor biomarkers. Autoantibodies are not only more sensitive and specific than antigens, but they have been present in all tumor types to date^[35,36] and can persist in the serum of cancer patients. With improvements in antibody detection technologies and detection limits, there will be growing utility for autoantibodies as diagnostic biomarkers for ESCC.

Increasing evidence shows that a single cancer-associated autoantibody biomarker has limited diagnostic value. The 70 studies summarized by Xu *et al*^[37] reported that the sensitivity of 49 autoantibodies ranged from 3.9% to 93.7%, with a specificity range of 78.7%-100%. Among the most studied individual markers in ESCC, there are autoantibodies against well-known TAAs such as p53, p16, c-Myc, survivin, NY-ESO-

1, and Hsp70 (Table 2)^[37-61]. The most commonly used antibody detection method is the ELISA. A meta-analysis by Zhang *et al.*^[61] summarized the diagnostic value of anti-p53 for EC, and found that the overall sensitivity and specificity of p53 autoantibodies to EC were 29.6% and 97.9%, respectively. Other studies have shown that anti-p16, c-Myc, survivin, and NY-ESO-1 autoantibodies have a high specificity but poor sensitivity (Table 2). Although one study reported that the sensitivity and specificity of Hsp70 autoantibodies could be as high as 93.7% and 100%, respectively, there were large-scale fluctuations between small samples and different studies^[60]. Overall, most anti-TAA autoantibody biomarkers are relatively less sensitive but more specific, indicating limited clinical utility for a single autoantibody. In general, shifting the cutoff toward a higher sensitivity leads to a reduced specificity and *vice versa*^[62].

Advances in technology have moved the field from investigations of individual candidate anti-TAA autoantibodies to high-throughput, larger-scale discovery efforts using methods such as serological proteome analysis^[63] and protein microarrays for the identification of novel anti-TAA autoantibodies^[64]. The emergence of these proteomics approaches has facilitated identification of promising anti-TAA autoantibodies. To the best of our knowledge, eight studies have been published on the diagnostic value of different ESCC-related autoantibody biomarker combinations^[37]. In these studies, the autoantibody signatures were able to distinguish ESCCs from healthy controls with a relatively high specificity and variable sensitivity. The sensitivity of autoantibody combinations ranged from 26.0% to 75.3%, and the specificity ranged from 81.0% to 98.8%. Xu *et al.*^[39] used two independent cohorts to study the combination of p53, NY-ESO-1, MMP-7, Hsp70, PRDX-6, and Bmi-1 autoantibodies with sensitivities/specificities of 57.0%/95.0% and 51.0%/96.0%, respectively. They also identified a simplified group of autoantibodies consisting of four anti-TAAs with a similar sensitivity and specificity in early stage ESCC. Another study reported an analysis of c-Myc, HCCR, p53, p62, IMP-1, and Koc in combination. The results showed that the sensitivity/specificity of this combination for distinguishing ESCC patients from the normal control group in the test and validation groups was 67.9%/86.7% and 67.7%/85.5%, respectively^[38]. Similar to the research strategy of Xu *et al.*^[39], Zhang *et al.*^[38] also identified a restricted panel of four TAAs that gave a similar sensitivity and specificity in early-stage ESCC. However, other than the above two studies, other literature did not report the diagnostic efficacy of the autoantibody panel for early-stage ESCC.

Although the above-mentioned anti-TAA autoantibody panel studies have shown satisfactory diagnostic value, due to the different research backgrounds, case characteristics (*e.g.*, diagnostic stage, tumor histology), cut-off values, and experimental methods, we observed that there were some differences in the diagnostic performance of these markers. Moreover, the age difference between the case and control groups in these studies was often large. It is known that the humoral immune response to self-antigens changes with aging^[65]. Therefore, the age imbalance between the cases and controls increases the selection bias and cannot be applied to the screening population. Moreover, current research on autoantibodies is lacking, and the anti-TAA autoantibodies we have identified so far may represent only a small fraction of the potential anti-TAA autoantibodies for diagnosis of ESCC. In fact, the diagnostic results of these biomarkers need to be validated in a larger multicenter cohort and evaluated in screening trials for high-risk populations. Therefore, standardized cross-validation studies are needed to validate and quantify the diagnostic potential of these markers^[66].

MICRORNAS

MiRNAs are highly conserved, non-coding single-stranded small RNA molecules encoded by endogenous genes, approximately 20 to 24 nucleotides in length^[67]. They can be involved in the regulation of a variety of biological functions, including cell differentiation, apoptosis, proliferation, and metabolism, by regulating the expression of target genes^[68]. In 2002, Calin *et al.*^[69] found that miRNAs are down-regulated in chronic B-lymphocytic leukemia, which is the first report of a relationship between miRNAs and tumors. It is currently believed that miRNAs mediate post-transcriptional gene expression regulation primarily by promoting both target mRNA degradation and protein translation inhibition. A growing number of studies have shown that different miRNAs play different roles in promoting cancer or tumor suppression, and these abnormally expressed miRNAs can unbalance the expression of oncogenic or suppressor genes in the body, eventually leading to tumor production^[70]. MiRNAs not only have abnormal expression in tumor tissues, but also have specific expression in patient serum. Recent studies have shown that tumor-

Table 2 Diagnostic performance of tumor-associated autoantibodies in esophageal squamous cell carcinoma

Target antigen of autoanti-body	Ref.	Year of publication	ESCC, <i>n</i>	Controls, <i>n</i>	All stages/early stage			<i>P</i> value	Detection method
					Sensitivity (%)	Specificity (%)	AUC		
p53	Zhang <i>et al</i> ^[38]	2016	324 (Training)	324 (Training)	55.9/-	89.5/-	0.784/-	< 0.001	ELISA
			186 (Validation)	186 (Validation)				< 0.001	ELISA
	Xu <i>et al</i> ^[39]	2014	388 (Test)	125 (Test)	30.0/-	98.0/-		< 0.0001	ELISA
			237 (Validation)	134 (Validation)	29.0/-	97.0/-		< 0.0001	ELISA
	Qin <i>et al</i> ^[40]	2014	174	242	21.8/-	96.3/-	0.6/-	< 0.05	ELISA
	Chai <i>et al</i> ^[41]	2014	157	85	22.9/-	100/-		< 0.01	ELISA
	Zhou <i>et al</i> ^[42]	2014	88	200	22.0/-	98.0/-		< 0.01	ELISA
	Cai <i>et al</i> ^[43]	2008	46	30	39.1/22.2	100/100		< 0.001	ELISA
	Looi <i>et al</i> ^[44]	2006	71	82	7.0/-	98.8/-		< 0.05	ELISA
	Müller <i>et al</i> ^[45]	2006	50	436	20.0/-	100/-		< 0.05	Western blot
	Megliorino <i>et al</i> ^[46]	2005	77	82	14.3/-	97.6/-		< 0.01	ELISA
	Shimada <i>et al</i> ^[47]	2003	301	205	30.0/-	95.5/-		< 0.05	ELISA
	Shimada <i>et al</i> ^[48]	2002	105	153	26.7/20.0	95.5/95.5		< 0.001	ELISA
	Ralhan <i>et al</i> ^[49]	2000	60	50		60.0/-	92.0/-	< 0.05	ELISA
	Shimada <i>et al</i> ^[50]	2000	35	69	40.0/-	100/-		< 0.001	ELISA
	Hagiwara <i>et al</i> ^[51]	2000	46	13	28.0/28.6	100/100		< 0.05	ELISA
	Shimada <i>et al</i> ^[52]	1998	57	208	58.0/-	99.0/-		< 0.05	ELISA
p16	Zhang <i>et al</i> ^[38]	2016	324 (Training)	324 (Training)	29.3/-	81.8/-	0.60/-	< 0.001	ELISA
			186 (Validation)	186 (Validation)				< 0.01	
	Jin <i>et al</i> ^[55]	2015	88	208				0.05	ELISA
	Qin <i>et al</i> ^[40]	2014	174	242	18.4/-	98.8/-	0.6/-	< 0.05	ELISA
	Zhou <i>et al</i> ^[42]	2014	88	200	11.0/-	97.0/-		0.004	ELISA
	Looi <i>et al</i> ^[44]	2006	71	82	14.1/-	98.8/-		< 0.05	ELISA
c-Myc	Zhang <i>et al</i> ^[38]	2016	324 (Training)	324 (Training)	49.1/-	81.5/-	0.699/-	< 0.001	ELISA
			186 (Validation)	186 (Validation)				< 0.001	ELISA
	Qin <i>et al</i> ^[40]	2014	174	242	15.5/-	98.8/-	0.6/-	< 0.05	ELISA
	Zhou <i>et al</i> ^[42]	2014	88	200	18.0/-	96.0/-		< 0.001	ELISA
	Looi <i>et al</i> ^[44]	2006	71	82	7.0/-	100/-		< 0.05	ELISA
	Megliorino <i>et al</i> ^[46]	2005	77	82	11.7/-	100/-		< 0.01	ELISA
Survivin	Xiu <i>et al</i> ^[56]	2018	159	362	14.5/-	90.0/-	0.327/-	0.524	ELISA
	Qin <i>et al</i> ^[40]	2014	174	242	12.1/-	99.6/-		< 0.05	ELISA
	Zhou <i>et al</i> ^[42]	2014	88	200	9.0/-	96.0/-		0.06	ELISA
	Megliorino <i>et al</i> ^[46]	2005	77	82	10.4/-	97.6/-		< 0.05	ELISA
NY-ESO-1	Oshima <i>et al</i> ^[57]	2016	172	74	32.0/16.0	100/100		< 0.001	ELISA
	Xu <i>et al</i> ^[39]	2014	388 (Test)	125 (Test)	26.0/-	100/-		< 0.0001	ELISA
			237 (Validation)	134 (Validation)	24.0/-	99.0/-		< 0.0001	ELISA
	Fujita <i>et al</i> ^[58]	2004	51	29	3.9/-	100/-		0.532	ELISA

Hsp 70	Xu <i>et al</i> ^[39]	2014	388 (Test)	125 (Test)	11.0/-	99.0/-	< 0.001	ELISA
			237 (Validation)	134 (Validation)	8.0/-	99.0/-	< 0.01	ELISA
	Zhang <i>et al</i> ^[59]	2011	69	76	39.1/-	92.3/-	> 0.01	ELISA
	Fujita <i>et al</i> ^[60]	2008	16	13	93.7/-	100/-	< 0.001	ELISA

derived miRNAs are resistant to endogenous ribonuclease activity, so it can exist in human serum in a stable form^[71]. In addition, serum miRNA expression levels are reproducible and consistent among individuals^[72], making them ideal candidates for diagnostic screening in blood. Since Zhang *et al*^[73] first reported serum miRNA levels in ESCC patients in 2010, several studies have investigated the differential expression of circulating miRNAs and explored their potential applications in ESCC^[74]. Therefore, circulating miRNA markers may contribute to the early diagnosis of ESCC.

To date, increasing studies have confirmed that c-miRNA can be used as a novel serum molecular marker to help early diagnosis of ESCC. Most of the research has focused on candidate miRNAs selected from prior ESCC tissue analysis, while other researchers used high-throughput technology to analyze miRNAs in the discovery sample datasets, and then performed qRT-PCR in an independent verification dataset to determine the diagnostic value of candidate miRNAs^[73]. A review by Yao *et al*^[75] of 33 manuscripts investigated a total of 43 different types of miRNAs in serum of ESCC patients. In these studies, the sensitivity, specificity, and AUC of miRNAs in the diagnosis of ESCC were 55.3%-96.9%, 47.4%-100% and 0.590-0.951, respectively^[75]. Among the most studied individual miRNAs in ESCC, there are well-known miRNAs, such as miR-21, miR-223, miR-375, miR-25, and miR-100 (Table 3)^[73-88]. Wang *et al*^[76] analyzed the diagnostic value of miR-21 and found that it has a good sensitivity and specificity for ESCC, being 71.0% and 96.9%, respectively. However, the number of ESCC patients included in the study was small, and the lack of validation studies of miR-21 limits extension to the clinic. In the current study, the article describes the analysis of the test and validation groups of serum miRNAs, which can serve as potential diagnostic biomarkers for ESCC^[73,87,89,90]. The combination of test cohort and validation cohort significantly improved the robustness of the diagnostic accuracy compared to many previous studies without a validation cohort. For example, the serum level of miR-1322 produced an area under the receiver operating characteristic (ROC) curve of 0.847 (95%CI: 0.795-0.890), which was used to distinguish between ESCC and healthy controls in the test group, and similar results were obtained in the validation group (area under the ROC curve: 0.845; 95%CI: 0.780-0.897)^[89].

Zhang *et al*^[73] measured the serum miRNA concentration by RT-qPCR and identified seven serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p) that were significantly up-regulated in the serum of ESCC patients compared to the control group. They showed that the seven-miRNA profile could be used as a biomarker for ESCC and, importantly, that it has the potential to predict early ESCC. In addition, this study demonstrated that the seven-miRNA panel was a more sensitive ESCC marker than traditional carcinoembryonic antigen biomarker. Sudo *et al*^[90] established a diagnostic model for serum miRNAs in 566 ESCC patients and 4965 control patients, the largest study to date in designing ESCC diagnostic models. This article^[90] used two independent cohorts to study the diagnostic model consisting of miR-8073, miR-6820-5p, miR-6794-5p, miR-3196, miR-744-5p, and miR-6799-5p. The sensitivities/specificities were 100%/98.0% and 96.0%/98.0%, respectively, with similar diagnostic value in early ESCC. In addition, Li *et al*^[91] reviewed 18 publications and investigated 39 different types of miRNAs in EC patients. The authors reported a relatively high sensitivity and specificity of combined and single miRNA markers, indicative of some value in diagnostic application^[91]. The results indicated that individual miRNAs showed no statistically significantly higher accuracy than multiple miRNA panels, which is contrary to some previous studies^[91]. However, since only two studies in this article compared panels of multiple miRNAs, this finding may not be sufficient to support such conclusion.

Numerous studies have shown that serum circulating miRNAs have potential clinical application as early tumor diagnostic markers, but further clinical data and mechanistic studies are needed for confirmation. The current understanding of miRNA can be summarized as follows. First, the transcription of one miRNA may require the regulation of multiple miRNAs at the same time. On the other hand, one miRNA may be involved in the regulation of the expression of multiple mRNAs at the same time^[92]. Obviously, this makes pathway studies of miRNAs more complicated. Second, the processing and detection methods of serum circulating miRNA still need to be standardized, and the selection of internal parameters needs further verification

Table 3 Diagnostic performance of microRNAs in esophageal squamous cell carcinoma

Micro-RNA	Ref.	Year of publication	ESCC, <i>n</i>	Controls, <i>n</i>	All stages/early stage			<i>P</i> value	Detection method
					Sensitivity (%)	Specificity (%)	AUC		
miR-21	Wang <i>et al</i> ^[76]	2018	31	32	71.0/-	96.9/-	0.88/-	< 0.001	qRT-PCR
	Sharma <i>et al</i> ^[77]	2018	24	21	83.3/-	57.2/-	0.692/-	0.027	qRT-PCR
	Zhang <i>et al</i> ^[78]	2018	125	125	74.0/-	78.0/-	0.80/0.86	< 0.001	RT-qPCR
	Lv <i>et al</i> ^[79]	2016	126	80			0.796/0.812	0.021	qRT-PCR
	Li <i>et al</i> ^[80]	2015	38	19			0.690/-	0.017	qRT-PCR
	Ye <i>et al</i> ^[81]	2014	100	50	/97.0	/56.0	/0.837	< 0.001	qRT-PCR
	Kurashige <i>et al</i> ^[82]	2012	71	39				< 0.001	qRT-PCR
	Wang <i>et al</i> ^[83]	2012	174	39	71.0/-	69.2/-	0.740/-	< 0.001	qRT-PCR
miR-223	Komatsu <i>et al</i> ^[84]	2011	50	20			0.618/-	0.022	qRT-PCR
	Zhang <i>et al</i> ^[78]	2017	125	125	0.68/-	0.68/-	0.73/0.83	< 0.001	RT-qPCR
	Zhou <i>et al</i> ^[85]	2017	137	155			0.649/-	< 0.001	RT-qPCR
	Wu <i>et al</i> ^[86]	2014	194	98			0.734/-	0.001	RT-qPCR
	Wu <i>et al</i> ^[87]	2014	20 (Test)	20 (Validation)			0.90/-	< 0.001	RT-qPCR
			63 (Test)	63 (Validation)			0.77/-	< 0.001	RT-qPCR
	Zhang <i>et al</i> ^[73]	2010	149	100	83.2/-	83.0/-	0.911/-	< 0.05	RT-qPCR
	Zhang <i>et al</i> ^[78]	2017	125	125	0.78/-	0.59/-	0.69/0.87	< 0.001	RT-qPCR
miR-375	Lv <i>et al</i> ^[79]	2016	126	80			0.712/0.739	0.023	qRT-PCR
	Li <i>et al</i> ^[80]	2015	38	19			0.921/-	< 0.0001	qRT-PCR
	Wu <i>et al</i> ^[86]	2014	194	98			0.720/-	0.007	RT-qPCR
	Komatsu <i>et al</i> ^[84]	2011	50	20			0.807/-	0.005	qRT-PCR
	Wang <i>et al</i> ^[76]	2018	31	32	71.0/-	68.8/-	0.72/-	< 0.001	qRT-PCR
miR-25	Zhang <i>et al</i> ^[78]	2017	125	125	0.54/-	0.57/-	0.55/-	0.025	RT-qPCR
	Wu <i>et al</i> ^[87]	2014	20 (Test)	20 (Validation)			0.94/-	< 0.001	RT-qPCR
			63 (Test)	63 (Validation)			0.78/-	< 0.001	RT-qPCR
	Wu <i>et al</i> ^[86]	2014	194	98	47.1/-	71.6/-	0.593/-	0.009	RT-qPCR
	Komatsu <i>et al</i> ^[88]	2014	20	50	85.0/-	86.0/-	0.856/-	< 0.0001	RT-qPCR
miR-100	Zhang <i>et al</i> ^[78]	2017	125	125	0.58/-	0.58/-	0.58/0.79	0.164	RT-qPCR
	Wu <i>et al</i> ^[87]	2014	20 (Test)	20 (Validation)			0.88/-	< 0.001	RT-qPCR
			63 (Test)	63 (Validation)			0.75/-	< 0.001	RT-qPCR
	Zhang <i>et al</i> ^[73]	2010	149	100	63.8/-	81.0/-	0.817/-	< 0.05	RT-qPCR

ESCC: Esophageal squamous cell carcinoma; AUC: Area under the curve.

and unification. Finally, most studies on serum circulating miRNAs, in the early diagnosis of tumors, involve small sample size, single-center studies, whereas large-sample, multicenter, prospective clinical studies are needed.

LONG NON-CODING RNAs

Long non-coding RNAs (lncRNAs) are non-coding RNAs that are greater than 200 bases in length, lack an open reading frame, and so have no protein coding ability^[93]. lncRNAs regulate gene expression at various levels (epigenetic, transcriptional, and post-transcriptional). lncRNAs regulate gene expression and function in a manner different from miRNAs, which not only affect the post-transcriptional regulation of protein translation, but also function through a variety of pathways that affect gene

transcriptional activity and protein degradation^[94,95]. A large body of evidence indicates that lncRNAs exert their cancer-promoting or anti-cancer effects by affecting the proliferation, invasion, metastasis, differentiation, apoptosis, and genomic stability of tumor cells^[96]. HOX-transcribed RNA (HOTAIR) is the first long non-coding RNA found to have trans-regulatory effects in primary and metastatic breast cancer^[97]. In addition, some studies have found that HOTAIR is also highly expressed in ESCC tissues, and the expression level is inversely correlated with degree of differentiation and positively correlated with TNM stage^[98]. In recent years, with the maturity and application of whole genome sequencing and lncRNA chips, more and more lncRNAs have been found in different types of tumors, and are closely related to the occurrence and development of tumors, suggesting that lncRNAs could be used as tumor biomarkers^[99].

Previous studies on lncRNAs initially focused on tumor tissue. In recent years, investigators have also studied the expression levels of lncRNAs in serum or plasma of tumor patients, and many studies have shown that lncRNAs can be present in extracellular fluids, including serum, plasma, and other body fluids, although the exact mechanism is unclear^[100]. In addition, studies by Arita *et al.*^[101] confirmed that lncRNAs can stably exist in circulating blood under certain conditions. Recently, a number of laboratories have proposed a variety of serum or plasma lncRNAs that may be used for early diagnosis and efficacy monitoring of ESCC (Table 4)^[102-105]. Wang *et al.*^[104] used qRT-PCR to detect HOTAIR in serum of ESCC patients, and found that the expression of HOTAIR is increased in serum of ESCC patients, with an area under the diagnostic curve of 0.793, sensitivity of 56.0%, and specificity of 90.0%. Moreover, the level of HOTAIR decreased in serum after ESCC surgery. These results suggest that serum lncRNA-HOTAIR may be a potential diagnostic molecular marker in ESCC^[104]. Some studies show that lncRNAs tested alone or in combination exhibit the same or even higher diagnostic performance than traditional cancer biomarkers. Tong *et al.*^[103] found that the levels of three lncRNAs, POU3F3, HNF1A-AS1, and SPRY4-IT1, in plasma of ESCC patients were significantly higher than those of normal controls, among which plasma POU3F3 showed the best diagnostic efficacy (area under the curve of 0.842, sensitivity 72.8%, and specificity 89.4%). It is noteworthy that in 147 ESCC and 123 healthy controls, combined detection of plasma POU3F3 and squamous cell carcinoma antigen (SCCA) showed better diagnostic performance (area under the curve of 0.926, sensitivity of 85.7%, and specificity of 81.4%) and an effective detection of 80.8% of patients with early ESCC, suggesting that the combination of POU3F3 and SCCA may be useful for screening early ESCC^[103].

Circulating lncRNAs are thought to be stable in blood because of encapsulation in microvesicles or exosomes^[99,101]. A better understanding of the transport of intracellular and intercellular lncRNAs and the underlying biology of cell-derived lipid vesicles may help to develop biomarkers for the detection of human diseases based on circulating lncRNAs. In addition, the detection of biomolecular markers in peripheral blood has the advantage of easy operation and is minimally invasive. Therefore, we expect that the search for new lncRNAs as molecular diagnostic markers in circulating blood will be a hot scientific issue in the field of biomarker research. In order to introduce circulating lncRNAs into clinical practice, further research and improvement should be carried out in the standardization of sample preparation protocols, the control of endogenous lncRNAs in body fluids, and the unification of extraction methods. The criteria for assessing the quality of lncRNAs and the reliability of qPCR results need to be more accurate and reliable, minimizing selection bias^[106]. Most of the current research is designed with small samples and thus lacks realistic clinical application at this point. Therefore, it is necessary to further expand the sample size and combine multi-center clinical validation studies to develop an lncRNA detection kit for marker detection in blood, thereby improving the early diagnosis and postoperative monitoring efficiency of lncRNAs in tumors.

CIRCULATING TUMOR DNA

In 1948, Mandel and Metais first reported the presence of circulating cell free-DNA (cf-DNA) in human peripheral blood^[107]. cf-DNA refers to extracellular DNA found in body fluids such as blood, cerebrospinal fluid, and synovial fluid, and is a degradation product of endogenous DNA in cells. In recent decades, many studies have found that cf-DNA levels are higher in cancer patients, especially in the advanced stages^[108,109]. Researchers first detected KRAS oncogenic mutations in the blood cf-DNA of patients with pancreatic cancer in 1994 by using PCR, which was consistent with that detected in tumor tissues. In other words, the small part of cf-DNA carrying tumor-specific mutations is indeed released by tumor cells^[110]. Thus,

Table 4 Diagnostic performance of long non-coding RNAs in esophageal squamous cell carcinoma

LncRNA	Ref.	Year of publication	ESCC, <i>n</i>	Controls, <i>n</i>	All stages/early stage			<i>P</i> value	Detection method
					Sensitivity (%)	Specificity (%)	AUC		
POU3F3	Hu <i>et al</i> ^[102]	2016	205	210			0.584/-	< 0.01	qRT-PCR
	Tong <i>et al</i> ^[103]	2015	147	123	72.8/69.2	89.4/-	0.842/-	< 0.001	qPCR
HOTAIR	Wang <i>et al</i> ^[104]	2017	50	20	56.0/-	90.0/-	0.793/-	< 0.01	qRT-PCR
HNF1A-AS1	Tong <i>et al</i> ^[103]	2015	147	123	32.7/-		0.781/-	< 0.001	qPCR
SPRY4-IT1	Tong <i>et al</i> ^[103]	2015	147	123	48.2/-		0.800/-	< 0.001	qPCR
linc00152	Hu <i>et al</i> ^[102]	2016	205	210			0.698/-	< 0.01	qRT-PCR
CFLAR-AS1	Hu <i>et al</i> ^[102]	2016	205	210			0.651/-	< 0.01	qRT-PCR
PGM5-AS1	Zhihua <i>et al</i> ^[105]	2019	41	26			0.894/-	< 0.001	qRT-PCR

ESCC: Esophageal squamous cell carcinoma; AUC: Area under the curve; lncRNA: Long noncoding RNA; POU3F3: POU class 3 homeobox 3; HOTAIR: HOX transcript antisense RNA; HNF1AAS1: Hepatocyte nuclear factor 1 homeobox A antisense RNA 1; SPRY4-IT1: SPRY4 intronic transcript 1; linc00152: long intergenic non-protein-coding RNA. CFLAR-AS1: CFLAR antisense RNA 1; PGM5-AS1: PGM5 antisense RNA 1.

tumor-associated mutations in cf-DNA can serve as tumor-specific markers, and these tumor-derived cf-DNA fragments carrying tumor characteristics are referred to as ctDNA^[110,111]. ctDNA is DNA fragments released by apoptotic or necrotic cells into the blood vessels, and is mainly present in extracellular plasma^[112]. The concentration of ctDNA in advanced tumors is between 0.1% and 10%, and is positively correlated with tumor stage and tumor volume^[113]. Because the content of ctDNA in total plasma DNA is small, the detection and quantification of ctDNA are very challenging. At present, the quantitative technology of ctDNA has developed from quantitative polymerase chain reaction to complex BEAMing and deep next-generation sequencing, thereby improving the sensitivity and specificity of ctDNA detection^[114]. With the development of sensitive technologies to detect rare mutations, the use of blood samples can determine tumor heterogeneity.

As a new molecular marker for tumors, ctDNA is being studied more and more extensively in the field of tumors. It shows great potential for clinical application in the early diagnosis of tumors, residual and recurrence monitoring, and prognosis, which has brought subversive changes to traditional tumor diagnosis and treatment. In recent years, ctDNA methylation has become a highly sensitive method for detecting landmark characteristics of tumors. Kawakami *et al*^[115] observed that high-level methylation of APC DNA occurs in 61% of ESCC patients, and its high expression is associated with poor prognosis. Moreover, Hibi *et al*^[116] detected abnormal methylation of the driver *P16* gene in 18% of ESCC patients. Liu *et al*^[117] evaluated the methylation status of Wnt antagonist family genes in EC patients by applying methylation-specific PCR to detect hypermethylation of the driving factors *SFRP-1/WIF-1*, *DKK-3*, and *RUNX-3* genes in plasma. Therefore, measuring abnormally high levels of methylation of drivers of cancer-related genes might be used for diagnosis of ESCC and monitoring recurrence. Increasing studies have confirmed that detection of ctDNA in the blood of tumor patients can also identify all driver gene mutations in the tumor tissue^[118,119]. Lebofsky *et al*^[120] performed in-depth sequencing analysis of plasma ctDNA and metastatic tumor tissue from 34 tumor patients (including 18 different tumor types), covering 6800 COSMIC tumor hotspot mutations in 46 genes. The results showed that in 27 patients, 28 (97%) of 29 mutant genes in metastatic tumor tissue were detected in paired plasma ctDNA^[120]. These results indicate that plasma ctDNA has the potential to replace tumor metastatic lesion tissue for the detection of mutant genes^[120,121]. Plasma ctDNA samples are easy to obtain, with good patient dependence, and the operations can be repeated. It is a feasible tumor molecular marker that might replace tissue biopsy for metastatic tumor gene mutation. Compared with tissue biopsy, ctDNA has the advantages of non-invasive operation and providing more comprehensive tumor genomics information^[122,123]. Another major clinical application of ctDNA detection is the dynamic monitoring of tumor burden. At the same time, ctDNA detection could detect tumor progression 5 to 10 mo in advance^[124,125], and detect disease progression earlier than traditional detection methods. However, the clinical application of ctDNA testing still has the following difficulties: (1) Detection technology is still immature and there is a lack of standardized ctDNA extraction and detection procedures; (2) Testing costs are expensive; and (3) There is a lack of large sample, prospective

clinical studies to evaluate the early diagnostic value in cancers. In the future, with the development of gene sequencing technology and precision medicine, the application of ctDNA technology in clinical practice will be just around the corner.

METABOLITES

Metabolomics is an emerging discipline that studies the composition, content changes, and interrelationships of all small molecule metabolites in biological samples at specific times or in given environments. In 1999, Nicholson *et al.*^[126] formally put forward the concept of “metabolomics”, the qualitative and quantitative analysis of dynamic changes of all metabolic components (intermediate products and end products) of a biological system under pathophysiological conditions. It is the continuation and development of genomics, transcriptomics, and proteomics, and is at the end of the regulation of life activities^[127]. In recent years, related research on metabolomics in tumors has also been given increasing attention. Pathological changes in tumor development often lead to significant changes in basic metabolism, resulting in changes in the relative level of small molecule metabolites, which ultimately show the difference between the metabolic spectrum of tumor patients and that of healthy controls^[128]. Metabolomics uses advanced analytical chemistry techniques to comprehensively measure a large number of small molecule metabolites in cells, tissues, and body fluids^[129]. At the same time, combined with bioinformatics and other methods, changes in the body's small molecule metabolites are analyzed during tumor development, and a tumor metabolism map is finally drawn^[130]. It is well known that small changes in gene and protein levels often lead to significant changes in metabolite levels, so metabolomics is a highly sensitive and direct method of disease detection. In recent years, with the development of metabolomics technology, the diagnosis and prognosis of tumors based on metabolomics analysis have been greatly improved^[128].

The study of metabolomics depends on the development of various related analytical chemistry technologies. At present, the spectroscopic techniques of metabolic analysis have been mainly limited to nuclear magnetic resonance and mass spectrometry (MS), the latter requiring a combination of separation techniques, to enable analysis by gas chromatography-MS (GC-MS) or liquid chromatography-MS^[128]. Recently, investigators have applied the latest metabolomics techniques to explore abnormal metabolic changes in tumors and found many metabolites that are abnormally elevated in specific tumors, such as glucose, serine, lactic acid, and polyamines^[131]. There have been many clinical advances in ESCC based on metabolomics^[132,133], most of which are non-targeted metabolomics studies focusing on the identification of diagnostic biomarkers for ESCC, but not prognosis for ESCC metastasis^[132]. Jin *et al.*^[132] used gas chromatography-MS to measure serum metabolome molecular marker levels in 60 ESCC patients and 30 normal controls. They developed a prediction model consisting of three metabolic molecules, valine, γ -aminobutyric acid, and pyrrole-2-carboxylic acid, which gave an area under the curve, sensitivity, and specificity of 0.964, 90.0%, and 96.67%, respectively. The diagnostic effectiveness of this predictive model was almost same as the validation set. Liu *et al.*^[134] conducted metabolomics analysis on the plasma of 53 ESCC patients and matched 53 normal controls, and found that 25 metabolites were up-regulated and 5 metabolites were down-regulated. Subsequent database verification identified 11 metabolites, of which 6 were the phospholipids phosphatidylserine, phosphatidic acid, lecithin, phosphatidylinositol, phosphatidylethanolamine, and sphinganine 1-phosphate. Ma *et al.*^[129] applied high performance liquid chromatography to analyze plasma free amino acids in patients with ESCC, and the results showed that there are many differences in plasma free amino acid metabolism profiles between ESCC patients and healthy controls, including Asp, Ala, Glu, Gly, and Thr, suggesting that plasma free amino acids may help distinguish ESCC from healthy controls.

The above-mentioned studies show that high-throughput detection methods of metabolomics can illustrate the whole picture of small molecule metabolic markers in tumor body, thus providing a new way to find ideal molecular markers for the early diagnosis of tumors. However, metabolomics is an emerging discipline, and its development faces many difficulties. First, it is unclear how many metabolites exist in the human metabolome. Second, the various substances produced by human metabolism are complex and involve different biochemical categories. Currently, no platform can achieve comprehensive identification and simultaneous measurement of all metabolites. Finally, the sources of metabolites in human samples are different, and changes in the same metabolite between different individuals will also be affected by multiple factors, which stands as a barrier for the implementation of metabolomics

research. Of course, at present, tumor metabolomics is still in the initial exploration stage. In future work, the clinical application of metabolomics still needs more experiments and clinical research for systematic and comprehensive verification.

CYTOKINES

Cytokines are a class of low-molecular-weight soluble substances with high activity and multifunctionality produced by various cells, such as immune cells activated by immunogens, mitogens, or other stimulants, most of which are peptides or small molecular glycoproteins^[135,136]. They play a role in intercellular communication and cell growth, and participate in cell differentiation, migration, and apoptosis^[137]. These mediators are involved in signal transduction between cells, and regulate the human immune response, promote hematopoietic and anti-inflammatory effects and antiviral immunity, participate in tumorigenesis and development, and are involved in various pathophysiological processes^[135,136]. According to their structure and function, cytokines can be divided into interleukins (ILs) (such as IL-6), interferons, chemokines (such as IL-8), growth factors [such as vascular endothelial growth factor (VEGF)], colony-stimulating factors, and the tumor necrosis factor superfamily. Some inflammatory cytokines are involved in different molecular mechanisms leading to canceration^[138]. It is well known that the process of malignant transformation of tumor cells involves the expression and activity of a variety of cytokines. These cytokines play an important role in tumorigenesis, angiogenesis, and induction of metastasis, and are also potential molecular markers for tumor diagnosis.

There is transient overexpression of cytokines in many disease states. In cancer, the changes in the production of cytokines increase with the progression of the disease, and participate in or even promote the progression of tumors. As a result, different cytokines are deregulated, and their altered local and systemic concentrations can be detected in body fluids as biomarkers of cancer. In recent years, more and more cytokines have been confirmed to be abnormally expressed in the serum of ESCC patients^[139-147], and may be used as molecular markers for ESCC diagnosis. The ESCC cytokine network is rich in pro-inflammatory cytokines, growth factors, and chemokines. The main ESCC-related cytokines are VEGF-A, VEGF-C, IL-6, and IL-8 (Table 5)^[139-147]. Kozłowski *et al*^[139] performed an analysis of 89 ESCC patients and 30 healthy controls and showed that the diagnostic sensitivity, specificity, and AUC of VEGF-A for ESCC were 83.0%, 70.0%, and 0.865, respectively. Another analysis of ESCC^[140] showed that IL-8, VEGF-C, and VEGF-A expression levels were significantly higher in 70 ESCC patients than in 42 normal controls. Combining both IL-8 and VEGF-C, the AUC that distinguishes ESCC from normal controls is better than that of IL-8 or VEGF-C tested alone. These results indicate that IL-8 and VEGF-C can potentially be used as cytokine molecular markers for the detection of ESCC. Those authors further analyzed the correlation between IL-8 and VEGF-C and VEGF-A, and found that IL-8 and VEGF-C are more closely related. Therefore, the authors speculated that IL-8 may work by stimulating the expression and secretion of VEGF-C^[140]. Łukaszewicz-Zajac *et al*^[147] measured the levels of serum IL-6 in 90 healthy controls and 30 ESCC patients, and found that IL-6 levels in ESCC patients were increased compared to the controls. Further ROC curve analysis results showed that the detection sensitivity of IL-6 was 87%, specificity was 92%, and AUC was 0.924, suggesting that IL-6 may be helpful for the diagnosis of ESCC.

The future potential of cytokines seems to be primarily related to their prognosis and predictive value^[139,146]. Cytokines can also be used as markers for monitoring treatment response and disease recurrence^[140-142]. At present, significant progress has also been made in exploring cytokines as molecular markers for the early diagnosis of tumors. However, most studies lack the evaluation of early tumor samples or samples before diagnosis. In the future, whether cytokines can be used clinically for early diagnosis of tumors still needs high-quality large samples and prospective studies for further confirmation.

CONCLUSION

Early diagnosis is one of the most effective ways to improve the survival rate and reduce the mortality of cancer patients. Clinically, endoscopy can detect early ESCC and its precancerous lesions. A recent large-scale prospective study confirmed for the first time that esophageal endoscopy screening and intervention can effectively reduce the incidence and mortality of ESCC^[14]. However, endoscopy is an invasive diagnostic and screening method, which limits its widespread use in the screening of

Table 5 Diagnostic performance of cytokines in esophageal squamous cell carcinoma

Cytokine	Ref.	Year of publication	ESCC, <i>n</i>	Controls, <i>n</i>	All stages/early stage			<i>P</i> value	Detection method
					Sensitivity (%)	Specificity (%)	AUC		
VEGF-A	Kozłowski <i>et al</i> ^[139]	2013	89	30	83.0/-	70.0/-	0.865/-	< 0.001	ELISA
	Krzystek-Korpacka <i>et al</i> ^[140]	2008	70	42	72.5/-	66.0/-	0.739/-	< 0.001	ELISA
	Krzystek-Korpacka <i>et al</i> ^[141]	2007	70	47	70.0/-	81.0/-	0.837/-	< 0.001	ELISA
	Ren <i>et al</i> ^[142]	2005	72	15				< 0.001	ELISA
	Shimada <i>et al</i> ^[143]	2001	96	24	79.0/-	48.0/-		0.001	ELISA
VEGF-C	Kozłowski <i>et al</i> ^[144]	2010	110	30	60.0/-	80.0/-		< 0.001	ELISA
	Krzystek-Korpacka <i>et al</i> ^[140]	2008	70	42	78.6/-	76.6/-	0.841/-	< 0.001	ELISA
IL-8	Tong <i>et al</i> ^[145]	2018	10	10				< 0.05	ELISA
	Krzystek-Korpacka <i>et al</i> ^[140]	2008	70	42	77.1/-	74.4/-	0.782/-	< 0.001	ELISA
	Ren <i>et al</i> ^[146]	2005	149	35				< 0.001	ELISA
	Ren <i>et al</i> ^[142]	2005	72	15				< 0.001	ELISA
IL-6	Tong <i>et al</i> ^[145]	2018	10	10				< 0.05	ELISA
	Łukaszewicz-Zajac <i>et al</i> ^[147]	2011	30	90	87.0/-	92.0/-	0.924/-	< 0.001	ELISA

ESCC: Esophageal squamous cell carcinoma; AUC: Area under the curve; VEGF-A: Vascular endothelial growth factor A; VEGF-C: Vascular endothelial growth factor C; IL-8: Interleukin-8; IL-6: Interleukin-6.

asymptomatic people, making the development and validation of non-invasive biomarkers important for the screening of ESCC. Although some new serological markers have been studied, these have not been translated into effective clinical tools.

In the field of biomarker research related to ESCC, although many studies have shown that biomarkers have diagnostic potential for early ESCC, on the whole, research on the diagnostic effects of these biomarkers on ESCC still have many limitations, such as small sample size, research design of a single population, lack of value for early diagnosis, lack of independent verification tests, and lack of pre-clinical data. Meanwhile, we note that some of the studies in this review did not include patients with early ESCC, so in future studies, the early diagnostic value of these markers needs to be further evaluated. Moreover, there is insufficient molecular profiling data on potential circulating biomarkers for ESCC diagnosis and prognosis. Therefore, in order to realize the clinical application of autoantibodies and the early diagnosis of ESCC, it is still necessary to further screen and identify biomarkers with better diagnostic efficiency and optimize the best combination. Moreover, results need to be confirmed for large sample sizes in multi-center prospective studies.

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