Review Article

Non-Invasive Detection of Esophageal Cancer using Genetic Changes in Circulating Cell-Free DNA

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Abstract

Cell free DNA (cfDNA) is a genetic biomarker that is present in serum or plasma in high concentration in many types of cancer. Identification of circulating cancer related DNA molecules in serum or plasma is a non-invasive tool for early diagnosis and prognosis in many cancer patients. For this review, study selection and data extraction were performed by the authors. Detection of point mutations, microsatellite alterations, DNA hypermethylations and losses of heterozygosity in circulating cell free DNA have been characterized in esophagus cancer. Application of circulating cell free DNA as a biomarker, provide the best opportunity for constructing non-invasive tests for early detection, prognosis and management of cancer patients, after therapy in many types of cancer.

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Introduction

The esophagus is a muscular tube that connects the throat to the stomach. Cancer of the esophagus, also called esophageal cancer, can occur any place along the liner of the tube. Among the diverse types of existent neoplasms, esophageal carcinoma is the eighth deadliest malignancy worldwide. Adenocarcinoma and squamous cell are liable for more than 95 percent of all esophagus cancers [(1)].

The predominant histological subtype in esophageal cancer is squamous cell carcinoma (SCC) which contributes to about 80% of all esophageal cancers in the world [(2)]. SCC has the highest incidence in Western countries with traditions of alcohol consumption, smoking or tobacco, hot drinks and malnutrition [(3-5)]. ADC is found in industrialized countries, with gastric esophageal reflux (which causes Barrett´s Esophagus), obesity, substances derived from the grain of moldy corn (fumonisins), alcoholism, and smoking (Table 1) [(3,6,7)].

Epidemiology and Pathology of Esophageal Cancer

Epidemiology

Epidemiologic data have shown variability in determining attitude in incidence of esophageal carcinoma malignancies worldwide. The ascending incidence of esophageal cancer over the past two decades conformed to the change in histological type and primary tumor location. An estimated 14,500 deaths from esophageal cancer occurred in the United States in 2010 [(8)].

The American Cancer Society's most recent estimates for esophageal cancer in the United States for 2011 are as follows: about 16,980 new esophageal cancer cases diagnosed (13,450 in men and 3,530 in women) and about 14,710 deaths from esophageal cancer (11,910 in men and 2,800 in women). This disease is 3-4 folds higher among men than women [(9)]. The incidence of esophageal cancer varies remarkably with geographic region,

Table 1. The most common risk factors affecting the development of esophageal carcinomas

Risk factors	SCC	ADC
Alcohol consumption	Moderate	Moderate
Obesity	Not significant	Moderate
Low socioeconomic class	High	Not significant
Excess fat consumption	Not significant	Moderate
Nitrosamine	High	Not significant
Gastroesophageal reflux disease (GERD)	Not significant	High
Lye ingestion	Moderate	Not significant
Polyaromatic hydrocarbons (PAH)	High	Not significant
Helicobacter pylori	High	Not significant
Tobacco smoking	High	Moderate
Barrett's esophagus	Not significant	High
Family history of cancer	Moderate	Not significant
Low education level	Low	Not significant
Hot beverage	Low	Not significant
Food habit (Excess energy consumption)	Low	Not significant
Poor oral hygiene	Low	Not significant
Viral agents	Low	Not significant

SCC, Squamous cell carcinoma; ADC, Adenocarcinoma. Information from references [3-7]

especially in developing nations. ADC is the most common type of esophageal malignancy in the United States and Western Europe [10]. In America, the reported incidence of esophageal cancer in patients is 3.2-4.0 per 100,000 persons [11], while in Europe and Asia, reports are 0.5-7.0 and 0.02-0.4 per 100,000 persons, respectively [12,13].

The incidence of SCC among the Asian countries is higher than that of ADC, especially in countries and areas of East Asia such as Korea and Japan with incidence of 8.2-21 per 100,000 persons [14,15]. Noticeable is the observation that the highest rates occurred in northern China and northern Iran, with incidence of about 40 per 100,000 persons in 2003 [13,16].

Pathology

Esophageal cancers are usually found with signs and symptoms that a person is having. The most common symptoms of esophageal cancer include: weight loss, difficulty in swallowing, chest pain, constant cough, bone pain, hiccups and pneumonia [17]. These symptoms are usually present for several months before medical treatment is sought and initially presents itself by having difficulty in swallowing dry foods. Furthermore, weight loss of 10 percent of normal body weight occurs in less than six months. About half of esophageal cancer patients present with locally advanced unresectable disease or distant metastasis.

ADC spreads via transverse penetration through the full thickness of the wall, whereas SCC tends to spread linearly in a submucosal fashion [18]. Esophageal cancer spreads through extensive lymphatic channels with a skip metastases pattern when observed in autopsy specimens [19].

Staging in esophageal cancer

The diagnostic evaluations of ADC and SCC are essentially identical. Several strategies and approaches are acceptable for diagnosis, staging, chemoradiotherapy, treatment, and surveillance of patients with esophageal cancer. These include endoscopy [20], bronchoscopy [21], thoracoscopy [22], laparoscopy [23], computed tomography [24], and surgery [25]. However, maybe some of these approaches and strategies are associated with painful experiences and cause discontent for cancer patients.

Recently, research focus has been in the development of non-invasive screening at the early stages of cancer. In most cases, early detection of cancers is extremely difficult because there are multiple uncertainties related to the location of the disease but it is neces-

Table 2. Summary of the most common alterations in esophageal cancers

	Gene	Location	Alterations	Authors/ Years	
				SCC	ADC
Oncogenes					
	Cyclin D_1	11q13	Gene amplification	Kawakubo et al; 2005.[49]	Izzo et al; 2007.[50]
	ErbB-2	17q21	Gene amplification	Friess et al; 1999.[51]	Andolfo et al; 2011.[52]
TSG					
	p53	17q13	LOH Mutation	Robert et al;2000.[31]	Putz et al; 2002.[30]
	p16	9p21	Mutation	Abbaszadegan et al; 2005.[34]	Ishii et al; 2007.[35]
	p15		LOH	Xing et al; 1999.[40]	--
	p14		Hypermethylation	Xing et al; 1999.[42]	Sarbia et al; 2004.[41]
	APC	5q21	LOH Hypermethylation	Fearnhead et al; 2001.[43]	Kawakami et al; 2000.[97]
DNA repair					
	MSH2	2p21	LOH	Ikeguchi et al; 1999.[53]	Montesano et al; 1996.[48]
	MLH1	3p21	LOH	Muzeau et al; 1997.[54]	Fitzgerald et al; 1998.[55]

SCC, Squamous cell carcinoma; ADC, Adenocarcinoma; LOH, loss of heterozygosity; TSG, tumor suppressor gene; APC, adenomatous polyposis coli; MLH1, mutL homolog1.

sary for full treatment and recovery. Currently, among the various approaches used for screening cancer patients, circulating molecular biomarkers have been found to be of the most convenient and useful non-invasive tool with esophageal carcinoma [(26)].

Molecular genetic changes in esophageal cancer

The most common genetic alterations in esophageal cancer involve genetic alterations in several oncogenes, tumor suppressor genes, and DNA repair genes. Furthermore, the most common alterations found in esophageal cancers such as deletions, amplifications and Loss Of Heterozygosity (LOH) has been reported to occur on several chromosomes listed in table 2 [(27)].

Mutations in oncogenes and tumor suppressor genes

Tumor suppressor genes *p53* and *p16* have been introduced to the primordial G1 cell cycle regulatory genes [(28)]. Alteration of these genes such as LOH and mutation can lead to the loss of regulation of cell growth, which is important in carcinogenesis [(29)]. Mutation of *p53* has been extensively investigated in both SCC and ADC [(30,31)]. Previous studies have reported the incidence of *p53* mutations (53 percent in ADC and more than 93 percent in SCC) [(32)] especially, in the exons 5-8 (encoding the DNA-binding domain of the protein) in SCC. In addition to mutations in *p53*, LOH at chromosome 17q13 is a significant event in both SCC and ADC.

In principle, the hypotheses were that *p53* alteration occurs frequently as an early event in the tumor progression of esophagus carcinoma [(33)]. However, inactivation of the *p16* gene, LOH, genetic mutation and aberrant DNA methylation in the coding and non-coding (promoter) regions also frequently occur in both SSC and ADC [(34,35)]. Tarmin et al, has reported the incidence of LOH in about 50 to 65 percent of patients with SCC at the 9p21 chromosomal locus [(36)]. However, aberrant *p16* hypermethylation in the promoter region is a common mechanism for the inactivation of this gene in SCC. Taghavi et al, showed the aberrant hypermethylation of this gene in 62 percent of SCC patients in Iran [(37)].

Previously, Abbaszadegan et al, had reported incidence of this aberration in SCC to be 73.3 percent in the northeastern Iran [(38)], while Hardie et al, reported that hypermethylation of the *p16* promoter is 85 percent in ADC [(39)]. Furthermore, inactivation of *p14* and *p15* genes has been observed in esophagus cancer. Aberrant methylation in the promoter regions of these genes was reported to be associated with loss of transcription in previous studies [(40,41)]. Xing et al, reported alteration of methylation patterns in the promoter of *p14* and *p15*

genes and incidence of LOH in *p14* and *p15* genes in SCC tumor samples [42].

The Adenomatous Polyposis Coli (*APC*) gene is a tumor suppressor gene located at chromosome 5q21 and is involved in esophagus cancer [43]. One of the alterations in this gene is LOH at 5q, occurring in about 55-80 percent of SCC and 20-55 percent of ADC [44,45] cases, but mutation of *APC* is under 10 percent in this cancer type [46]. The most common type of gene inactivation occurs via hypermethylation in the promoter region of the *APC* gene with an incidence of 92 percent in ADC and 50 percent in patients with SCC [47]. Therefore, the hypermethylation of this gene has noticeable roles in both SCC and ADC.

The *cyclin* D_1 gene, located at chromosome 11q13, encodes a protein that is required for controlling the cell cycle [48]. Previous studies have analyzed *cyclin* D_1 expression in patients with esophagus cancer and aberrant overexpression of this gene in 23-73 percent of patients with SCC has been reported [49,50]. Furthermore, Metzger et al, showed an overexpression and gene amplification of *cyclin* D_1 in 22-64 percent of patients with ADC [27].

The erbB-2 (HER2) is one of the members of Epidermal Growth Factor Receptor (EGFR) family, which acts as tyrosine kinase receptor. This receptor has an intracellular tyrosine kinase activity and extracellular binding domains. The *erbB-2* oncogene encodes a truncated form of EGFR which contains continuous tyrosine kinase activity [51]. The overexpression and gene amplifications of *erbB-2* has been demonstrated in patients with SCC and ADC with 20 to 60 percent frequency [52].

Microsatellite instability

Microsatellites are short tandem repeats of nucleotide sequences found at about 5000 base pair intervals. Microsatellite Instability (MSI) has been recognized as a length mutation that occurs especially in microsatellites. The two genes studied in this alteration include *MSH2* and *MLH1* which are located at chromosomes 2p and 3p, respectively. The functions of these genes are essential in DNA mismatch repair and reduction of genomic replicative error rate. Several studies have reported 10 to 20 percent frequency of MSI in patients with ADC and SCC, with a higher frequency in SCC patients [53-55].

Molecular Biomarkers

Circulating molecular biomarkers

Currently, progress of proteomics has opened the door to cancer-related biomarker discovery. Proteomics is the complete descriptiton of all proteins encoded by the genome, called the proteome [56]. Advances in proteomic technologies such as the development of quantitative proteomic methods, high-resolution and high-throughput methods have been used to identify and understand pathophysiology of carcinomas. Rapid advances in the field of proteomics promise discovery of biomarkers which could potentially aid in early diagnosis, prognosis and accurate prediction of outcomes during cancer treatments and management [57-62].

Fortunately, biological fluids such as urine, blood, serum or plasma contain many biomarkers originating in many different tissues of the body. In the past few decades different biomarkers have been tested as screening tools for cancer patients. Proteins are probably the first-generation of biomarkers to have been investigated and discovered in the biological fluids. For example Prostate Specific Antigen (PSA), CA19-9, CA125, squamous cell carcinoma antigen (SCC) and cytokeratin 19 fragment (CYFRA) are clinically applied as biomarkers [63-66]. However, these conventional biomarkers have low sensitivity and insufficient specificity. Thus, researchers have looked toward the other types of tumor biomarkers [67].

Recent advances in analytical assay technologies have allowed development of amplification techniques that are based on circulating nucleic acids (RNA and DNA) as biomarkers in serum or plasma of cancer patients [68]. Over the past decade, tremendous amount

of information has been accumulated which are related to cancer-specific changes such as gene mutation, Single Nucleotide Polymorphism (SNP), gene deletion, LOH, epigenetic alterations [69], genome instability [70] and aberrant at the expression level at RNAs and protein levels [71,72].

In recent years, efforts in many laboratories throughout the world have been focused on the utilization of genetic and expression abnormalities in circulating biomarkers for early detection, prognosis and assessment of cancer patients after therapy [73].

Biological characteristics of cfDNA

In 1948, Mandel and Metais reported the existence of circulating extracellular nucleic acids in human blood [74]. Thereafter, Stroun et al, showed biological characteristics of circulating cell free DNA [75]. Circulating cfDNAs are small fragments of genomic DNA present in the plasma or serum [76]. The mechanisms of the occurrence of cfDNA in blood under normal and pathological conditions remain unknown. Two main mechanisms of release cfDNA in the plasma or serum have been postulated: (a) cells detach and extravasate into the blood stream where they undergo lysis and (b) cells undergo apoptosis or necrosis in cancer tissues in vivo and their DNA is released in the blood stream [77]. Both molds simultaneously contribute to cfDNA production in cancer patients.

In the programmed enzymatic apoptosis, low molecular weight DNA fragments of about 185-200 base pairs are released in the necrosis products, much of the high molecular weight DNA fragments of about 400 base pairs were detected in serum and plasma [78,79]. The role of macrophages in degradation of DNA fragments and then release into the bloodstream has been suggested to probably occur through engulfing process of necrotic or apoptotic cells [80].

Isolation and quantification of cfDNA

The cfDNA is extracted using phenol/chloroform or standard commercially available DNA kits such as glass-milk-based methods, nucleospin blood kit and PureGene DNA isolation kit from serum or plasma tumor patients. Furthermore, a variety of DNA quantification methods is utilized to measure including DipStick (Invitrogen), fluorometry with SYBR-green and real-time PCR [76]. Diehl et al, developed a technique called BEAMing (beads, emulsion, amplification and magnetics) for quantification of circulating mutant cfDNA in serum or plasma of tumor patients [81]. However, with the appearance of novel approaches incorporating automated DNA isolation and amplification with multiplex quantitative fluorescent PCR of circulating cfDNA, the potential to utilize cfDNA as a screening tool for many types of cancer exists.

Non-Invasive Screening of Esophagus Cancer using cfDNA

Alterations at the cfDNA level

Leon et al reported that the average cfDNA concentration in the serum or plasma of cancer patients is higher than in normal subjects [82]. This is because the increase of cfDNA levels in the serum or plasma of tumor patients are contributed by both tumor DNA and non-tumor DNA. However, meta-analysis data has shown high discrepancy in the cfDNA concentrations between studies. And this probably depends on variables such as number of patients in a given study, cancer subtypes, tumor size, stages, location, and other risk-related factors [75].

Unfortunately, high variability in the abnormal cfDNA concentrations has prevented it from becoming more than a critical biomarker in each of malignancies [83]. One of the complicating facts is that an increased plasma cfDNA level has been also observed due to inflammation [84], trauma [85], premalignant states [86], after exercise [87], and in patients suffering from acute or chronic illnesses [88,89].

Several investigators have reported circulating cfDNA levels to be significantly higher in patients with malignancies such as lung, breast, colon, hepatocellular, ovarian, pro-

state, and melanoma than in healthy individuals [90-93]. In another study, Banki et al, reported that cfDNA level in patients with esophageal cancer was significantly higher and after complete resection of the tumor, the cfDNA level returned to normal [94]. Chikawa et al, reported similar results in which the average circulating cfDNA concentrations from esophageal cancer patients were shown to be significantly higher than those in healthy controls [95]. An earlier study had shown that the average concentration of cfDNA in serum was 13 *ng/mL* in a healthy individual and 180 *ng/mL* in various cancer patients [80]. Detection of differences in cfDNA levels may be a good approach and potentially a valuable tool for early detection and for the evaluation of the prognosis of patients with esophagus carcinoma.

Methylation analysis

Aberrant DNA methylation patterns as a measurement of epigenetic changes are frequently found in several types of cancer. The epigenetic changes are described as changes in hypomethylation and hypermethylation levels in special gene regions (*e.g.* promoter regions). For example, tumor suppressor genes have been demonstrated to be hypermethylated at an early stage of tumorigenesis and hypermethylation process is known to silence regulatory genes involved in the cellular pathways related to cancer. Esteller et al, proposed detection of aberrant methylation changes of cfDNA in the serum or plasma in tumor patients and suggested it can be used as a tool for early detection and monitoring of the efficacy of therapy in tumor patients [96].

Kawakam et al, observed hypermethylation in *APC* DNA, with incidence of 6.3 percent in the plasma of SCC patients and 25 percent in plasma of ADC patients [97]. Furthermore, Hibi et al, detected aberration in methylation level of the promoter of *p16* gene in serum DNA of 18 percent of SCC cancer patients [98]. Liu et al, assessed the methylation status *of Wnt* antagonist family of genes in esophageal cancer patients and found that hypermethylation of promoters for *SFRP-1*, *WIF-1*, *DKK-3*, and *RUNX-3* genes could be detected in plasma DNA using Methylation-Specific PCR method [99]. Therefore, detection of aberrant promoter hypermethylation of cancer related genes in serum may be useful for esophageal cancer early diagnosis and detection of recurrence.

Microsatellite analysis

The presence of microsatellite alterations in the serum or plasma could be used as a prognostic indicator. Microsatellite analysis of circulating cfDNA represents an appearing group of biomolecular tumor markers, where as control subjects show no serum alterations [100]. Claus et al, analyzed several microsatellite markers which are commonly altered in serum of patients with SCC of the esophagus. These markers were located at chromosomes 9p (*p16*), 17p (*p53*) and 5q *(APC* gene) and he observed that in 96.4 percent of cases, at least one alteration was found in the serum DNA specimen of esophagus cancer patients [101]. Identification and analysis of microsatellite alterations in the serum DNA from SCC patients may be a valuable tool for evaluation at early stage and follow-up studies.

Conclusion

Early diagnosis of cancer provides much promise for full recovery of patients. Unfortunately, conventional methods of cancer screening are often invasive and expensive. Sensitivity and specificity of these methods are also insufficient for diagnosis of cancer at an earlier stage. For this reason, many researchers are attempting to increase sensitivity and specificity of methods for early detection and monitoring of tumor recurrence.

In the past decade, there has been a revolution in the number of studies analyzing cfDNA of tumor, as a reliable substitute for tissue analysis and a possible tool for the prognosis and diagnosis of cancer patients. At the present time, the major advantage of cfDNA as a biomarker, is the easy accessibility and its stability in plasma or serum specimens.

Another advantage is the use of minimally invasive method for obtaining blood to analyze cfDNA and this may have the potential to replace the existing cancer tissue biomarkers in the future. The combined observations that a correlation exists between molecular alterations such as mutations, microsatellite instability and aberrant methylations in cfDNA and clinical data in esophageal cancer patients, is a strong indicator that cfDNA would likely play a role in early diagnosis and prognosis of esophageal cancer patients in the future.

Furthermore, recent developments of sequencing methods and characterization of circulating cfDNA obtained from a variety of cancer patients in different conditions would increase our knowledge about the mechanisms and functions of circulating cfDNA. Exploration for novel cancer biomarkers with improved diagnostic sensitivity and specificity should help clinicians to apply therapeutics more efficiently and effectively during the management of the cancer disease.

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