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Circulating DNA for detection of gastric cancer

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Abstract. – OBJECTIVE: Gastric cancer (GC) is one of the most common malignant tumors worldwide, particularly, prevalent in China. Despite the decreasing incidence of GC in China, the 5-year survival rate is still not over 30% yet. Therefore, early diagnosis and therapeutic outcome evaluation of GC remains as the issue to be resolved in a clinical setting.

MATERIALS AND METHODS: Recent studies have found the presence of a certain amount of circulating DNA in the peripheral blood of patients with malignant tumor and shown that these free DNA bear tumor-specific genetic information. The circulating DNA detection includes quantitative and qualitative methods and analysis. Combined monitoring of changes in circulating DNA levels and aberrant alteration of relevant tumor genes is likely to provide comprehensive real-time information to patients.

RESULTS: Under normal conditions, oncogene presents in the form of proto-oncogene such as K-ras, which is in non-carcinogenic status under the influence of tumor suppressor gene. When tumor suppressor gene is damaged or mutated of oncogene itself is induced for instance P53, oncogene is then activated and induces tumorigenesis. However, compared to gene mutation detection, the detection of DNA methylation is relatively more well-developed and stable.

CONCLUSIONS: This article reviews the current status of the research on circulating DNA in the diagnosis, assessment of response to therapy and prognostic evaluation in GC. In addition, the advantage, current issue and prospect of using circulating DNA as tumor marker are also analyzed.

Key Words: Gastric cancer, cfDNA, ctDNA, Oncogene.

Introduction

Gastric cancer (GC) is the most common malignant tumor worldwide, and the incidence of GC varies significantly across the world with the highest incidence in eastern Asia followed by Central and Eastern Europe and the lowest incidence in North America and West Africa[1]. GC is prevalent in China with an incidence rate of 679.1/1000 persons and mortality rate of 498.0/1000 persons, which is second only to that of lung cancer, ranking the second most malignant cancer and the most malignant tumor of the gastrointestinal tract. High incidence of GC is observed in East China, Southwest China, North China and Central China. In these areas, morbidity and mortality are two times higher in males than in females, and higher in rural areas than in urban areas[2]. Due to non-significant symptoms being present at the early stage of GC, over half of the patients have missed the opportunity for radical surgery at diagnosis. For these patients, chemotherapy is the most common option of treatment. Unfortunately, the efficacy of chemotherapy is often limited due to congenital and acquired drug resistance. Despite the decreasing incidence of GC in China, the 5-year survival rate is still less than 30%. Therefore, early diagnosis and therapeutic evaluation of GC is urgent an issue to be resolved. In this regard, researchers have been searching for ideal tumor markers, and increasing attentions have been drawn to the prospect of using circulating DNA in clinical diagnosis and treatment of malignant carcinomas.

Circulating cell-free DNA (cfDNA) is cell-free extracellular DNA in the forms of single-stranded (ss) or double-stranded (ds) DNA, or a mixture of ss DNA and ds DNA, presenting in the blood (the plasma or the serum), synovial fluid and other body fluid of animal and human. In 1948, Mandel and Metais[3] detected cfDNA in human blood for the first time. However, the knowledge of cfDNA was limited to its relationship with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and glomerulonephritis, as well as its association with chronic inflammation, such as pancreatitis, inflammatory bowel disease and hepatitis[4,5]. Until 1977, Leon et al[6] showed that circulating plasma DNA is significantly increased in cancer patients and demonstrated the utility of assessing DNA levels in peripheral blood in the therapeutic evaluation and prognostic prediction. These findings were subsequently verified by many other investigations[7,8]. Moreover,

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tumor-related mutations have been found in thise DNA. Currently, the utility of circulating tumor DNA (ctDNA) in tumor management is the most extensively and most intensively studied issue in cfDNA research.

cfDNA is released from normal cells and abnormal cells (such as tumor cells) or produced as viral DNA in the blood of vial carriers. In addition, fetal cfDNA can be found in peripheral blood of a pregnant woman. In healthy subjects, cfDNA is mainly released from apoptotic lymphocytes and other nucleated cells. Apoptosis[9] results in the generation of small and homogeneous DNA fragments with a length of less than 180 bp and a concentration of 3.6-5.0 ng/ml. However, in a cancer patient, cfDNA mainly comes from tumor cells and its concentration is ten times greater than that of health subject[10]. The mechanisms by which circulation tumor DNA (ctDNA) is produced in tumor patients are not yet completely understood. However, most studies suggested that ctDNA of tumor patient can be produced in the following ways: (1) Lysis of tumor cells in circulating blood or micrometastatic loci[11]. (2) Necrosis or apoptosis of tumor cells[12]. (3) DNA release from tumor cells into blood circulation[13]. (4) Presence of DNase inhibitors that inhibit DNA degradation in the plasma results in the accumulation of DNA in the blood of tumor patient[14]. Taken together, the increase in ctDNA in tumor patient is likely to be the result of the collective effect of multiple ways, in which only a small amount of ctDNA is released from tumor cells, whereas the majorities of ctDNA may be produced by cells in the microenvironment of the tumor[15].

Isolation of ctDNA using tools of modern molecular biology and analysis of aberrant genetic and epigenetic alterations may provide critical information for the early diagnosis, therapeutic evaluation, relapse monitoring and prognostic prediction of the tumor. This type of blood-based analysis has become a hot topic of tumor research. With the deepening knowledge of tumor initiation and development as well as the improvement in the sensitivity and specificity of detection technology, ctDNA detection including the detection of DNA in other body fluids is expected to become an important focus of future research.

Methods and Analysis

ctDNA Detection in GC Patient

ctDNA detection includes quantitative and qualitative methods. The former involves quantification of total blood/serum DNA while the latter involves detection of tumor-specific genetic alteration in the blood/serum. Studies have shown that ctDNA level can, to a certain extent, reflect tumor burden[16]. Some study also showed that ctDNA level is closely associated with tumor size and TNM stage of the tumor, and its level is negatively correlated with 3-year disease-free survival as well as overall survival[17]. Moreover, the qualitative study also demonstrated that ctDNA bears same molecular and genetic alterations as the DNA inside tumor (such as genetic mutation[18], hypermethylation in promoter of tumor suppressor gene[19], microsatellite instability and loss of heterozygosity[20], etc.). These findings indicate that tumor detection by genetic investigation only using tumor sample is expected to be replaced by blood analysis, and this novel analytic strategy combined with drug susceptibility testing may be used in clinical settings to facilitate personalized medicine.

Quantitative Analysis

ctDNA quantification has not been standardized and assessment methods vary, as a result, research findings are difficult to be compared. Earlier ctDNA quantification involved fluorescent colorimetric assay, hemagglutinin inhibition, complement fixation and agarose diffusion experiment. However, these methods resulted in low sensitivity and specificity. The later RNA and DNA hybridization, radioimmunological assay and fluorescent method allow to detect ctDNA in nanogram. In the past few years, ctDNA detection using quantitative PCR analysis has become a major focus of research, which enables ctDNA quantification in pictogram (pg). Of note, studies showed that DNA level is higher in the serum than in the plasma, which is likely due to mor genomic DNA released from necrotic white blood cells. In this regard, a plasma sample is the more reliable sample to be studied.

In the field of GC research, studies have shown that different levels of circulating DNA are observed between GC patients and healthy controls. For example, Haruhisa et al[21] measured circulating DNA levels in 53 GC patients preoperatively and 21 healthy controls using quantitative real-time PCR (qrtPCR), and the results showed that levels of circulating DNA are significantly higher in GC patients than in healthy controls ($p=0.03$). Furthermore, Park et al[22] demonstrated that levels of plasma cfDNA in GC patients are 2.4 times higher than those in healthy controls. Subsequently, Sai et al[23], by Kolesnikova et al[24], and Wu et al[25] all showed that ctDNA levels are significantly higher

in GC patients than in patients with benign gastric tumor as well as in healthy controls ($p<0.05$); however, no significant differences were observed in ctDNA levels between patients with benign gastric tumor and healthy controls ($p>0.05$). Wu et al[25] further demonstrated that no significant differences are observed in plasma cfDNA levels in GC patients between tumor size, tumor differentiation and lymphatic metastasis and classification ($p>0.05$). Moreover, the sensitivity of cfDNA detection is significantly higher than those of conventional tumor markers, such as CEA, CA-199 and CA-724. However, no significant differences were found in the specificities of these detections. These findings suggest that quantitative analysis of circulating DNA has certain implications in guiding laboratory GC diagnosis as well as differential diagnosis of benign and malignant tumors.

Subsequently, Kyongchol et al[26] reported that levels of serum cfDNA are increased in patients with advanced GC followed by patients with early GC and healthy controls with significant differences ($p<0.05$). Besides, the study also showed that cfDNA level is closely associated with tumor size, TNM stage of the tumor and postoperative events related to radical tumor resection. cfDNA levels in the blood were significantly declined 24h postoperatively compared to preoperative levels ($p<0.05$). This work indicated that changes in cfDNA level are closely associated with GC initiation and development, surgical outcomes and prognosis.

Qualitative Analysis

Apart from quantitative analysis, various tumor-specific genetic alterations can also be detected in ctDNA through PCR method, including mutation of oncogenes and tumor suppressor genes, microsatellite alterations and some methylation of the promoter of cancer-related genes. Tumor initiation and development, as well as the emergence of resistance to chemotherapy, are closely associated with genetic alterations, which may provide predictive markers with better sensitivity and specificity for tumor surveillance and individualized therapy.

Results

Oncogene and Tumor Suppressor Gene

Under normal conditions, oncogene presents in the form of a proto-oncogene, which is in non-carcinogenic status under the influence of tumor suppressor gene. When tumor suppressor gene is damaged or mutated or mutation of oncogene itself is induced, oncogene is then activated and induces tumorigenesis. In 1989, Stroun et al[27] performed sequencing of circulating DNA for the first time in cancer patients. The first cancer-related mutation detected in the blood involves Ras[28] and p53[29].

K-ras, the gene encoding p21 protein, is the most common activated proto-oncogene of humor tumor. Point mutation of this gene has been observed in various tumors, and three mutation hotspots occur at codons 12, 13 and 61, respectively. The mutation at codon 12 has been frequently seen in pancreatic cancer and colon cancer. The presence of K-ras mutation in various tumors limits the specificity of its detection; however, its detection plays an important role in differentiating benign and malignant tumors. Although some genetic mutations can be detected both in healthy and benign tissues, it will not be present in the plasma or the serum.

p53 is by far the most commonly mutated gene found in human cancer, and p53 mutations are distributed widely in most coding exons of the p53 gene (from exon 4 to exon 10). Inactivation of p53 can cause genomic instability, induce tumorigenesis, and block major apoptotic pathways. Point mutations of p53 have been detected in plasma or serum DNA in patients with colorectal cancer, lung cancer, liver cancer, breast cancer as well as head and neck cancer.

Chen et al[30] reported that p53 mutations are detected in peripheral blood samples from seven out of 73 GC patients with a positive rate of 9.6% and K-ras gene mutations are detected in five patients with a positive rate of 6.8%. However, neither K-ras nor p53 gene mutations are observed in peripheral blood of controls. In addition, p53 and k-ras mutation rates in the peripheral blood were significantly different between GC patients with and without metastasis ($p<0.05$), but these mutation rates are not significantly different between GC of various degrees of differentiation and different clinical stages ($p>0.05$). Hamakawa et al[31] reported that p53 mutations are detected in cfDNA from 3/10 of GC patients. This study suggested that cfDNA level cannot always reflect the extent of the disease, but ctDNA fragment is closely associated with disease status. However, the level of mutated DNA is very low in the peripheral blood due to the low incidence of gene mutation. Besides, gene mutations frequently occur in small cfDNA fragments, which are easily lost during

DNA isolation. Taken together, these conditions can result in low positive rate and false-negative rate for detection. Therefore, mutations detected in many tissues cannot serve as a biomarker of peripheral blood. Moreover, due to the complexity and high likelihood of variation of tumor genes, detection of mutations of a single gene is limited in clinical settings.

Epigenetic Abnormalities

Compared to gene mutation detection, the detection of DNA methylation is relatively more well-developed and stable.

DNA methylation, a process by which a methyl group is transferred from S-adenosyl-methionine to the pyrimidine ring of cytosines within the context of CpG dinucleotides catalyzed by DNA methyltransferase[32], is one of the most intensely studied epigenetic events. DNA methylation often occurs in a specific region of tumor genes, that is, CpG island in the promoter region. DNA methylation causes aberrant expression of genes related to cell proliferation and differentiation through genetic and epigenetic mechanisms, resulting in the loss of regulation of normal cell differentiation followed by cell transformation and eventually leading to tumorigenesis. DNA methylation presents widely in tumors and occurs across all stages of carcinogenesis. This alteration shows a certain degree of tissue-specificity and some gene methylation can be sued as a diagnostic marker for early tumor detection. Aberrant gene methylation involves hypomethylation of tumor gene, hypermethylation of tumor suppressor gene, genomic instability and gene imprinting. Of these alterations, hypermethylation of tumor suppressor gene, which is an important mechanism of inactivation of the gene and plays an important role in tumor initiation and development, can serve as a marker for early detection of GC.

Detection of gene methylation in cfDNA was first reported in 1999[33]. Subsequently, many known methylated gene markers, including APC, DAPK, GSTP1, MGMT, p16, RASSFIA and RAR, have been detected in cfDNA of tumor patients. Currently, the most commonly used method for detecting gene methylation is methylation specific PCR, which exhibits high sensitivity and allows for the detection of 1/1000 DNA methylation in a background of DNA. In fact, hypermethylation also occurs in healthy tissue, but plasma/serum DNA hypermethylation only occurs in malignancies. Therefore, its diagnostic specificity is valuable. However, the low frequency of hypermethylation detected in cancer as well as the correlation between hypermethylation and disease staging limited the value of using hypermethylation detection as early diagnosis of cancer.

Lee et al[34] conducted a study on 54 patients with gastric adenocarcinoma and reported that the rates of methylation at the promoter regions of DAP-kinase, E-cadherin, GSTP1, p15 and p16 detected in primary cancer are 70.3%, 75.9%, 18.5%, 68.5% and 66.7%, respectively, whereas the rates detected in serum DNA were 48.1%, 57.4%, 14.8%, 55.6% and 51.9%, respectively. However, none of these methylations was detected in healthy controls. This study indicated that DNA methylation in peripheral blood can reflect, to some extent, the methylation of gastric cancer with diagnostic significance. Kolesnikova et al[24] studied 20 GC patients and reported that methylation rates of MGMT, p15 and hMLH1 in cfDNA were 50%, 70% and 25%, respectively, whereas these rates in patients with stage III, stage IV and distant metastases were 90%, 90% and 60%, respectively. However, these methylations were not detected in 22 healthy controls. These findings suggested that combined detection of these markers results in higher sensitivity. Bernal et al[35] assessed the methylation of 24 genes in the peripheral blood of GC patients and found that methylation of seven genes was significantly different between cancer patients and healthy subjects. Further analysis in peripheral blood of early GC patients showed that Reprimo methylation rates were 97.7% (42/43), 95.3% (41/43) and 9.7%(3/31) in GC tissue, peripheral blood of GC patients and healthy subjects, respectively. Moreover, Wang et al[36] studied 69 GC patients using nested methylation-specific PCR and reported rates of methylation at promoters of p16 and MGMT of 30.4% and 17.4%, respectively. No methylation was detected in controls. Cheung et al[37] observed that methylation of RNF180 can be detected in GC tissue but not significantly detected in normal gastric tissue, colon cancer and liver cancer. In addition, the sensitivity and specificity of RNF180 methylation detected in the peripheral blood of GC patients reache 63% and 91%, respectively, indicating a better sensitivity and specificity of RNF180 detection. Furthermore, Ling et al[38] reported that XAF1 methylation is detected in cfDNA of 69.8% of GC patients, and XAF1 methylation is closely associated with GC prognosis.

Taken together, methylation of circulating DNA can be used as a marker for GC surveillance and combined detection of multiple DNA

methylations can improve diagnostic efficiency; however, further study is required to explore the optimal combination of gene detections.

Discussion

The major obstacle to the diagnosis, individualized treatment and surveillance of solid tumor as GC is the need for frequent obtaining of sufficient tumor tissue and tumor tissue derived does not completely represent entire tumor. The ideal goal of physicians is to monitor tumor initiation and development using a simple blood analysis. The advancement in molecular and biological technology enables the quantitative analysis of traces of free DNA in peripheral blood, breaking the limit associated with the inability of ex vivo proliferation of large molecules including protein, lipid and carbohydrate detected in body fluid. So, it significantly improved the sensitivity of tumor diagnosis and surveillance. Compared to the limitations of conventional biopsy that results in significant trauma and generates small sample size, ctDNA detection exhibits several advantages including convenient sampling, minimal invasiveness and high repeatability. Obtaining entire fragment of tumor genes from plasma ctDNA of patients, referred to as liquid biopsy, instead of directly performing a biopsy of the tumor can reflect more comprehensively genetic alterations of tumor gene, thereby facilitating the assessment of gene mutations and gene clones of the current tumor[39]. ctDNA may be more reliable than protein tumor markers, such as CEA, CA19-9 and CA72-4, which exhibit a sensitivity of <40% in detecting GC. Moreover, ctDNA has been shown to be more sensitive than circulating tumor cell (CTC). The concentration of ctDNA is higher than that of CTC. ctDNA detection results in lower false-positive rate and ctDNA carries more comprehensive genetic information. In addition, the half-life of ctDNA is less than two hours and its expression depends on the origin of ctDNA. Therefore, ctDNA reflects the current condition of the tumor and its detection allows for dynamic monitoring of tumor development.

ctDNA detection is quite likely to be of great importance in the early diagnosis, treatment monitoring and prognosis evaluation of malignant tumor. However, large sample, multi-center and prospective study is still lacking. Results of conventional qualitative are not consistent with those of quantitative studies. The variations are attributed to the following reasons: (1) Difference in patient selection. Pathological types and staging of GC vary between studies. (2) Difference in control selection. Controls are not matched by relevant factors such as age and gender. (3) Difference in sample processing, including storage, the number and speed of centrifugation of blood sample and DNA isolation procedure. Besides, the efficiency of DNA isolation is not reported. (4) Quantification methods vary across different studies. Quantitative PCR widely used results in a detection of lower levels of circulating DNA in the blood compared to conventional methods. (5) Different internal control housekeeping genes selected in different studies also affects results.

Therefore, current issues to be resolved are listed as follows. (1) Identifying origin of ctDNA. (2) Improving the sensitivity and specificity of ctdNA quantification with a goal to develop defined and standardized testing method, diagnostic standard and follow-up indicators. (3) Currently, ctDNA detection relies mainly on PCR technique, which is prone to generate false-positivity. Besides, it can also result in false-negativity due to the heterogeneity of tumor. Due to the lack of an effective method of ctDNA isolation from blood, excessive background DNA that is often observed in ctdNA studies can affect study result. (4) Precise definition of inclusion conditions of enrolled patients and rational selection of controls are required.

Conclusions

ctDNA is of great importance in the diagnosis and treatment of GC patients. Despite the fact that tumor-specific gene targeting GC has not yet discovered, ctDNA detection is expected to be applied in clinical settings and open a new era of cancer detection using minimal invasive method, with the advance in the study of molecular and biological mechanisms of tumor, advancement in ctDNA detection method and continuous improvement of genetic detection from single gene detection to multiple gene detection.

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Conflicts of interest

The authors declare no conflicts of interest.

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