The Role of Circulating Tumor DNA and Cell-Free DNA in the Management of Germ Cell Tumors: A Narrative Review

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Abstract

Liquid biopsy has demonstrated success as a diagnostic, prognostic, and therapy response monitoring tool in various cancers and could represent a rapid and minimally invasive alternative or complementary test for testicular germ cell tumors (GCTs). This article aims to review the current state of the research into circulating tumor DNA (ctDNA) and cell-free DNA (cfDNA) in testicular GCTs.

Studies have confirmed the presence of ctDNA and cfDNA can be identified in peripheral blood samples of patients with testicular GCTs. Further research has attempted to optimize the methods for ctDNA detection in plasma to improve the sensitivity of these tests; however, a single method with high sensitivity and reliability has yet to be established. Previous studies have employes different methods for detecting cfDNA, including spectrophotometry, capillary electrophoresis, quantitative polymerase chain reaction (PCR), reverse transcription-polymerase chain reaction (RT-PCR), and whole genome sequencing. These studies have various elements of cfDNA examined such as total cfDNA quantity, methylation patterns, and specific mutations. Additional studies have investigated the efficacy of cfDNA detection in combination with other tests including miRNA analysis.

The application of cfDNA as a biomarker has been rapidly expanding in several malignancies. However, there is a relative paucity of research on the clinical utility of cfDNA in testicular cancer, and many questions remain about the significance and feasibility of this biomarker in GCTs. Cell-free DNA shows promise as a biomarker to enhance detection and disease monitoring in testicular cancer, but robust studies are needed to develop an optimal and reproducible method for cfDNA detection in order to determine its clinical application in testicular cancer.

Introduction

The diagnostic evaluation of testicular cancer typically involves serum tumor markers including alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), and lactate dehydrogenase (LDH)[1]. However, these markers are elevated in only about 40% of patients with testicular cancer[2]. Testicular biopsy is typically avoided because of the associated risk for tumor cell seedling and altered patterns of metastases[3]. To overcome these limitations, liquid biopsy has emerged as a recent development, providing a rapid, accurate, and noninvasive alternative to tissue biopsy and radical orchiectomy[4].

Key Words

Testicular cancer, germ cell tumors, cell-free DNA, circulating tumor DNA, liquid biopsy

Competing Interests

None declared.

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Abbreviations

AFP alpha-fetoprotein
bp base pairs
CTCs circulating tumor cells
cfDNA cell-free DNA
ctDNA circulating tumor DNA
GCTs germ cell tumors
hCG human chorionic gonadotropin
LDH lactate dehydrogenase
miRNA microRNA
PCR polymerase chain reaction
qPCR quantitative polymerase chain reaction
RT-PCR reverse transcription-polymerase chain reaction
TGCTs testicular germ cell tumors

Liquid biopsy involves the extraction and analysis of biological samples, such as blood, urine, or saliva. Unlike tissue biopsy, which provides information confined to a specific region and fails to capture complex tumor heterogeneity, liquid biopsy isolates and analyzes tumor-derived or tumor-associated components that circulate in the bloodstream: circulating tumor cells (CTCs), circulating leukocytes, and tumor-derived circulating nucleic acids, such as cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), microRNA (miRNA), and noncoding RNA. This enables longitudinal monitoring of cancer progression[4].

Cell-free DNA refers to fragmented DNA in the noncellular component of blood. Circulating tumor DNA consists of DNA fragments of 150 to 200 base pairs (bp) released into the peripheral blood from cancerous cells as a result of apoptosis, necrosis, or secretion[5]. The presence of tumor-specific genetic and epigenetic alterations in ctDNA makes ctDNA a promising biomarker. The concentration of ctDNA in blood plasma varies among cancer patients based on the type, location, and stage of the cancer and is typically low. The detection of somatic mutations, frequently single base-pair changes, copy number variations, or chromosomal rearrangements in ctDNA shows promise for early cancer diagnosis, tumor dynamics assessment, minimal residual disease identification, and therapy monitoring.

Cell-free DNA isolated from cancer patients can be used for various downstream applications, such as the investigation of mutations, copy number variations, gene fusions, and DNA methylation using various methods including sequencing- or PCR-based approaches. ctDNA analysis can potentially overcome tumor heterogeneity, which may not be captured by tissue sampling.

Currently, there are 3 FDA-approved liquid biopsy tests: 2 cfDNA-based tests (Cobas EGFR Mutation Test v2 and Epi proColon) and one CTC-based test (CellSearch)[5]. Numerous ongoing clinical trials are employing cfDNA analysis in urologic malignancies. Mutations in fibroblast growth factor receptor-3 (FGFR3) in cfDNA were monitored in patients with advanced urothelial carcinoma undergoing treatment with an FGFR antagonist[6]. Patients with a decrease in mutations in cfDNA were found to correlate with a greater decrease in tumor size. Additionally, analysis of FGFR3 mutations in cfDNA were shown to predict disease progression ahead of imaging with computerized tomography (CT) scans. cfDNA has also been used in a study on a programmed death-ligand 1 (PD-L1) inhibitor in urothelial cancer by Powles et al.[7]. Those authors found a correlation between mutations found in ctDNA with mutations found in tumor DNA, suggesting a role for plasma-based biomarker screening in predicting immunotherapy response[7].

Cell-free and circulating tumor DNA are currently being used in clinical trials for urothelial carcinomas. For example, Huang et al. are recruiting for a study using ctDNA and urine tumor DNA as biomarkers for minimal residual disease and upper urinary tract urothelial carcinoma recurrence[8]. Van der Heijden et al. are monitoring ctDNA during follow-up of patients with urothelial carcinoma treated with ipilimumab and nivolumab[9]. Lolkema and van der Veldt are collecting ctDNA as part of their study on biomarkers in patients with advanced urothelial cancer treated with pembrolizumab[10]. In this paper, we review the current state of research into liquid biopsies in GCTs.

Methods

A narrative review was conducted on September 24, 2022, by searching the PubMed and Cochrane Review databases without any limitation on the date range. Articles addressing cfDNA and ctDNA in testicular cancer were included in the review. The search terms used included all Medical Subject Heading (MeSH) entry terms for testicular cancer and cell-free DNA–related terms (**Table 1**). Relevant articles were also identified through the references of the included articles. All types of studies related to ctDNA and cfDNA in testicular cancer were included. Duplicate articles were excluded. Three reviewers (I.D., S.C., D.P.) independently screened the articles for inclusion. Any discrepancies were discussed to determine the final inclusion.

Circulating Tumor DNA and Cell-Free DNA in Testicular Cancer

Testicular cfDNA is thought to result from apoptosis during spermatogenesis. This can occur in both physiological and pathological conditions, including malignancy. In the case of malignancy, cfDNA is considered to be tumor specific and uniquely identifiable, and its analysis can provide insights into disease burden and progression. Of the various cfDNA characteristics, cfDNA methylation and mutation analysis hold the most promise as cancer biomarkers[11].

TABLE 1.

List of testicular cancer MeSH entry terms and cell-free DNA–related search terms used to identify relevant literature in the Cochrane Review and PubMed databases

Testicular cancer MeSH entry terms	Cell-free DNA–related search terms
Testicular Neoplasm	Biopsies, Liquid
Neoplasm, Testicular	Biopsy, Liquid
Testicular Tumors	Liquid Biopsies
Neoplasms, Testis	Cell-Free Nucleic Acids
Neoplasm, Testis	Nucleic Acids, Cell-Free
Testis Neoplasm	Circulating Cell-Free Nucleic Acid
Testis Neoplasms	Circulating Cell-Free Nucleic Acid
Testicular Tumor	Circulating Nucleic Acids
Tumor, Testicular	Acids, Circulating Nucleic
Tumors, Testicular	Nucleic Acids, Circulating
Neoplasms, Testicular	Cell-Free Nucleic Acid
Cancer of Testis	Cell-Free Nucleic Acid
Testis Cancer	Nucleic Acid, Cell-Free
Cancer, Testis	Circulating Cell-Free Nucleic Acids
Cancers, Testis	Circulating Cell-Free Nucleic Acids
Testis Cancers	Circulating Nucleic Acid
Cancer of the Testes	Acid, Circulating Nucleic
Cancer of the Testis	Nucleic Acid, Circulating
Testicular Cancer	Cell-Free DNA
Cancer, Testicular	Cell-Free DNA
Cancers, Testicular	DNA, Cell-Free
Testicular Cancers	cfDNA
Tumor of Rete Testis	cirDNA
Rete Testis Tumor	Cell-Free Deoxyribonucleic Acid
Rete Testis Tumors	Acid, Cell-Free Deoxyribonucleic
Testis Tumor, Rete	Cell-Free Deoxyribonucleic Acid
Testis Tumors, Rete	Deoxyribonucleic Acid, Cell-Free
	Circulating DNA
	DNA, Circulating
	Cell-Free RNA
	Cell-Free RNA
	RNA, Cell-Free
	cfRNA
	cirRNA
	Cell-Free Ribonucleic Acid
	Acid, Cell-Free Ribonucleic
	Cell-Free Ribonucleic Acid
	Ribonucleic Acid, Cell-Free
	Circulating RNA
	RNA, Circulating
	DNA, Circulating Tumor
	Tumor DNA, Circulating
	Cell-Free Tumor DNA
	Cell-Free Tumor DNA
	DNA, Cell-Free Tumor
	Tumor DNA, Cell-Free

cfDNA: cell-free DNA; cirRNA: circulating tumor DNA; MeSH: Medical Subject Heading.

Several studies have identified cfDNA in plasma samples of testicular cancer patients. Ellinger et al. collected serum samples from patients with seminomas or nonseminomas and from healthy patients and isolated the cfDNA[1]. The investigators used RT-PCR to examine beta-actin DNA fragments (106 bp, 193 bp, and 384 bp) and found these fragments to be significantly increased in patients with testicular germ cell tumors (TGCTs) compared to disease-free controls. Boublikova et al. investigated total cfDNA using spectrophotometry, capillary electrophoresis, and quantitative polymerase chain reaction (qPCR) in the peripheral plasma of patients with TGCTs and controls[12]. They found that patients with TGCTs had significantly higher total cfDNA compared to controls.

Studies have shown CTCs can also be detected in the peripheral samples of patients with TGCTs. Bokemeyer et al. detected CTCs in apheresis samples from 58% of patients with metastatic TGCTs undergoing high-dose chemotherapy and peripheral blood stem-cell (PBSC) transplantation[13]. Fan et al. detected beta-hCG mRNA in apheresis products using PCR[14], suggesting the presence of a significant number of CTCs or cell-free DNA rather than tumor cells. All patients with circulating mRNA exhibited elevated serum beta- hCG levels, whereas only 46% of patients without circulating mRNA tested positive for beta- hCG. The findings also revealed a correlation between positive PCR results and higher serum beta- hCG levels at diagnosis[14]. Similarly, in a study by Hautkappe et al., all patients with circulating beta- hCG or AFP mRNA had elevated serum beta- hCG levels, while only 40% of patients with negative PCR results had positive serum beta- hCG[15].

A study conducted by Hildebrandt et al. demonstrated that reverse transcription-polymerase chain reaction (RT-PCR) targeting germ cell alkaline phosphatase is highly sensitive in detecting residual GCT cells in peripheral blood. This method enabled the detection of one tumor cell in at least 106 mononuclear cells[16]. Yuasa et al. also used RT-PCR to detect malignant cells in the bloodstream by measuring the expression of AFP in the peripheral blood of patients with advanced stage testicular cancer. They found that this assay could detect a single cancer cell in 106 peripheral blood stem cells[17].

Further advancements in research have led to the development of techniques that enhance the detectability of ctDNA and CTCs in GCTs. Nastały et al. developed a new assay using a label-free enrichment technique based on the physical properties of tumor cells. By using

keratins 8, 18, and 19, and EpCAM as epithelial cell markers and SALL4 andOCT3/4 as germ cell markers, they achieved the detection of CTCs in 17.5% of peripheral blood samples from testicular cancer patients, compared to only an 11.5% detection rate using the CellSearch assay[18]. Ruf et al. demonstrated the detection of CTCs through alkaline phosphatase enzymatic activity and the use of anti-keratin, anti-EpCAM, and anti-SALL4 antibodies in immunocytochemistry[19].

Taken together, these studies highlight the wide variety of tests developed to measure cfDNA and ctDNA in the peripheral samples of patients with TGCTs. However, the inability to designate a single test as the gold testing standard hinders comparison of cfDNA and ctDNA levels in TGCT patients across different studies. The variation in testing methods poses a significant challenge in establishing clinical application of cfDNA and ctDNA in testicular cancer care.

Clinical Utility of Circulating Tumor DNA and Cell-Free DNA in Testicular Cancer

Circulating tumor and cell-free DNA offer multiple clinical applications in the context of testicular cancer, showing promise as diagnostic and prognostic tools. Various detection methods have been described in the literature. For example, Ellinger et al. used RT-PCR to isolate beta-actin DNA fragments in cfDNA, achieving a sensitivity of 84% and specificity of 97% in distinguishing patients with GCTs from healthy controls[1]. Even among patients with normal ranges of serum tumor markers, cfDNA exhibited a sensitivity of 84% and specificity of 97%, suggesting its potential utility as a biomarker for GCTs, particularly in those patients who do not have elevations in other serum tumor markers[1].

Investigations have also focused on total cfDNA and its 2 main fragments (360 bp and 180 bp) as targets for detection. Boublikova et al. used spectrophotometry, capillary electrophoresis, and qPCR to identify total cfDNA and found that the quantity of cfDNA does not offer a clear threshold to distinguish between TGCT patients and controls, thus limiting the sensitivity of this marker. However, they found that longer cfDNA fragments (360 bp) were present in 58% of patients with TGCTs but absent in controls[12], suggesting that targeting longer cfDNA fragments instead of total cfDNA may be a viable approach. Further studies involving larger samples are necessary to establish an acceptable cutoff value.

Methylation patterns in serum samples of patients with TGCTs have also shown promise as a biomarker. Lobo et al. evaluated hypermethylated RASSF1A in cfDNA as a diagnostic marker for testicular GCTs[20]. RASSF1A is a tumor suppressor gene that exhibits inactivation in many cancers. Promoter hypermethylation of RASSF1A has been a useful marker in several solid malignancies and has also been detected in tissue samples of TGCTs[20]. To quantify hypermethylated RASSF1A, Lobo et al. used a novel droplet digital PCR method to analyze 102 serum samples from patients with GCTs and 29 samples from healthy young adult men[20]. They found that hypermethylated RASSF1A exhibited a sensitivity of 86.7%, surpassing the 65.5% sensitivity of AFP and hCG markers. They also found that combining hypermethylated RASSF1A with miR-371a-3p, a reliable microRNA biomarker for TGCTs, exhibited a sensitivity and specificity of 100%. This study identified a cfDNA target with high sensitivity and specificity, particularly in combination with a microRNA marker.

Ellinger et al. also examined hypermethylation at RASSF1A, as well as methylation at APC, GSTP1, PTGS2, p14(ARF), and p16(INK)[21] using RT-PCR to evaluate samples from 73 patients with TGCTs and 35 individuals without disease. They found significantly higher rates of hypermethylation in the patients with testicular cancer across all examined gene sites. When combining multiple gene sites, the test achieved a sensitivity of 67% and specificity of 97%, surpassing the 58% sensitivity of conventional biomarkers (AFP, hCG, PLAP, LDH). In patients with normal laboratory results, the hypermethylation pattern test demonstrated a sensitivity of 97%, indicating its potential as a set of methylation markers particularly useful for identifying disease in patients with normal laboratory markers.

Kawakami et al. identified another potential cfDNA biomarker, the 5' end of the XIST gene expressed in male germ cells[22]. This gene has been found to be hypomethylated in TGCTs. The investigators analyzed plasma samples from 24 patients with TGCTs and 24 controls and found 64% of patients with TGCTs had detectable unmethylated signals compared to none of the control samples. The presence of unmethylated signals was more common in patients with metastatic tumors (88%) compared to patients with nonmetastatic tumors (53%). Further research is needed to improve the sensitivity of tests for this marker and establish unmethylated XIST fragments as a diagnostic tool for TGCT. Ellinger et al. also investigated circulating cell-free mitochondrial DNA in the serum of patients with seminoma and nonseminomas and found that cell-free mtDNA exhibited a sensitivity of 59.6% and 94.3%, respectively, in identifying patients with GCTs[23]. For patients without elevated serum tumor markers, cell-free mitochondrial DNA demonstrated a sensitivity of 64.5% and specificity of 91.4%.

Studies have investigated the use of the cell-free DNA tumor fraction and the identification of somatic mutations. Tsui et al. identified cfDNA mutations in plasma samples and employed whole genome sequencing to estimate the cfDNA tumor fraction[24]. By using cf-IMPACT, a tumor test capable of detecting genomic alterations in specific cancer-related genes, the researchers found somatic mutations in 60% of samples from patients with TGCTs (n = 118). For the remaining samples without detected alterations and low tumor fraction, a more sensitive but less comprehensive test, MSK-ACCESS, was used for re-analysis. This latter test showed 14 additional samples with somatic mutations. Whole exome sequencing was then performed for samples without detected alterations but high tumor fraction, leading to the identification of 5 more samples with somatic mutations. In total, 90 of 118 samples (79%) exhibited somatic mutations across all 3 methods. Overall, this study showed that cfDNA tumor fraction can guide further testing for somatic mutations in cfDNA to maximize mutation identification. Moreover, the identification of specific mutations has the potential to inform the selection of precision medicine treatments.

The prognostic implications of the presence of cfDNA and ctDNA in GCTs remain unclear. Ellinger et al. found that levels of cell-free mtDNA did not correlate with disease severity, including tumor stage or lymphovascular invasion, and Bokemeyer et al. showed that the presence of CTCs in transplanted PBSC apheresis products did not affect overall survival[13,23]. On the other hand, Hautkappe et al. demonstrated that 41.2% of patients with stage IIc/III GCTs had detectable mRNA of either AFP or beta- hCG compared to only 23.5% of patients with stage I disease[15].

In a clinical trial conducted by Reid et al., cell-free DNA is being investigated to determine whether cfDNA is detectable in the plasma of patients with platinum refractory/resistant GCTs. The researchers are further analyzing patients with detectable cfDNA for changes in cfDNA that are associated with clinical resistance to platinum (NCT0398087)[25].

The clinical management of TGCT has experienced limited progress in recent decades in integrating molecular diagnostic and prognostic tools into targeted therapies for affected patients[26]. Total cfDNA and changes in cfDNA levels have been shown to be associated with specific treatment responses, prognoses, and survival rates in various tumors including lung, pancreatic, colorectal, and other malignancies.

While the utility of cfDNA in GCTs remains uncertain, circulating miR-371a-3p has recently captured attention as a robust biomarker with significant potential to address clinical gaps in GCT management. In 2011, Murray and colleagues reported detectable serum miR-371a-3p in a 4-year-old boy with disseminated yolk sac tumor[27]. Subsequent to this, a large body of literature has consistently illustrated the utility of miR-371a-3p in differentiating GCTs from controls[28]. In the largest report to date, Dieckmann et al. analyzed a cohort of 616 GCT patients and found that miR-371a-3p exhibited a sensitivity of 90.1% and specificity of 94% for the diagnosis of GCT[29]. miR-371a-3p also demonstrated a sensitivity of 83% and specificity of 96% for identifying relapses, indicating its expanded clinical utility beyond diagnosis[29]. The predictive capability of miR-371a-3p for identifying relapses has been substantiated by Nappi et al. and Lafin et al.[30,31]. Leão et al. expanded on previous work by demonstrating that miR-371a-3p could also detect residual viable GCT after chemotherapy, with an area under the curve of 0.874, and with levels declining in response to treatment[32]. Apart from identifying teratomas, miR-371a-3p shows potential as an important adjunct in the clinical management of GCTs. Currently, 2 clinical trials (NCT04914026, NCT04435756) are exploring the role of miR-371 in the management of GCTs[33,34].

Discussion

The utility of circulating tumor DNA and cell-free DNA as diagnostic, prognostic, and therapeutic markers has been well-documented in various cancers. While the application of cfDNA as a biomarker has been rapidly expanding in several malignancies, relatively little research exists on the clinical utility of cfDNA in testicular cancer, leaving many questions unanswered about the significance and feasibility of this biomarker in TGCTs.

Studies have demonstrated the presence of ctDNA and cfDNA in peripheral blood samples of patients with TGCTs. Efforts have been made to optimize the methods for ctDNA detection in plasma to enhance the sensitivity of these tests; however, a single method with high sensitivity and reliability has yet to be established. Previous studies have employed diverse methods for detecting cfDNA, including spectrophotometry, capillary electrophoresis, qPCR, RT-PCR, and whole genome sequencing. These studies have also examined various aspects of cfDNA, such as total cfDNA quantity, methylation patterns, and specific mutations (**Table 2**). Some studies have evaluated the efficacy of cfDNA detection in combination with other tests including miRNA analysis.

Many questions remain about the implementation of cfDNA and ctDNA in the clinical setting. To achieve the clinical validity and utility of liquid biopsy tests, it is necessary to assess the analytical validity of these tests, and then conduct prospective studies using the protocols that resulted in analytical validity. Analytical validity encompasses the accuracy, sensitivity, specificity, and robustness of the liquid biopsy test and relies on pre-analytical variables and protocols used for sample

TABLE 2.

Summary of studies included in this narrative review highlighting the various detection methods and targets

Study	Detection method	Target
Bokemeyer et al.[13]	RT-PCR	beta- hCG, fibronectin (EDB variant), EGFR, CD44 (v8 to 10 variant), germ cell and placental alkaline phosphatase, human endogenous retrovirus type K (ENV and GAG), and XIST
Boublikova et al.[12]	Spectrophotometry, capillary electrophoresis, qPCR	Total cfDNA and its 2 main fragments (360 bp and 180 bp)
Ellinger et al.[23]	RT-PCR	cell-free mitochondrial DNA
Ellinger et al.[1]	RT-PCR	106 bp, 193 bp, and a 384 bp beta-actin DNA fragment
Ellinger et al.[21]	RT-PCR	Hypermethylation at RASSF1A; methylation at APC, GSTP1, PTGS2, p14(ARF), and p16(INK)
Fan et al.[14]	PCR	beta- hCG mRNA
Hautkappe et al.[15]	RT-PCR	beta- hCG mRNA, AFP mRNA
Hildebrandt et al.[16]	RT-PCR	Germ cell alkaline phosphatase
Kawakami et al.[22]	PCR	Methylation of 5′ end of the XIST gene
Lobo et al.[20]	Droplet digital PCR	Hypermethylated RASSF1A
Nastały et al.[18]	Label-free enrichment technique, immunocytochemical staining	Germ cell tumor (anti-SALL4, anti-OCT3/4) and epithelial cell–specific (anti-keratin, anti-EpCAM) antibodies
Ruf et al.[19]	Immunocytochemistry	Anti-keratin, anti-EpCAM, and anti-SALL4 antibodies
Tsui et al.[24]	cf-IMPACT, MSK-ACCESS, whole genome sequencing	cfDNA tumor fraction and somatic mutations
Yuasa et al.[17]	RT-PCR	AFP

cfDNA: cell-free DNA; mRNA: messenger RNA; PCR: polymerase chain reaction; RT-PCR: transcription-polymerase chain reaction.

preparation and biomarker detection[35]. Pre-analytical variables are factors not associated with the disease that can impact the integrity of the bodily fluids or biomarkers present therein and hence influence the analysis results. These variables can have a technical, biological, or environmental origin. The impact of pre-analytical variables is based on the biomarker and nature of the bodily fluid studied and should be thoroughly assessed for each newly developed liquid biopsy test[36,37]. Standard operating procedures for the complete workflow of liquid biopsy tests are critical for advancing their clinical implementation[5].

Despite the success of cfDNA in multiple cancers, particularly lung and colon cancers, only 3 liquid biopsy tests have received FDA approval. The lack of reproducibility in preclinical research may contribute to this poor clearance rate by the FDA. Future directions may focus on developing standardized methods for measuring cfDNA in plasma to ensure reliable results that provide clinical value. Standardization efforts may also help establish cutoff values for screening and prognostic purposes. Addressing the challenges of cfDNA detection, such as the low quantities of cfDNA in plasma and its rapid clearance of cfDNA to circulation, is crucial for the successful implementation of standardized methods. Cell-free DNA holds promise as a biomarker to enhance detection and disease monitoring in testicular cancer, but robust studies are needed to establish an optimal and reproducible method for cfDNA detection before determining its clinical application in the context of testicular cancer.

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