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Tumor heterogeneity: biological basis, evolutionary dynamics, emerging technologies, and clinical implications

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Abstract

Tumor heterogeneity is a fundamental hallmark of cancer and one of the major challenges of modern oncology. Genetic and epigenetic alterations, as well as interactions with the tumor microenvironment, drive the continuous emergence of distinct cellular subpopulations, generating dynamic intratumor heterogeneity mediated by clonal evolution. These processes promote tumor progression, metastatic dissemination, therapeutic resistance and disease recurrence. Recent advances in next-generation sequencing, liquid biopsy, single-cell sequencing and spatial transcriptomics have substantially improved our knowledge of tumor evolution and enabled more extensive characterization of tumor diversity. This review summarizes the biological mechanisms underlying tumor heterogeneity, the major evolutionary models of tumor development and current technologies used to investigate tumor evolution. In addition, we discuss the clinical implications of tumor heterogeneity, such as treatment selection, resistance mechanisms and longitudinal disease monitoring. Finally, we discuss emerging technologies and future perspectives that could facilitate the integration of tumor evolution into precision oncology and support the development of more effective personalized therapeutic strategies.

Keywords: tumor heterogeneity, tumor evolution, cancer

Introduction

According to GLOBOCAN, there are approximately 20 million new cases of cancer each year, resulting in 9.7 million deaths (1). Tumors are not homogeneous entities but are highly heterogeneous due to the accumulation of genetic and epigenetic alterations caused by genomic instability, carcinogen exposure as well as interactions with the tumor microenvironment. Molecular diversity can occur at both the intertumoral and intratumoral levels. In other words, differences can exist between tumors from different patients as well as within a single tumor. This is manifested as cancer cell phenotypic differences that are continuously shaped by evolutionary processes, including natural selection, mutations and genetic drift, as well as through epigenetic changes and external factors conferring cancer cell plasticity (2-4). As a consequence, tumor heterogeneity contributes to tumor progression, metastasis, therapeutic resistance, and poor clinical outcomes. Despite the major progress made in the understanding of tumor initiation, progression, intercellular communication and interaction with the immune microenvironment obtained through next-generation sequencing (NGS) technologies, tumor heterogeneity and clonal evolution are still poorly understood, particularly concerning their contribution to clinical outcome and treatment response (5-7).

This review outlines the biological basis of tumor heterogeneity, the evolutionary dynamics, technologies to characterize tumor heterogeneity and its clinical implications.

Biological basis of tumor heterogeneity

Tumor heterogeneity is reflected in differences between tumors of the same type in different patients (interpatient heterogeneity), between different tumors within the same patient (intertumor heterogeneity), and between distinct regions of the same tumor (intratumor heterogeneity – ITH). These differences arise from genetic and epigenetic alterations, as well as from interactions with the tumor microenvironment, phenotypic plasticity, and various external factors. Together, these mechanisms contribute to the continuous generation of cellular diversity, driving tumor progression, metastatic potential, and therapeutic resistance (8).

Genomic instability is a hallmark of most cancers (9). Depending on the underlying mechanism, it can be manifested as an increased rate of nucleotide mutations (mutator phenotype), or copy number alterations (CNA), chromosomal instability (CIN), or microsatellite instability (MSI) and CpG island mutator phenotype (CIMP) (10, 11).

In hereditary cancers, genomic instability mainly occurs due to pathogenic variants in DNA repair genes, such as BRCA1, and BRCA2, which increase the risk of developing breast and ovarian cancer. In tumors carrying these variants, additional functional loss of homologous recombination repair (HRR), can lead to a homologous recombination repair deficiency (HRD) phenotype, which is associated with characteristic genomic "scars", including increased rates of loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST). Similarly, pathogenic variantsin mismatch repair genes (MMR) in colorectal cancer can lead to widespread MSI (12, 13). In contrast, the molecular basis of genomic instability in sporadic cancers is multifaceted and ranges from increased burden of single nucleotide variants (SNVs) to whole genome duplication events. Defects in the proofreading pathway (PRD), mismatch repair deficiency (MMRd) or direct exposure to carcinogens from the external environment can lead to accumulation of mutations and very high tumor mutational burden (TMB) which acts as a substrate for tumor evolution (14, 15). Chromosomal instability is a result of errors in chromosome segregation leading to changes in the number of chromosomes (16). CIN is a catalyst for genomic, epigenomic and transcriptional heterogeneity, fueling cancer progression. Chromothripsis is a particular form of CIN characterized by focal complex rearrangements resulting from a single burst of catastrophic fragmentation. One consequence of chromothripsis may be the formation of self-propagating extrachromosomal DNA (ecDNA) carrying oncogenes or drug resistance genes, that follow a non-Mendelian inheritance mode through mitosis (17).

As genomic instability increases, so does the rate of genetic, epigenetic and structural alterations, giving rise to increased phenotypic diversity within a tumor cell population (**Figure 1**). These changes are not gained by all tumor cells simultaneously, leading to genetically heterogeneous subpopulations (18-20). For example, multiregion sequencing studies have shown that different areas of the same tumor can contain different subclonal populations with different driver alterations, providing direct evidence for spatial intratumoral heterogeneity and branched tumor evolution (21). Intratumor heterogeneity is not reflected exclusively at the genetic level (22). Examples of non-genetic heterogeneity include not only epigenetic changes at the level of DNA methylation (e.g. CpG island mutator phenotype), changes in chromatin conformation driven by mutations in chromatin modifier genes or 3D architecture, but also in the form of transcriptional stochasticity, or asymmetric distribution of molecules during mitosis (23). External factors also contribute

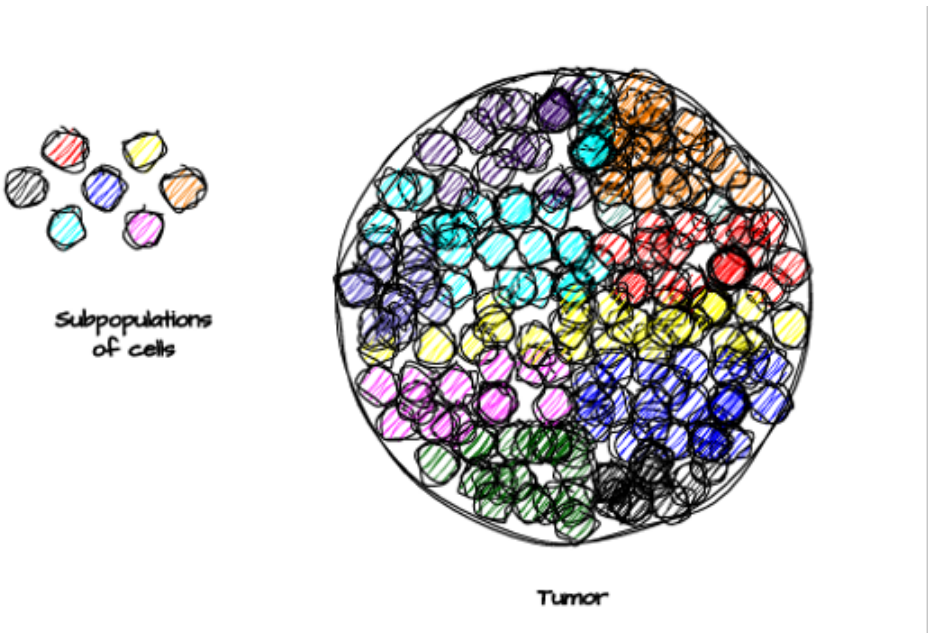


Figure 1. Schematic representation of intratumor heterogeneity with phenotypically diverse tumor cell subpopulations

to tumor heterogeneity by causing changes in gene expression through the influence of the tumor microenvironment, even though the genetic makeup remains the same, conferring adaptive phenotypes. This phenotypic plasticity may be driven by the tumor microenvironment states such as hypoxia, acidosis, extracellular matrix, inflammatory states, immune-predation, or exposure to cancer therapeutics (6, 24).

Among these factors, hypoxia has emerged as one of the strongest promoters of phenotypic plasticity. Hypoxia induces metabolic reprogramming, cancer stem cell-like properties, epithelial–mesenchymal transition and therapeutic resistance through the activation of hypoxia-inducible factors (HIF-1α and HIF-2α), which promote tumor progression and intratumor heterogeneity (25). Yabo et al. also reported that in glioblastoma, phenotypic plasticity allows transitions between cellular states without the acquisition of new genetic changes, and that the tumor microenvironment represents one of the main regulators of phenotypic plasticity through hypoxia, vascular niches, immune cells, and intercellular communication. These microenvironment cues induce stemness, metabolic adaptation and transitions towards more aggressive, therapy-resistant phenotypes (26).

Evolutionary Dynamics of Tumor Heterogeneity

Genomic instability leads to the accumulation of new mutations and contributes to tumor heterogeneity, especially ITH, represented by the coexistence of genetically and phenotypically distinct populations of cancer cells within the same tumor. Selective pressure constantly acts on subpopulations of cells, allowing the evolution of those with the highest fitness. Here, fitness refers to the relative ability of a cell or clone to survive and proliferate under specific environmental conditions. Those subpopulations of cells that do not have sufficient fitness are eliminated by therapy or outgrown by other subpopulations; however, some subclones harbor mutations that allow them to resist therapy and continue to expand after treatment, leading to disease relapse (18, 20, 27).

Tumor evolution follows Darwin's evolutionary principles, where under selective pressure, mutations and genetic drift tumor cell subpopulations with different fitness levels, gain a selective advantage over others. Based on these evolutionary trajectories, several models of tumor evolution have been proposed: Linear, Branched, Neutral, and Punctuated (parallel) evolution (28-30). The differences are presented in **Table 1**.

The linear model of evolution proposes a scenario in which new driver mutations provide a strong selective advantage to tumor cell populations, allowing them to expand by outcompeting pre-existing clones and leading to the expansion of one dominant clone, with some intermediate clones remaining from previous selective sweeps (**Figure 2A**). Compared with other evolutionary models, this model is characterized by lower intratumoral heterogeneity, as the tumor population is mainly dominated by a single successful clone. Although linear evolution is generally associated with lower clonal diversity, recent studies suggest that this evolutionary pattern may also be linked to aggressive clinical behavior. For example, Lv et al. demonstrated that breast cancer patients exhibiting a linear evolutionary trajectory experienced more rapid disease progression, indicating that limited clonal diversity does not necessarily predict a favorable clinical outcome (28, 29, 31).

The branched model of evolution is characterized by a common ancestral clone from which, due to the accumulation of driver mutations, new subclones develop and all evolve in parallel within a single tumor (**Figure 2B**). There are indications that distinct subclonal populations interact with each other, thereby promoting tumor growth (32-34). Branched evolutionary trajectories have been described in several solid tumors, including breast cancer and non-small cell lung cancer (NSCLC). While Lv et al. reported improved survival among breast cancer patients exhibiting a branched evolutionary pattern compared with those following a linear trajectory, Frankell et al. showed that recent subclonal expansion in NSCLC was associated with significantly poorer disease-free survival. The results indicate that the clinical relevance of branched evolution is context-dependent and may vary according to the tumor type, stage and timing of subclonal diversification (31, 33, 35).

The neutral evolution model is founded on the premise of a very high ITH, which is generated by the accumulation of passenger mutations that are not strongly selected and do not significantly influence tumor fitness (**Figure 2C**). The accumulation of these mutations is a consequence of genetic drift, which can lead to the random expansion of certain subclones. Williams et al. postulated that not all tumors follow this evolutionary model, but only approximately one-third of tumors analyzed from The Cancer Genome Atlas (TCGA) database, especially colorectal cancer. However, their study was based on low-depth exome sequencing, which may introduce limitations related to sequencing sensitivity and the detection of subclonal populations. Also, neutral evolution is a concept derived from molecular evolution and mathematical calculations and explains how genetic diversity can arise in the absence of strong selective pressure through genetic drift. However, in tumor biology, it is not considered to directly contribute to tumor growth, resistance to therapy, or the fitness of the tumor cell populations. Therefore, the theory proposed by Williams et al. should not be considered as the sole model explaining tumor evolution (29, 36, 37).

The punctuated model of evolution proposes that multiple genomic aberrations arise in the early stages of tumor development over a relatively short period of time. ITH is extremely high in the earliest stages, after which a limited number of dominant subclones form the majority of the tumor mass (**Figure 2D**). This suggests that early catastrophic genomic events may contribute to the acquisition of invasive, metastatic and therapy-resistant features in tumor cells. This hypothesis is experimentally supported by the presence of copy number variations and structural rearrangements that can be restricted to a single chromosome or span the entire genome, leading to chromosomal instability and aneuploidy (28, 29). Evidence for punctuated evolution was provided by Gao et al., who, through single-cell sequencing of 12 untreated triple-negative breast cancers, showed that most copy number aberrations were established early and then maintained during clonal expansion (38). Similarly, Baka et al., in whole-genome sequencing analyses of 57 prostate cancers, showed that complex genomic rearrangements often occur in coordinated, catastrophic events, supporting the concept that multiple genomic changes may arise during discrete bursts rather than by gradual accumulation (39). Comparison of the major models of tumor evolution is given in **Table 1**.

Table 1. Comparison of the major models of tumor evolution

Evolutionary model	Main characteristic	Intratumoral heterogeneity	Selective pressure	Clinical relevance
Linear	Sequential clonal replacement	Low	High	Lower adaptability
Branched	Parallel expansion of multiple subclones	High	High	Therapy resistance, metastasis
Neutral	Genetic drift predominates	Very high	Low	Limited direct clinical impact
Punctuated	Early burst of genomic alterations	High initially	Early selection	Rapid progression

Technologies for Characterizing Tumor Heterogeneity

Intratumor heterogeneity cannot be fully captured using conventional tissue biopsy as only a part of the tumor is sampled. This is not enough to accurately characterize the heterogeneity of the tumor or guide the selection of the most appropriate therapy, as we cannot say with certainty whether a mutation may be present in the unsampled region of the tumor. Furthermore, tissue biopsy only provides a static snapshot of a constantly evolving disease. The development of high-throughput technologies has allowed us to analyze genomic, transcriptomic and epigenomic changes in great detail. Although no single technology can capture the complexity of tumor evolution, each technology provides complementary information on various aspects of tumor biology (40).

Liquid biopsy is one of the most promising tools to study tumor heterogeneity and clonal evolution. Analysis of circulating tumor DNA (ctDNA), circulating tumor cells (CTCs) and other circulating biomarkers obtained from blood and other body fluids, such as urine or saliva, allows minimally invasive, longitudinal monitoring of tumor dynamics. This strategy allows early resistance detection, clonal evolution monitoring, treatment response assessment, and minimal residual disease (MRD) detection (40, 41). For example, Lv et al. used ctDNA sequencing to reconstruct clonal evolution and track temporal dynamics of metastatic breast cancer, which demonstrated the potential of liquid biopsy for longitudinal assessment of tumor evolution (31).

By monitoring somatic mutations of the tumor over time, or at different regions of the tumor, it is possible to identify subclonal populations that undergo clonal expansion over the course of tumor progression. In this way, we can reconstruct tumor evolution. The reconstruction of tumor evolution within a single tumor is based on high-depth sequencing, followed by bioinformatic data processing and the estimation of mutant allele frequencies (MAF). Based on MAF estimates, computational approaches for clonal deconvolution (e.g., PyClone, SciClone, CITUP) can normalize and cluster mutation frequencies to identify clonal subpopulations and to determine how ITH evolves during tumor progression, metastatic dissemination or treatment (42).

In addition to liquid biopsy, approaches for studying tumor heterogeneity include bulk sequencing, as well as single-cell

sequencing and spatial transcriptomics. Bulk sequencing allows reconstruction of clonal architecture and comparison of primary and metastatic tumors. Jamal-Hanjani et al. used multiregion bulk sequencing to uncover extensive intratumoral heterogeneity and branched evolution of NSCLC and reconstruct its evolutionary history (33).

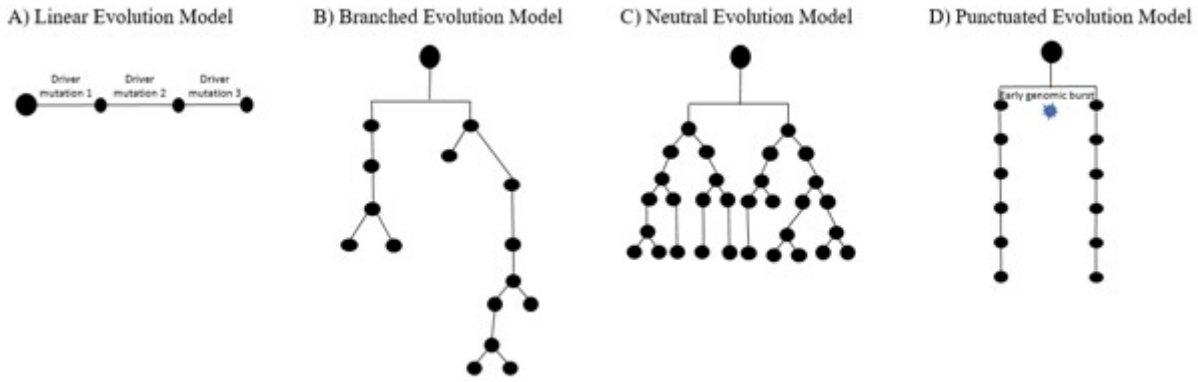


Figure 2. Schematic representation of the major models of tumour evolution. (A) Linear evolution is characterized by the sequential acquisition of driver mutations, resulting in the progressive replacement of one dominant clone by another. (B) Branched evolution involves the parallel diversification of multiple subclones originating from a common ancestral clone. (C) Neutral evolution is driven primarily by genetic drift and the accumulation of passenger mutations, generating extensive subclonal diversity without strong selective pressure. (D) Punctuated evolution is characterized by an early burst of genomic alterations followed by the expansion of a limited number of dominant subclones.

Single-cell sequencing provides a cellular-resolution analysis of tumors to identify rare subclonal populations and characterize tumor evolution. Accordingly, Maynard et al. used single-cell RNA sequencing to characterize therapy-induced tumor evolution and identify resistant cellular populations in NSCLC (43). Spatial transcriptomics (ST) provides information on the spatial organization of tumor cells, cell–cell interactions, and the influence of the tumor microenvironment (TME) (40). By preserving the spatial context of molecular indicators, we can reveal spatially distinct cellular populations that could be obscured by bulk sequencing. In breast cancer, spatial transcriptomics has demonstrated that the immune repertoire of intraepithelial T and B cells exhibits lower diversity and higher clonal potential compared with that of stromal T and B lymphocytes (44).

These approaches provide complementary information on tumor heterogeneity. Liquid biopsy enables a minimally invasive longitudinal approach for monitoring tumor progression and treatment response, but its sensitivity is limited by tumor burden and ctDNA loss. Bulk sequencing offers good information about the clonal architecture but averages molecular signals from heterogeneous cell populations and may miss rare subclonal mutations and introduce biases from tumor purity and copy number variations (CNVs). Single-cell sequencing overcomes many of these limitations, but is still technically challenging, expensive and prone to technical noise and dropout events that may generate false-negative results. Spatial transcriptomics offers information on the TME and the spatial arrangement of cells, but current limitations include cost, resolution and complexity of bioinformatic tools. Therefore, the complementary integration of technologies provides the most complete understanding of tumor heterogeneity and clonal evolution (37).

Clinical Implications of tumor heterogeneity

Due to the selective pressure imposed by therapy, sensitive subclonal populations may be eliminated, while resistant clones can survive, expand, and contribute to disease progression. The emergence of resistance reduces the efficacy of targeted therapies, ultimately leading to treatment failure and disease relapse (5). A well-characterized example of this process is observed in NSCLC, where treatment with EGFR tyrosine kinase inhibitors does not have an effect on resistant subclones harboring secondary EGFR alterations, such as the T790M mutation, allowing the survival of tumor cells during therapy and driving disease progression (45, 46).

Genetic alterations associated with therapy resistance generally consist of SNVs, CNAs, as well as epigenetic changes, most commonly DNA methylation. These alterations continuously accumulate, leading to stochastic changes in gene expression and increased metastatic potential of tumor cells. Although the mechanisms underlying the development of ITH are not fully understood, numerous studies have shown that higher levels of ITH are associated with poorer patient prognosis (18, 47).

A better understanding of tumor heterogeneity can facilitate identification of a patient's unique molecular profile and thus enable a more precise choice of targeted therapies. Characterization of the clonal architecture of tumors

enables identification of truncal driver mutations as well as clinically relevant subclonal populations that may influence therapeutic response and development of resistance. This knowledge facilitates more informed therapeutic decision making and may enable design of combination treatment strategies, thus delaying or preventing disease progression (5, 48).

As tissue biopsy only represents one region of a spatially heterogeneous tumor, clinically relevant subclonal alterations may be missed. Therefore, integration of tissue biopsy with complementary molecular approaches may allow more comprehensive characterization of tumor heterogeneity and improve treatment selection.

Technologies based on liquid biopsy provide a non-invasive approach for longitudinal monitoring of tumor evolution through the analysis of ctDNA. As ctDNA is released from multiple primary and metastatic tumor sites, liquid biopsy can capture spatial and temporal tumor heterogeneity more comprehensively than a single tissue biopsy. Longitudinal ctDNA analysis enables detection of emerging resistant clones, monitoring of treatment response and identification of molecular relapse prior to radiological progression. In patients with EGFR mutant NSCLC, serial ctDNA analysis can identify the emergence or disappearance of the EGFR T790M resistance mutation, thus guiding the use of third-generation EGFR tyrosine kinase inhibitors such as osimertinib (49). While osimertinib was initially approved for patients with acquired T790M-positive disease after progression on earlier-generation EGFR TKIs, it has since become an established first-line therapeutic choice for patients with EGFR-mutant advanced NSCLC. Longitudinal analysis within the TRACERx study showed that serial ctDNA profiling captures the dynamic evolution of tumor subclones and provides a non-invasive approach for monitoring intratumor heterogeneity and the emergence of therapy-resistant mutations over time (7). Likewise, in ALK-rearranged NSCLC, repeat molecular profiling can identify specific ALK resistance mutations and guide the selection of second-generation ALK inhibitors (50). In addition, liquid biopsy allows for the detection and monitoring of MRD, and therefore the enables earlier detection of relapse and timely adjustment of therapeutic strategies (5, 51). All these advances support a more dynamic and personalized approach to cancer management, in which therapeutic decisions can be continuously refined according to the evolving molecular landscape of the tumor.

Future perspectives

Although our understanding of tumors, tumor heterogeneity and tumor evolution has advanced significantly over the decades, there are still many unknowns in this field because of current technical limitations. With the development of new technologies, there will be a deeper understanding of the molecular profile of tumors, and therefore more effective treatment strategies. Technologies such as spatial transcriptomics, spatial proteomics, as well as single-cell sequencing, will enable more precise mapping of intratumoral heterogeneity and the interactions between tumor cells and the tumor microenvironment. Moreover, longitudinal monitoring of the disease using ctDNA to track tumor evolution will enable a better therapeutic approach for patients. Machine learning and artificial intelligence are currently rapidly evolving and are expected to improve our understanding of mutation patterns, as well as facilitate the integration of genomics, proteomics, transcriptomics, radiomics, and pathology with the aim of better predicting tumor evolution, resistance, and response to therapy.

Advances in technology are also improving the clinical aspects of therapy, monitoring, early diagnosis and prevention. There is a growing trend toward a personalized approach, but there are currently some limitations that need to be addressed. In particular, longitudinal monitoring of disease and tumor evolution during therapy has the potential to predict resistance, control the disease, and eradicate all tumor cells. However, there are limitations, such as sampling strategies, since there is not always the possibility of taking a sample from a patient, as well as the possibility of patient's death during the course of the treatment. Regarding monitoring, while it could allow personalized mutation detection, implementation is challenging due to costs and the appearance of new clones which could be overlooked. Early diagnosis could identify genetic changes that require intervention, but differentiation of these mutations from those originating in normal tissue remains challenging and there is possible lack of impact on clinical outcomes. Finally, prevention is possible through the identification of mutational signatures that could reveal the etiological factors driving tumorigenesis. However, exogenous factors cannot always be prevented, and some tumors, like those occurring in children, could not be prevented. In addition to these challenges, there is also a lack of standardized analytical workflows and assay protocols, differences in sample processing and sequencing platforms, and the need for robust bioinformatic pipelines that can handle increasingly complex and heterogeneous datasets.

Many challenges remain, but rapid progress is being made in each of these areas to provide the best possible care to patients according to their specific conditions, as tumors are extremely heterogeneous and dynamic (52).

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