Molecular Subtypes of Clear Cell Renal Cell Carcinoma Are Associated with Sunitinib Response in the Metastatic Setting

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Abstract

Purpose: Selecting patients with metastatic clear-cell renal cell carcinoma (m-ccRCC) who might benefit from treatment with targeted tyrosine kinase inhibitors (TKI) is a challenge. Our aim was to identify molecular markers associated with outcome in patients with m-ccRCC treated with sunitinib.

Experimental Design: We performed global transcriptome analyses on 53 primary resected ccRCC tumors from patients who developed metastatic disease and were treated with first-line sunitinib. We also determined chromosome copy-number aberrations, methylation status, and gene mutations in von Hippel-Lindau (VHL) gene.

Molecular data were analyzed in relation with response rate (RR), progression-free survival (PFS), and overall survival (OS). Validation was performed in 47 additional ccRCC samples treated in first-line metastatic setting with sunitinib.

Results: Unsupervised transcriptome analysis identified 4 robust ccRCC subtypes (ccRCC1 to 4) related to previous molecular classifications that were associated with different responses to sunitinib treatment. ccRCC1/ccRCC4 tumors had a lower RR (P = 0.005) and a shorter PFS and OS than ccRCC2/ccRCC3 tumors (P = 0.001 and 0.0003, respectively). These subtypes were the only significant covariate in the multivariate Cox model for PFS and OS (P = 0.017 and 0.006, respectively). ccRCC1/ccRCC4 tumors were characterized by a stem-cell polycomb signature and CpG hypermethylation, whereas ccRCC3 tumors, sensitive to sunitinib, did not exhibit cellular response to hypoxia. Moreover, ccRCC4 tumors exhibited sarcomatoid differentiation with a strong inflammatory, Th1-oriented but suppressive immune microenvironment, with high expression of PDCD1 (PD-1) and its ligands.

Conclusions: ccRCC molecular subtypes are predictive of sunitinib response in metastatic patients, and could be used for personalized mRCC treatment with TKIs, demethylating or immunomodulatory drugs.

Introduction

Targeted therapies (TT) have significantly improved the prognosis of patients with metastatic clear-cell renal cell carcinoma (m-ccRCC). Sunitinib is a tyrosine kinase inhibitor (TKI) targeting VEGFR that significantly prolongs progression-free survival (PFS), but not overall survival (OS), as compared with IFNα (1, 2). Currently, it is an approved first-line treatment option for patients with m-ccRCC. However, in the pivotal phase III trial, 7% of patients experience progressive disease (PD) upon RECIST at the first evaluation, and virtually all patients ultimately develop PD (2). In routine clinical practice, primary PD rate is higher and may reach 20%. Although several prognostic factors of survival in patients with m-ccRCC receiving TT have been described and several resistance mechanisms have been proposed (3), no reliable biomarkers of sunitinib sensitivity or primary/secondary resistance have been identified.

Recent unsupervised gene expression analysis of 48 ccRCCs identified two molecular ccRCC subtypes (ccA and ccB; ref. 4). A meta-analysis of 480 ccRCCs confirmed this classification and identified a third subtype, cluster_3, associated with a wild-type von Hippel-Lindau (VHL) gene profile (5). The Cancer Genome Atlas (TCGA) project subsequently highlighted four ccRCC...
subtypes (6). In both studies, postnephrectomy survival was related to ccRCC subtype. A poor prognosis was also associated with somatic mutations in BAP1 and SETD2 (7, 8) and with chromosome amplifications or losses at 8q, 14q, or 9p (9–12). However, no associations have been made between molecular features and outcome in patients with m-ccRCC on TT (13).

We hypothesized that an integrated genomic analysis of primary ccRCCs might help to identify subgroups of ccRCCs more sensitive or resistant to anti-VEGFR TT. Thus, the aim of our study was to determine whether expression profiles, genomic abnormalities, mutational status, and epigenetic changes were related to outcomes after first-line sunitinib in patients with m-ccRCC.

Materials and Methods

Included patients

Primary ccRCC specimens were collected from 121 patients undergoing nephrectomy in 19 French and one Belgian hospitals from 1994 to 2011 (patient characteristics in Supplementary Table S1). For inclusion in the study, patients had to have developed synchronous or metachronous metastases, received sunitinib (50 mg/day, 4 weeks on/2 weeks off) as first-line treatment in the metastatic setting (prior cytokine therapy was allowed), and undergone their first CT scan assessment.

Drug schedule and dose-reduction policy complied with local practice guidelines. Follow-up chest/abdomen CT scans were performed every 2 cycles of treatment. Study endpoints were response rate (RR) according to RECIST 1.0, PFS, and OS. Four expert genitourinary pathologists blinded to patient outcome reviewed all nephrectomy pathology slides. The protocol was approved by the medical ethics review boards of all participating institutions. Signed consent was obtained from all patients in accordance with French and Belgian legislations. Frozen biologic material from deceased patients was used when prior agreement for such use had been given by the institutional review board.

Datasets and preprocessing

Database for transcriptome (E-MTAB-3267), SNP (E-MTAB-3269) and methylome (E-MTAB-3274) are available at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/).

Transcriptome data. Transcriptomic profiling was performed using HuGene 1.0ST Affymetrix array for 53 ccRCC samples and 6 adjacent normal tissue samples (NT). Biotinylated single-strand cDNA targets were prepared with 200 ng of total RNA, using the Ambion WT Expression Kit and the GeneChip WT Terminal Labeling Kit according to Affymetrix recommendations.

Methylome data. Whole-genome DNA methylation was analyzed in 102 ccRCCs and 5 adjacent NTs using the Illumina Infinium HumanMethylation450 assay that examines the DNA methylation status of 485,000 CpG sites (covering 99% of RefSeq genes and 96% of CpG islands). Genomic DNA was extracted using either the Manual-MagNa Pure LC DNA II Kit (Roche) or the Manual-Genta Puregene Kit (Qiagen) and bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research) by Integragen SA (http://www.integragen.com) and processed according to the Illumina protocol.

Genome data. For chromosome gain/loss profiling, 103 ccRCCs and 5 adjacent NTs were analyzed with Illumina OmniExpress chips, containing 731,442 probes. Hybridization was performed by Integragen, according to the manufacturer’s instructions. The absolute copy numbers and genotype status of segments were determined using the genome alteration print (GAP) method (14). Segments with an absolute copy number above (respectively below) the ploidy of the sample were considered as gains (respectively losses). The Genomic Identification of Significant Targets In Cancer (GISTIC) methodology (15) was used to identify significantly recurrent chromosome aberrations.

Quantitative RT-PCR. qRT-PCR reactions were performed on 98 ccRCCs and 5 NTs with low density array (LDA) for 65 genes and individual probes for 5 genes as previously described (16). Genes were selected from the differential analysis of the Affymetrix data (ANOVA q-value less than 0.05 and an absolute fold change greater than 1.5). Primers and probes for all genes were obtained from Applied Biosystems Taqman Gene Expression Assays. Supplementary Table S2 provides the gene symbol, gene name, and Applied Biosystems Assay ID number for the genes studied.

For details on overlap between samples used for each omics, see Supplementary Table S3.

Mutation analysis

Tumors were screened for VHL and PBRM1 mutations (Supplementary Table S4) using direct sequencing (primers and protocols available on request).

Omens analysis

Unsupervised classification. For unsupervised class discovery within the four omics, three methods were used: the recursively partitioned mixture model (RPMM; ref. 17) and two consensus clustering methods (18, 19). Only the results obtained with the third method were described in the article. Nevertheless, we showed a strong association between the three methods (Fisher exact P values from 3.9e-19 to 2.92e-21). We used the gap statistic to determine the number of clusters (20).
Differential analysis. We used moderate T tests to identify genes differentially expressed between groups of samples, using limma R-package. ANOVA models were used for multigroup comparison. To control for multiple testing, we measured the local FDR using the Benjamini and Hochberg method (R-package stats).

Association analysis. We evaluated the association between unsupervised or supervised subgroups and the bioclinical factors using χ² or Fisher exact test. For each clinical characterization, all covariates were analyzed but only the significant covariates were shown (P ≤ 0.05). The bioclinical factors included tumor–node–metastasis, Eastern Cooperative Oncology Group Performance Status (ECOG-PS), International Metastatic Renal Cell Carcinoma Database Consortium (IMDC), Fuhrman, or Memorial Sloan Kettering Cancer Center (MSKCC) scores, systemic treatment, Kettering Cancer Center (MSKCC) scores, systemic treatment, rhabdoid, and sarcomatoid phenotypes.

Survival analysis. Survival time was calculated from the first sunitinib treatment. Patients who were lost to follow-up or alive at the time of the study were treated as censored events. Survival curves were calculated according to the Kaplan–Meier method (function Surv, R-package survival, V2.29), and differences between curves were assessed using the log-rank test (function survdiff, R-package survival).

To find clinical criteria related to PFS or OS, univariate models were performed on all the pathologic and clinical covariates (function coxph, R-package survival). Covariates showing a significant association to prognosis (log-rank P < 0.05) at the univariate level were selected to be analyzed in multivariate models, after the exclusion of redundant covariates (ex MSKCC- and IMDC scores).

Classifier building. The training set was composed of 51 samples with Affymetrix and qRT-PCR, and the validation set was composed of 47 samples with only qRT-PCR.

A first centroid-based predictor was built to assign a sample to one of the three following groups ccrrc1&4/ccrrc2/ccrrc3 using a subset of the differentially expressed genes between these three groups. A gene was defined differentially expressed in a subtype when the ANOVA P value was < 0.05 and when the absolute fold change with the other subtypes was > 0.5. The optimal subset of 27 genes was obtained by a step-by-step strategy by optimizing the success rate. The DLD2-dissimilarity measure was then used to predict the subtype (Supplementary Table S2).

For samples predicted as ccrrc1&4 (using the predictor mentioned above), a second centroid-based predictor was built to assign each sample to one of the two subtypes ccrrc1 or ccrrc4. A subset of the differentially expressed genes between ccrrc1 and ccrrc4 subtypes was used. A gene was defined differentially expressed between the two subtypes when the ANOVA P value was < 0.05 and when the absolute fold change was > 3. The optimal subset of 8 genes was obtained by a step-by-step strategy by optimizing the success rate. The DLD2-dissimilarity measure was then used to predict the subtype.

The obtained centroid-based predictors were applied on two public datasets: The Brannon expression dataset (GEO-website, GSE33093; ref. 5) and TCGA dataset (6).

Use of immune metagenes
The datasets mentioned by Bindea and colleagues (21) were downloaded and normalized separately using the fRMA Biocductor package. Cancer cell lines dataset GSE5720 was also retrieved from Gene Expression Omnibus and normalized using fRMA. Samples GSM133550, GSM133594, GSM133638, GSM133635, which correspond respectively to ccRCC cell lines ACHN, SN12C, UO-31, and Caki-1, were added. All probesets included identified in the five metagenes used in our work (NK CD56dim, Cytotoxic cells, T cells, B cells, macrophages) were checked for expression by kidney cancer cell lines. One hundred thirty-eight of 142 probesets were completely specific and sensitive to predict immune cell–type against ccRCC cell lines samples, and 134 of 142 probesets had a right-tailed t test P value < 0.05 when compared with ccRCC cell lines samples. To compute average metagene values in our dataset, log, expression values for each probesets were first mapped to gene symbols using average expression of all the corresponding probesets, then this value was centered, and scaled across our dataset. Finally, the mean of all the scaled expressions was taken to represent the signature.

Immunohistochemistry
Formalin-fixed and paraffin-embedded tissue sections (5 μm) were stained with an anti-CD8 (5.0 μg/ml; clone SP16; Spring-bioscience) with an autostainer Link 48 (Dako) as previously described (22). Deparaffinization, rehydration, and epitope retrieval were performed in a PT-Link (Dako) in a high pH solution as described by the manufacturer. Tissue sections were first incubated with anti-CD8 (SP16; Springbioscience) and posteriorly with a biotin–streptavidin-coupled antibody. Revelation was done with 3-amino-9-ethylcarbazole substrate (Vector Laboratories).

Results
Patient characteristics and clinical predictors of sunitinib response
In our series of 121 patients, after a median follow-up of 55 months (range, 1.5–86), median PFS and OS were 13 and 27
months, respectively. Forty-seven percent of patients experienced a complete or partial response (CR/PR), 36% stable disease (SD), and 17% early PD following RECIST 1.0. By comparing patients with extreme phenotype (CR/PR vs. PD), we identified several clinical and biologic features associated with a poor sunitinib response: baseline neutrophilia, thrombocytosis, anemia, sarcomatoid differentiation, synchronous metastases at initial diagnosis, poorer ECOG-PS, Heng (23), and MSKCC scores (24, 25; Fig. 1A and B). However, in the overall series of patients, using all the clinical, pathologic, and molecular features and a variety of predictive algorithms (PAM, DLDA, DQDA), supervised analyses failed to identify robust factors predictive of sunitinib response (Supplementary Table S5). As this result could be due to the molecular subtypes and sunitinib response molecular diversity of the tumors, we investigated the relationships (Supplementary Table S5). To better understand how each molecular subtype could influence response to sunitinib, we characterized our cohort for somatic mutations in PBRM1 and VHL genes (n = 117 tumors), methylation profiling exploring 485,000 nucleotide sites (n = 102), and copy number targeting 731,442 genomic loci (n = 103). Transcriptome and methylome profiles of the four subtypes were compared using pathway analysis methods, and recurrent copy number aberrations were delimited from the SNP profiles.

Characterization of the m-ccRCC molecular subtypes
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IL10 as well as inhibitory receptors LAG3 and PD-1 (PDCD1) and PD-1 ligands PD-L1 and PD-L2 were also highly expressed (Supplementary Fig. S1). These results suggest that ccrcc4 tumors display a strong inflammatory, Th1-oriented but suppressive immune microenvironment. We then assessed the expression of immune population-specific transcripts using gene signatures previously published (Supplementary Fig. S2; ref. 21). ccrcc4 samples contained high amounts of B, T, and cytotoxic cells-specific transcripts, but not of natural killer cells-specific transcripts, suggesting that the cytotoxic signature in ccrcc4 is mostly due to CD8+ T-cell infiltration. We analyzed 46 samples by immunohistochemistry and confirmed a high infiltration of CD8+ cells in ccrcc4 tumors (Supplementary Fig. S3). Sample contamination with diploid cells was estimated to be slightly higher in ccrcc4 tumors, which could reflect immune infiltration (Supplementary Fig. S5C).

Overall, 45% of the tumors were VHL-mutated and 32% PBRM1-mutated. Whereas rare mutations in VHL and PBRM1 were found in ccrcc4 tumors, they were frequent in ccrcc1 and ccrcc2 tumors but without relationship with sunitinib response (Fig. 4A and Table 2).

At a global methylation level, ccrcc1/ccrcc4 tumors showed more hypermethylated probes in CpG islands compared with the other subtypes (Supplementary Fig. S4A). Pathway analysis of the related hypermethylated genes showed a strong enrichment of polycomb targets (hypergeometric test, \( P < 8 \times 10^{-14} \)), and corresponding genes (PRC2, SUZ12, and H3K27m3) were found downexpressed (Supplementary Fig. S4B), suggesting a stem-cell phenotype for these two subtypes. The ccrcc1/ccrcc4 subtypes were indeed less differentiated, displaying 76% of Fuhrman grade 4 compared with 56% in ccrcc2/ccrcc3 tumors.

The ccrcc4 subtype showed several characteristic copy-number aberrations, the most significant being 2p12, 2p22.3, and 8q21.13 amplifications (Fig. 4D; Supplementary Fig. S5; Supplementary Table S9), and both ccrcc1 and ccrcc4 subtypes overexpressed MYC targets (Fig. 4B). Amplification in the upstream region of MYC was found in >40% of ccrcc1 and ccrcc4 tumors compared with <22% in the other subtypes. Also, a CpG island was hypomethylated in the body gene of MYC in ccrcc1/ccrcc2/ccrcc4 tumors as compared with ccrcc3/NTs (ANOVA \( P < 0.0001 \)), suggesting that both DNA copy number and methylation aberrations could contribute to MYC overexpression in the ccrcc1 and ccrcc4 subtypes (Supplementary Fig. S6).

Finally, the expression profile of ccrcc3 samples was similar to that of NTs concerning metabolic pathways and transporter activities, consistent with the cluster_3 described by Brannon and colleagues (ref. 5; Fig. 4B). ccrcc3 tumors also showed a methylation profile similar to that of NTs (Supplementary Fig. S4C). Despite the "normal-like" characteristics of ccrcc3 tumors, pathologic review confirmed the tumoral nature of these samples and
their clear cell histology. The ccrcc2 subtype was not characterized by specific pathways; it always showed an intermediate expression signature, comprised between ccrcc3 and ccrcc1/ccrcc4-related profiles (Fig. 4B). ccrcc2 tumors showed the highest mutation rate for VHL. In ccrcc2 tumors, the "cellular response to hypoxia" pathway was less activated than in the ccrcc1/ccrcc4 subtypes (Fig. 4B).

Based on these molecular characteristics, we renamed our subtypes as follows: ccrcc1 "c-myc-up," ccrcc2 "classical," ccrcc3 "normal-like," and ccrcc4 "c-myc-up and immune-up" (Table 2).

Validation using the TCGA dataset

We further predicted our four subtypes in the public TCGA samples (Supplementary Fig. S7; ref. 6) with our 35-gene classifier (Supplementary Table S6). Like in our series, ccrcc3 tumors showed "normal-like" transcriptome and methylome profiles. Somatic PBRM1 mutations were most frequently identified in ccrcc1/ccrcc2 tumors but rarely found in ccrcc3/ccrcc4 tumors. In both series, somatic VHL mutations were more frequently distributed in ccrcc1/ccrcc2 tumors. The BAP1 and SETD2 mutations (data not available in our series) also showed association with the molecular subtypes: BAP1 was most mutated in the ccrcc4 tumors (P value = 0.0098) and SETD2 was most mutated in the ccrcc1 tumors (P value = 0.06). At a methylation level, in the TCGA samples, ccrcc1/ccrcc4 tumors also showed more hypermethylated probes in CpG islands compared with the other subtypes. Pathways involved in immune response and mitotic cell cycle were activated in ccrcc4 tumors, meanwhile in ccrcc3 tumors, pathways involved in hypoxia were not activated. Finally, in the TCGA cohort, 2p11.2, 8q12.1, and 8q24.3 amplifications were mostly found in ccrcc1/ccrcc4 tumors. Supplementary Fig. S8 shows the survival analysis according to our classification: ccrcc2/ccrcc3 tumors display the best survival, ccrcc1 tumors an...
intermediate survival, and ccrcc4 tumors the poorest survival ($P < 0.0001$).

### Discussion

Our multi-omics analysis revealed that molecular tumor subtypes are germane to predict response, PFS, and OS, in patients with m-ccRCC treated with sunitinib. We identified 4 robust molecular subgroups of ccRCCs based on mRNA expression data. ccrcc3 ("normal-like") and ccrcc2 tumors ("classical") showed better sunitinib responses than ccrcc1 ("c-myc-up") and ccrcc4 tumors ("c-myc-up and immune-up"). The ccrcc4 subtype was associated with the poorest sunitinib response. Subtype classification was the only significant covariate in multivariate analyses for PFS and OS. Survival was significantly longer for ccrcc1/ccrcc4 tumors compared with ccrcc1/ccrcc4.

Comparison with previous ccRCC molecular classifications showed a high correlation of our four ccRCC groups with the three groups cCA, cCB, and cluster_3 described by Brannon and colleagues (4, 5; Supplementary Table S10). In particular, ccrcc3 tumors shared several characteristics of cluster_3 (5); it included all NTs and showed a transcriptomic signature closest to normal samples (Fig. 2B and C). Although the series of Brannon and colleagues and the TCGA included both patients that were cured with nephrectomy alone as well as patients that eventually reached the metastatic setting, whereas in our series, only patients with metastatic disease were included, our subgroups are tightly related to the previously identified classification with an over-representation of the poor-prognosis cCB group, and its refinement in two subgroups, ccrcc1 and ccrcc4. Subtypes were identified using a 35-gene signature that could be useful in future tumor screening.

As a consequence, the following factors were included in the multivariate analysis, both for PFS and for OS: IMDC (Heng) score, the presence of bone metastases, sarcomatoid dedifferentiation, and showed a transcriptomic signature closest to normal samples (Fig. 2B and C). Although the series of Brannon and colleagues and the TCGA included both patients that were cured with nephrectomy alone as well as patients that eventually reached the metastatic setting, whereas in our series, only patients with metastatic disease were included, our subgroups are tightly related to the previously identified classification with an over-representation of the poor-prognosis cCB group, and its refinement in two subgroups, ccrcc1 and ccrcc4. Subtypes were identified using a 35-gene signature that could be useful in future tumor screening.

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others have found no association between the presence or absence of VHL alterations and prognosis or adverse clinical and pathologic features.

The ccrcc1/ccrcc4 subtypes, which were more closely linked with nonresponders to sunitinib, shared common molecular characteristics such as upregulation of MYC targets or a hypermethylated status strongly correlated with a polycomb stem-cell phenotype. However, ccrcc4 tumors showed specific pathologic features such as a more inflammatory and sarcomatoid phenotype, an upregulation of cellular immune pathways, and an omnipresent 8q21.13 amplification. These findings are consistent with several publications showing the negative impact on outcome of an elevated baseline C-reactive protein level, a marker of inflammation, and of the presence of sarcomatoid differentiation in m-ccRCC treated with anti-VEGFR TT (25–31).

Integrative analyses of the different omic experiments suggested a possible gradient of tumor progression in the following order: normal samples/ccrcc3/ccrcc2/ccrcc1/ccrcc4. We observed a metabolism switch with increasing deregulation along the ordered subtypes; similar results are obtained for the polycomb stem-cell phenotypes, the hypermethylated profile or the MYC target activation (Supplementary Fig. S9). These features fit the transcriptional and epigenetic sequential changes responsible for cellular reprogramming leading to acquired pluripotency by fibroblasts (32): apoptosis blockade, cell cycle activation, metabolic switch, polycomb stem-cell phenotype with the involvement of MYC in this cell reprogramming. Inversely, activation of the immune pathway in ccrcc4 tumors seemed more linked to a switch on/off than to a gradient. Similarly, in ccrcc4, few VHL/
PBRM1 mutations were detected, indicating that probably other gene alterations might be involved in the pathogenesis of ccrcc4 tumors.

Hypoxia pathways are not activated in ccrcc3 tumors and less activated in ccrcc2 tumors than in resistant ccrcc1/4cc4 tumors (Fig. 4B). Hypoxia is associated with tumor aggressiveness through higher HIF levels and expression of genes involved in tumor proliferation, vasculature, invasion, and metastatic spread leading to a poor prognosis. Through the reduction and normalization of blood vessels, anti-VEGF TT leads to better oxygen delivery in the tumor. Thus, lowering hypoxia might be an important part of the mechanism of action of anti-VEGF TT. However, tumor hypoxia was recently assessed before the start and after one month of sunitinib by a PET-CT scan with 18F-fluoromisonidazole, which accumulates in hypoxic cells. Patients with initially hypoxic targets had shorter PFS than patients with nonhypoxic targets (36). Thus, the balance of neoangiogenesis versus hypoxia could be a major trigger of response to anti-VEGF TT (37).

In a first step, as we worked on a limited number of patients, our findings should be validated in an independent patient cohort. Once validated, this molecular subtyping of tumors could probably help treatment personalization. Because patients with ccrcc4 tumors have a short PFS under sunitinib treatment, hypomethylating agents targeting epigenetic defects (38) or immunomodulatory antibodies (39) should be preferentially tested in this subgroup of patients (40). Moreover, as even in the poor-prognosis ccrcc4 subgroup, 20% of the patients experienced a PR, and as a placebo-treated subgroup of patients is not available for comparison, our classification does not permit us to preclude any subgroup of patients from treatment with sunitinib. Further validation of these findings is warranted in future clinical trials integrating molecular subtyping in their design.

Disclosure of Potential Conflicts of Interest

B. Beuselinck reports receiving a commercial research grant from Pfizer. E. Becht is a consultant/advisory board member for SOTIO. S.M. Oudard is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Other (performed central histologic review): V. Verkarre

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