Novel *XLF/Cernunnos* mutation linked to severe combined immunodeficiency, microcephaly and abnormal T and B cell receptor repertoires

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Short title: XLF/Cernunnos mutation - associated immunodeficiency

Abstract

Background: During the process of generating diverse T and B cell receptor (TCR and BCR, respectively) repertoires, double strand DNA breaks are produced. Subsequently, these breaks are corrected by a complexed system led mainly by the non-homologous end-joining (NHEJ). Mutations in proteins involved in this process, including the *XLF/ Cernunnos* gene, cause severe combined immunodeficiency syndrome (SCID) along with neurodevelopmental disease and susseptability to inoizing radiation.

Objective: To provide new clinical and immunological insights on XLF/Cernunnos deficiency, arising from a newly diagnosed patient with severe immunodeficiency.

Methods: A male infant, born to consanguineous parents, suspected of having primary immunodeficiency underwent immunological and genetic work up. This included a thorough assessment of T cell phenotyping and lymphocyte activation by mitogen stimulation tests, whole exome sequencing (WES), TCR repertoire Vβ repertoire via flow cytometry analysis and TCR and BCR via next generation sequencing (NGS).

Results: Clinical findings included microcephaly, recurrent bacterial viral pneumonia and failure to thrive. Immune workup revealed lymphopenia, reduced T cell function and hypogammaglubolinemia. A skewed TCR Vβ repertoire, TCR gamma (TRG) repertoire and BCR repertoire were determined in the patient. Genetic analysis identified a novel autosomal recessive homozygous missense mutation in XLF/Cernunnos c. A580Ins.T; p. M194fs. The patient underwent a successful hematopoietic stem cell transplantation (HSCT).

Conclusions:

A novel XLF/Cernunnos mutation is reported in a patient presented with SCID phenotype that displayed clonally expanded T and B cells. An adjusted HSCT was safe to ensure full T cell immune reconstitution.

Keywords: NHJE1, Severe combined immunodeficiency (SCID), NGS, TCR repertoire, XLF/Cernunnos

Abbreviations:

NHEJ- Non-homologous End-Joining

NGS- Next-generation sequencing

PBMCs - peripheral blood mononuclear cells

SCID - severe combined immunodeficiency

TRECs - T cell receptor excision circles

TCR - T cell receptor

IGH- Immunoglobulin heavy chain

HSCT - hematopoietic stem cell transplantation

Introduction

The unique ability of the adaptive immune system to produce a polyclonal diverse repertoire of antigen receptors is critical for its function. In most species, this is obtained through the natural course of T and B cell maturation process that includes recombination of different V(D)J gene segments (1). This complex process starts with double strand DNA breaks (DSBs) and ends when new coding joints are generated. The process subsequently results in development of new and specific antigen receptors, which are obligatory for host survival. Many proteins take part in this cascade, among them are the recombination-activating gene (RAG)-1 and -2 proteins which act during the initial stage when DSBs are formed (2). The repair of the damaged DBSs is performed using two cellular mechanisms: the Non-Homologous End-Joining (NHEJ) and the Homologous Recombination Repair (HRR). NHEJ, the major pathway includes DNA-end binding complex Ku70-Ku80, DNA-PKcs, Artemis, DNA Ligase IV and XLF (XRCC4 like factor), also called Cernunnos proteins (3-5). During the NHEJ pathway, XLF/Cernunnos interacts with XRCC4-Ligase IV, stimulates its ligase activity, thereby repairing the broken DNA (6-7). It is well known that genetic defects in proteins involved in the V(D)J recombination process can cause severe type of combined immunodeficiency (SCID). Such SCID is usually characterized by T cell-negative (T-), B cell-negative (B-), and natural killer cell-positive (NK+) immune phenotype (8-11). Regardless of the genetic defects, SCID patients require a curative treatment with hematopoietic stem cell transplantation (HSCT) from an optimal matched donor, in order to achieve restoration of their immune system. The amount of donor's cells that is needed for complete immune recovery after the HSCT procedure is not always clear. Only few cases of SCID due to NHEJ pathway mutations, and specifically mutations causing defects in XLF/Cernunnos protein have been described so far (12-17). These patients were characterized by recurrent infections (mainly bacterial and opportunistic), growth retardation, microcephaly and few autoimmune manifestations. The ability of such patients to produce T and B cell receptor (TCR, BCR) repertoire has been rarely investigated (18,19).

Herein we describe a patient harboring a novel *XLF/Cernunnos* mutation who underwent successful HSCT, yet with engraftment of low but selective amounts of donor's cells. We further extend the knowledge about the immunodeficiency

associated with mutation in *XLF/Cernunnos* by specifically deciphering the patient's TCR and B cell receptor (BCR) repertoire using next generation sequencing (NGS).

Methods

Clinical data

Patient information was obtained from the electronic medical record of our hospital. The guardians were interviewed and the patients were examined by the authors. Informed written consent was obtained, and all procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration.

Immune function.

Cell surface markers of peripheral blood mononuclear cells (PBMCs) were determined by immunofluorescent staining using flow cytometry (FACS, NAVIOS, Beckman Coulter) with antibodies purchased from Beckman Coulter. Lymphocyte proliferation was done in response to Phytohemagglutinin and anti-CD3 (using tritiated thymidine incorporation). The cells were harvested three days after collection, and samples were counted in a liquid scintillation counter. All assays were performed in triplicate, and a stimulation index was calculated as the ratio between stimulated and unstimulated lymphocyte responses. The resultant stimulation index was compared with the stimulation index obtained from normal controls. Serum concentration of immunoglobulins was measured by nephelometry.

Quantification of TRECs

TREC analysis was performed using DNA extracted from the study patients' PBMCs. The amount of signal joint TREC copies per DNA content was determined by real-time quantitative PCR. Reactions were performed using 0.5-mcg genomic DNA and PCRs contained TaqMan universal PCR master mix (Applied Biosystems), specific primers (900 nM) and FAM-TAMRA probes (250 nM). RQ-PCR was carried out in step one plus (Applied Biosystems).The number of TRECs in a given sample was estimated by comparing the cycle threshold value obtained with a standard curve obtained from PCRs performed with 10-fold serial dilutions of an internal standard. Amplification of RNAseP (Taq-Man assay, Applied Biosystems) served as a quality control to verify similar amounts of genomic DNA that were used in the assays.

TCR repertoire

Representatives of specific TCR-Variable b families were detected and quantified using patients' PBMCs with flow cytometry (NAVIOS, Beckman Coulter) according to the manufacturer's instructions (Beta Mark TCR Vβ repertoire kit, Beckman Coulter). Normal control values comprised of 58 healthy people were obtained from the kit.

Next-generation sequencing (NGS)

TCR and IGH libraries were generated from patient and control genomic DNA using primers for conserved regions of V and J genes in the *TRG* (TCR-gamma) locus and IGH, respectively, according to the manufacturer's protocol (Lymphotrack; Invivoscibe Technologies®, Carlsbad, CA, USA). Quantified libraries were pooled and sequenced using Mi-Seq Illumia technology®. FASTA files from the filtered sequences were submitted to ImMunoGeneTics (IMGT) HighV-QUEST webserver® (http://www.imgt.org), filtered for productive sequences only (no stop codons or frame shifts) and analyzed (20). Analyses were performed on CDR3 amino acid sequences. For TCR repertoires, V and J gene usage patterns were analyzed. Repertoire diversity was calculated using Shannon's and Gini-Simpson's diversity indices (21).

Exome sequencing analysis and SANGER sequencing

High throughput sequencing for whole exome sequencing was performed on genomic DNA patient samples, coding regions were enriched with a SureSelect Human All Exon V5 Kit (Agilent) and then sequenced as 100-bp paired-end runs on an Illumina HiSeq 2500 (Illumina Inc.). We used the BWA mem algorithm (version 0.7.12) (22) for the alignment of the sequence reads to the human reference genome (hg19).
The HaplotypeCaller algorithm of GATK version 3.4 was applied for variant calling, as recommended in the best practice pipeline (23). KGG-seq v.08 was used for annotation of identified variants (24) and in-house scripts were applied for filtering, based on family pedigree and local dataset of variants detected in previous sequencing projects. The XLF/Cernunnos mutation was validated by dideoxy Sanger sequencing in the patients and carriers. Data were evaluated using Sequencer v5.0 software (Gene Codes Corporation).

Results

Clinical description

The patient was born prematurely, at 34+5 weeks of gestation. His parents were healthy and were first-degree cosines. He had a clinically healthy 3-year-old brother. Since birth, the patient suffered recurrent skin rash and from the age of 4 months, he had started to have frequent episodes of pneumonia. Preliminary evaluation demonstrated lymphopenia and hypogammaglobinemia and therefore he was referred to our hospital at the age of 9 months for further evaluation of a possible immunodeficiency. On preliminary examination he was extremely cachexic, tachypnea and dyspneic, and noted to have dysmorphic features with microcephaly (head circumference below 3rd percentile), triangle shaped face and developmental delay. There were signs of chronic respiratory involvement and clubbing of his fingernails. A diffused maculopapular rash was seen with suspected fungal origin. Initial infectious inquiry revealed evidence of clinical bilateral pneumonia with sustained *Respiratory syncytial virus* infection, and later on diagnosed with bacterial pneumonia due to *Klebsiela Pneumonia* infection. Further lymphoproliferative, metabolic and autoimmune investigations were all normal.

Immunological evaluation

Initial immunologic investigation revealed normal leukocyte count with lymphopenia and low IgG levels and with normal IgA and IgM levels (Table 1). Lymphocytes immuno-phenotyping showed abnormal representation for T, B and NK cells, with significant deficiency of $CD3^{+}$and $CD20^{+}$ cells, and increased percentage of $CD56^{+}$ cells, consistent with a T^{-} B^{-} NK^{+} SCID phenotype. Lymphocyte proliferation was markedly reduced following stimulation with Phytohemagglutinin (PHA) or anti CD3 (Table 2). Thymus activity, determined by quantification of T cell receptor excision circles (TRECs) was absent. (Table 1). The constellation of his physical findings and immune workup were consistent with a diagnosis of SCID. Nevertheless, the patient had still few residual T cell that we attempted to characterize. Therefore, analysis of the TCR repertoire was performed using both flow-cytometry analysis and NGS.

The TCR repertoire was first assessed with the TCR-Vβ assay using flow cytometry. This resulted in a skewed repertoire with clonally expanded two TCRs (vβ 5.1 and vβ

13.1) and underrepresentation of 10 different TCRs (Figure 1). In order to further characterize this abnormal TCR repertoire, high-throughput immune sequencing of the TRG (T cell receptor γ chain) repertoire was performed on PBMCs from the patient and a healthy age-matched control. The patient expressed a restricted repertoire with clonal expansion cells as can be observed by the Tree-maps, which graphically represent the TRG repertoire (Figure 2A). In addition, similar restriction and clonal expansion was observed in the Treemaps of the patient in the IGH, representing the BCR repertoire (Figure 2B). This is further observed in the unique and total number of sequence, which were about 10 fold less in the patient compared to the controls for both TRG and IGH repertoires (Figure 2, C and D). Lastly, the Shannon's H diversity index, which account for both the total number of sequences and the overall level of clonal expansions, was lower for the patient compared to the controls (Figure 2E). Calculation of the Simpson's D index, which measures the unevenness of the repertoire, showed unevenness in the patient's TRG and IGH repertoires, compared to controls (Figure 2F). Taken together, these abnormal TCR and IGH repertoires indicate a possible defect in the V(D)J gene recombination process and/or abnormal T and B cell selections within the thymus or bone marrow, respectively.

Exome sequencing analysis and Sanger sequencing

Due to familial consanguinity, a homozygous recessive disease trait was suspected. Genetic analysis by whole exome sequencing identified a novel bi-allelic homozygous frame shift mutation in the *NHEJ1* gene (*XLF/Cernunnos*) (c.A580Ins.T; p. M194fs). This was confirmed by direct Sanger sequencing. Segregation of the identified mutation with the disease phenotype, was confirmed within patient's family (Figure 3) with both parents being carriers for the mutation and a healthy brother who harbor a wild-type *XLF/Cernunnos* gene. The WES did not reveal any other mutation in a gene that is known to cause immunodeficiency or immune-dysregulation. The *XLF/Cernunnos* novel variant was not found in our in-house exomes (n=1500), and was not present with a minor allele frequency (MAF) ≥ 0.01 in either the 1000 Genomes Project (1KG; http://browser.1000genomes.org/ index.html) or dbSNP 135 database or the NHLBI Exome Sequencing Project(ESP;http://evs.gs.washington.edu/ EVS/). This *XLF/Cernunnos* mutation is rare and different from previously described

patients (16). As mentioned above, our patient shared several clinical and laboratory characteristics described in previous human cases (12-15) and animal model (25-27), thereby supporting the hypothesis that this *XLF/Cernunnos* variant is a disease-causing mutation.

Clinical outcome

At age of 14 months, the patient underwent bone marrow transplantation from a matched related donor. Bone marrow graft contained total nucleated cell count of 3 $^{x}10^{8}$ per kg of recipient, $CD34^{+}$ 10 $^{x}10^{6}$ per kg of recipient and $CD3^{+}$ 0.3 $^{x}10^{8}$ per kg of recipient. Due to his underlying genetic defect, which deserves a special conditioning protocol (28), HSCT was performed using reduced intensity conditioning, with Cyclophosphamide and Fludarabine. Immunosuppressant treatment for graft versus host (GVHD) prevention was given (Cyclosporine and Mycophenolate mofetil). During the post transplantation period, patient presented several clinical issues, including mild skin graft versus host disease, chronic lung disease which required maintenance therapy of inhaled bronchodilators and inhaled corticosteroids, feeding difficulties and failure to thrive which required oral feeding tube. Gastrointestinal GVHD was ruled out via gastric and rectal endoscopy and biopsy. During the course of his hospitalization there was a clinically and laboratory improvements. He had begun gaining weight, there were no severe infections and lymphocytes counts improved with $CD4^{+}$ and $CD8^{+}$ cell counts elevation but with no signs of $CD20^{+}$ cell engraftment (Table 1). Functional assay for evaluating T cell immune reconstitution using repeated measurements of TREC level, as well as the presentation of his TCR which was normal, demonstrated significant improvement when comparing to pre transplantation status (Figure 1).

Despite his fairly good clinical condition and signs of immune restoration, his microsatellites test in peripheral blood lymphocytes post transplantation revealed low level and mixed chimerism status, ranging from 30-50% of donor cells. Yet, further investigation of these cells by preforming sorted T cells microsatellites evaluation, resulted in a 100% donor cells, suggesting full recovery of his T cell compartment. This was consistent with his good clinical condition and blood work.

Immunosuppression therapy was gradually decreased and the patient was discharged on the 130-day post transplantation with preventive antibiotics, anti-viral and anti-

mycobacterial treatment. During his post transplantation follow up period, he was clinically well and after signs of B cell engraftment, he was weaned of regular prophylactic treatment and immunoglobulins replacement therapy. The patient is currently two years after the transplantation, doing well and free of infections.

Discussion

Here we report a patient with a clinical and immunological phenotype of SCID. The patient harbored a novel autosomal recessive mutation in *XLF/Cernunnos*, a gene that is part of the NHEJ complex. We characterized his TCR and BCR repertoires using flow cytometry assay and NGS in order to demonstrate the effect of XLF/Cernunnos mutation on the ability to produce normal diverse TCR and BCR repertories. The patient underwent a successful HSCT, which was in correlation to full engraftment of T cells that detected only by a specific split chimerism test.

Human mutations in NHEJ complex cause T and B lymphocytes maturation arrest, sensitivity to IR, and result in clinical and laboratory T^- B^- NK^+ SCID phenotype (11, 12, 29-32). Mutations in DCLRE1C (Artemis) (11) and XRCC4/Ligase IV (32, 33) may also include microcephaly and developmental delay and where described in association with leukemia and lymphoma (34, 35). In 2006 the role of a newly identified protein, XLF/Cernunnos, in this complex mechanism, was elucidated (12). Since then, several other studies deciphered XLF function (36, 37) and it is now known that this protein is actually a key player during DNA repair. Its interaction with XRCC4-Ligase IV, (and by that stimulating its ligase activity), is a crucial element in the process. Another important role was identified later on, in contributing to junctional diversity during V(D)J recombination, by stimulating N-nucleotide insertion (16). The latter may also be responsible for an abnormal TCR diversity.

Mutations in *XLF/Cernunnos* have been previously described, first in 2006 (12) with the description of five patients presented with clinical and laboratory presentation of severe combined immunodeficiency. These patients shared clinical features of microcephaly growth retardation. They also demonstrated immune phenotype of T^- B^- NK^+ SCID and *in vitro* sensitivity to ionized radiation. Interestingly, as opposed to other forms of SCID, few of these patients were diagnosed at a fairly older age, some even as teenagers, most probably due to leaky mutation or partial function of residual cells. Since then more than a dozen patient with similar mutations in *XLF/Cernunnos*

were described. (13-17) These patients also shared similar clinical manifestation of susceptibility to opportunistic infections, microcephaly (except for one patient (13)) and growth retardation. An association with autoimmunity was also described (12-15, 17). In these cases, patients presented fairly later in life, and survived several years despite suffering from infections otherwise lethal in other forms of SCID. Except for one case (17), all patients, as seen before, demonstrated immunological profile suitable of $T^-B^-NK^+$ SCID. The former had normal B cell count and normal IgM and IgA levels, suggesting perhaps a leaky form of this deficiency. Further immune work up in several patients (16) revealed skewed TCR repertoire and reduced N-nucleotides insertion during V(D)J recombination. Of what is known so far regarding the patients aforementioned, 3 patients died from sepsis and 17 survived, among them, 11 received stem cell transplantation (16).

It seems that the effect of mutations in *XLF/Cernunnos* result mainly in extreme sensitivity to IR, genome instability, failure to thrive and microcephaly. The effect on immunity is perhaps milder (12, 13) than other forms of SCID. This discrepancy was demonstrated in vitro in a mice model (38) were mutation in *XLF/Cernunnos* caused only a mild defect in lymphocyte maturation, although cells were severely impaired in their ability to support V(D)J recombination. In this study it was suggested the existence of compensatory mechanisms for the dysfunction of XLF during V (D) J recombination. This might explain why many patients, sharing mutations in *XLF/Cernunnos*, present later on in life and survive the risk of severe infections as mentioned earlier. Yet, a curative treatment for patients with *XLF/Cernunnos* may be HSCT. In 2018, a study analyzing the outcome of HSCT in PID patients with DSB repair disorders, was published (39). Of 87 patients in the study, 17 were genetically diagnosed with *XLF/Cernunnos* mutation. Sixty-nine percent of patients who received conditioning survived, with better survival rates in those receiving reduced intensity chemotherapy protocol. The latter may be explained by the DNA instability and radio sensitivity, known in these patients, and previously discussed. A review on HSCT in PID patients that was published in 2019 (40), conclude that the decision making on HSCT should be done after taking in consideration the specific diagnosis and indication, timing of the procedure and perhaps most importantly, the risks of HSCT against the risks of disease progression.

Here in we describe a new diagnosed patient carrying a novel homozygous mutation in *XLF/Cernunnos*. This patient presented since birth with microcephaly and recurrent infections. The infant shared clinical and immunological features described in the previously reported cases, suggestive of lymphocyte maturation arrest and developmental delay. As in previous cases, patient had increased susceptibility to viral and bacterial infections, microcephaly and failure to thrive. Immunologic profile demonstrated impaired cellular and humoral immunity feature common to previously described patients. As previously shown, profiling the TCR and BCR repertoires, using NGS, revealed a skewed repertoire (18,19). While these TCR and BCR development abnormalities could be attributed to secondary environmental triggers (e.g. infections) that drives clonally expended cells, it is clear that they reflect the underlying genetic defect which unable the production of diverse repertoire. As many patients described in previous cases (13-17), our patient underwent successful bone marrow transplantation from a matched related donor (sibling). Close clinical and laboratory follow up demonstrated cellular immune reconstitution with normal TCR and normal lymphocyte proliferation (Table 1 and 2) While post HSCT is important to achieve high amount of donor cells, simple detection in the peripheral blood is not always sufficient and a better assessment of the engraftment quality mandates cell split tests.

As mentioned above, the *NHJE* defects in general and in particular, *XLF/ Cernunnos* have been associated with neurodevelopmental problems and IR susceptibility (16). As in previous cases, our patient presented with microcephaly andneurodevelopmental delay. Obviously, these factors will not be corrected by HSCT, which only replace factors of immunity, but do not change the course of the developmental, neurological disease, and do not reduce the IR sensitivity of these patients. Continues follow up and monitoring is required.

In summary, we presented a patient with a novel *XLF/ Cernunnos* mutation that displayed typical features. HSCT cured his immune symptoms including the abnormal TCR repertoire. This was in correlation to a full T cells engraftment, which was detected using a specific split chimerism assay. The latter is an important and informative test that should be used in special cases in order to verify the success of the HSCT procedure.

Declarations

The authors have no conflict of interests.

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Table 1: Immunological workup pre and post HSCT of *XLF/Cernunnos* mutated patient

Status at presentation	Pre HCST	Post HCST	Normal range *
CBC (Cells/ml x $10^{-3)}$			
WBC	3.9	4.9	5.2–11
Monocytes	0.4	0.65	0.2–1
Lymphocytes	1.27	1.8	1.5–6 5
Hemoglobin (g/dl)	11.8	12.4	11–14
Platelets	308	180	150–400
Eosinophils	0.02	0.07	0-0.7
Lymphocyte subsets (Cells/mm3 (%))			
Lymphocytes	0.643 (22.4)	1.617	2.3–5.4
T ($CD3^+$)	0.135 (21)	1.035	1.4–3.7 (60-85)
T helper ($CD4^+$)	0.148 (23)	0.437	0.7–2.2 (36-63)
T cytotoxic ($CD8^+$)	0.148 (23)	0.647	0.49–1.3 (15-40)
B ($CD\ 20^+$)	0.019	0.065	0.05-0.3
NK (CD16+CD56+) (%)	70	30	6-30
Serum immunoglobulin (mg/dl)			
IgA	41	< 26	38–222
IgM	119	27.7	56–208
IgG	376	462	590–1430
IgE	< 4.8	Not available	0 -90
TRECs (Copies per 0.5 µg DNA)	0	5280	> 400
TCR	Restricted	Polyclonal	

* Healthy donors, aged 1–2 years, with percentages/counts presented as median (10th and 90th percentiles) (41). HSCT – hematopoietic stem cell transplantation; TRECs - T-cell receptor excision circles; TCR- T cell receptor repertoire.

Table 2. Lymphocyte proliferation assay

	Patient	Control
No stimulation (CPM)	198	607
No stimulation (SI)	1	1
PHA6 (SI)	24	130
PHA25 (SI)	22	168
CD3 $^{+}$Ab (SI)	4	56.5

CPM- counts per minute

SI -All values of stimulation with mitogens are presented as stimulation index- number of counts per minutes divided by number of counts per minutes without stimulation

Legends

Table 1. **Immune workup**

Pre and post HSCT immune workup in the reported patient with *XLF/Cernunnos* mutation

Table 2. **Lymphocyte proliferation assay**

Thymidine incorporation assay documented reduced T cell proliferation upon stimulation with anti-CD3, as compared to healthy donors (HD), in patient's cells compared to healthy control.

Figure 1. **T cell receptor Vβ repertoire analyses.**

Flow cytometry analysis of surface membrane expression of 24 T cell receptor β chain's variable gene families, in our *XLF/Cernunnos* deficient patients, (black bars), compared with healthy controls (white bars), before (upper panel) and after (lower panel) the HSCT.

Figure 2. Characteristics of the TRG and IGH repertoire determined by NGS

Graphical presentation of the TRG repertoire using Tree map program where each square represents a specific clone and the size of the square represent the frequency of the clone, for TRG (A) and IGH (B) repertoires. Scatter dot plot presenting the unique (C) and total (D) number of sequences for TRG and IGH repertoires. Scatter dot plot presenting Shannon's H diversity index (E) and Simpson's Index of Unevenness (F) for TRG and IGH repertoires. TRG and IGH repertoire analysis from a total of four controls were compared to our patient's data.

Figure 3. **Validation of a *Non-homologous End-Joining 1(*XLF/Cernunnos*)* mutation**

Sanger sequencing confirmed the presence of frame shift mutation in peripheral blood mononuclear cells of the patient (PBMCs). There was a heterozygous state for both parents. (c.A580Ins.T; p. M194fs). The blue arrow indicates insertion of extra T nucleotide in the patient two alleles and in one allele of a parent.

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