Genetic variation in TGFB1 gene and risk of idiopathic recurrent pregnancy loss


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Submitted on September 10, 2012; resubmitted on January 13, 2013; accepted on February 5, 2013

ABSTRACT: Transforming growth factor β1 plays a significant role in pregnancy outcome. We investigated the association of TGFB1 exon 1 (rs1800471, rs1800470) and promoter region (rs1800469, rs1800468) polymorphisms with recurrent pregnancy loss (RPL) in 675 Tunisian women: 304 women with a history of three consecutive pregnancy losses of unknown etiology with the same partner and 371 age-matched multiparous control women. TGFB1 genotyping was done by TaqMan assays. Higher minor allele frequency for rs1800471 (P < 0.001), but not for rs1800470, rs1800469 or rs1800468 was found in RPL cases compared with controls. A significant difference in the distribution of rs1800471 genotypes was seen between the RPL cases and control women, irrespective of the genetic model used. Increased RPL risk was seen with rs1800471 allele C in the heterozygous state and to a greater degree in the homozygous state, thus establishing a dose-dependent effect. Haploview analysis revealed differential linkage disequilibrium between the TGFB1 single-nucleotide polymorphisms analyzed. TGFB1 haplotype analysis identified eight common haplotypes (rs1800471/rs1800470/rs1800469/rs1800468) with three (GT TG, Pc = 0.02; CCTG, Pc = 0.02 and CT TG, Pc = 0.02) positively associated with RPL and one (GCCG, Pc = 0.009) negatively associated with RPL. This study provides the first evidence that the TGFB1 genotype may influence RPL.

Key words: haplotype / polymorphisms / spontaneous miscarriage / TGFB1

Introduction

Recurrent pregnancy loss (RPL) affects 1% of couples and almost half of cases are without an identified etiology. Several genes are candidates for this increased susceptibility and have been tested in polymorphism association studies with conflicting results (Daher et al., 2012). Most of these genes produce cytokines, hormones and angiogenic factors. We decided to focus on transforming growth factor β1 (TGFB1) because of its important role in reproduction (Ingman and Robertson, 2009).

Three TGFB-β isofoms encoded by three distinct genes (TGFB1, TGFB2 and TGFB3) have been identified. TGFB1, TGFB2 and TGFB3 are reportedly expressed in the gonads, and implicated in secondary sex organ development, spermatogenesis and ovarian function, as well as immunoregulation in pregnancy, embryonic implantation and placental development. Although almost every cell produces some quantity of the pleiotropic cytokine TGFB-β1, it is mainly produced by the leukocytes, exerting a predominantly anti-inflammatory effect and thereby contributes to maintaining immune tolerance (Li et al., 2006; Taylor, 2009). The human TGFB1 gene is located on chromosome 19q13, and split into seven exons (Lawrence 1996; Clark and Coker, 1998). TGFB-β1 is secreted in an active form (Taylor, 2009) and as a latent complex in which TGFB-β1 binds latency-associated peptide and latent TGFB-β-binding protein. Owing to its anti-inflammatory and immunosuppressive capacities, TGFB-β1 has been shown to regulate several aspects of pregnancy (Goodwin et al., 1998; McLennan and Koishi, 2004; Ingman and Roberston, 2009). TGFB-β1 levels increase in maternal plasma during pregnancy, but decrease quickly after delivery (Ogasawara et al., 2000; Power et al., 2002).

A successful pregnancy was originally depicted as dependent on the delicate balance between T helper (Th)1 and Th2 cells, since (anti-inflammatory) Th2 cells and their mediators are required for successful pregnancy, while (pro-inflammatory) Th1 cells and cytokines are associated with adverse pregnancy outcomes (Wegmann et al., 1993; Liu et al., 2011; Giannubilo et al., 2012). This ‘Th1—Th2 paradigm’ was then expanded to include regulatory T (Treg) and Th17 cells, which have recently been shown to contribute as regulator and effector...
cells to the establishment and maintenance of pregnancy, respectively (Lee et al., 2012). As such, an imbalance between the effector and regulator cells would lead to reproductive failure and related pregnancy disorders (Lee et al., 2012). As a key regulator of Th17 cells, TGFB-1 has been implicated in the regulation of trophoblast uterine invasiveness (Graham et al., 1994; Bischof et al., 2000). Reduced dosages of TGFB-1 and its receptors, of TGFB-2 and of TGFB-3 have been found in placentas after miscarriage (Cheng and Cao, 2005; Giannubilo et al., 2012), while other studies have found conflicting results: unaltered immunolocalization of TGFB and TGFB-2 and TGFB-3 levels in the plasma of spontaneous abortion cases (Ball et al., 2007). It should be noted that TGFB-1 has both endocrine and paracrine actions and so in situ placental expression is more relevant than analysis of plasma levels (Ingman and Roberston, 2009).

The expression of TGFB1 is influenced by genetic determinants (Grainger et al., 1999). Several single-nucleotide polymorphisms (SNPs) throughout the TGFB1 gene have been described; some of them associated with elevated TGFB-1 serum levels (Cambien et al., 1996; Awad et al., 1998). Several associations between TGFB1 polymorphisms and inflammatory conditions have been reported in cardiovascular disease (Yokota et al., 2000; Gewaltig et al., 2002; Morris et al., 2012), pulmonary fibrosis (Awad et al., 1998), liver fibrosis (Gewaltig et al., 2002), osteoporosis (Yamada et al., 2001) and cancer (Dunning et al., 2003).

Despite the importance of TGFB-1 in the regulation of pregnancy, only a few studies have tested the hypothesis of an association between TGFB1 polymorphisms and RPL (Prigoshin et al., 2004; Amani et al., 2005; Von Linsingen et al., 2005). In this study, we investigated the association of TGFB1 exon 1 (rs1800471, rs1800470) and promoter region (rs1800469, rs1800468) SNPs with RPL in 675 Tunisian women. These SNPs were selected owing to their previous association with pregnancy complications (pre-eclampsia/eclampsia, RPL) and their frequency among Tunisians (MAF > 0.05).

Subjects and Methods

Subjects

For this case–control study, a total of 675 women were recruited from the outpatient maternity clinics of Hôpital Farhat Hached (Sousse, Tunisia) for 30 months starting in January 2009. Data collection procedures were the same for patients and control subjects. Study subjects were a homogenous population of Tunisian Arabs, originating from central Tunisia. After obtaining each participant’s signed, informed consent, blood samples were collected in EDTA-containing tubes. The University of Monastir’s Research and Ethics Committee, who approved the study protocol and the consent process, granted IRB approval.

For the 304 women with confirmed RPL, inclusion criteria were three or more consecutive pregnancy losses of unknown etiology, with the same partner and with no classical risk factors. These pregnancy losses occurred during the first trimester of gestation, gestational age being calculated on a clinical basis in weeks of amenorrhea. Every case had endometrial biopsies to evaluate luteal phase defect (morphological assessment of endometrial development), pelvic ultrasound scan for assessment of ovarian morphology and the uterine cavity and hysteroscopy or sonohysterography to evaluate anatomic uterine abnormalities. Exclusion criteria were anatomical abnormalities, previously known systemic disease, endocrine disorders including diabetes mellitus, Rhesus incompatibility and any reported personal or family history of thromboembolism (defined if one or more first-degree family member had had a previous venous or arterial thromboembolic event). Inclusion and exclusion criteria were screened for during a standardized interview and consultation of medical records.

Whenever possible, chromosomal aberrations were ruled out before inclusion in the study. Due to economic constraints, karyotype analysis of fetal products or blood lymphocytes of both partners were available only for 119 (39.1%) cases. All subjects included were confirmed to be free of active infection for the TORCH agents (Toxoplasma gondii, rubella, cytomegalovirus, HSV-1, HSV-2, varicella zoster virus, HIV-1 and HIV-2). In addition, we excluded women aged over 40 at their first miscarriage and those with preclinical miscarriages and/or biochemical pregnancies (i.e. very early termination of pregnancy before delayed menses), or pre-eclampsia in previous pregnancies [elevated systolic and diastolic blood pressure (BP) >145/95 mmHg, or rise in systolic/diastolic BP >30/15 mmHg on at least two occasions]. In total, 136 patients (45.2%) had had no live births, 99 (32.9%) had had one live birth, 41 (13.6%) had had two live births and the remaining 28 (8.3%) had had three live births.

Controls comprised 371 consecutively recruited multiparous, age- and ethnically matched (self-reported) women who were examined during a routine check-up after an uncomplicated pregnancy, who had had at least two live births and no miscarriages (spontaneous or induced), and did not report any family history of RPL.

TGFB1 genotyping

Total genomic DNA was isolated from peripheral blood lymphocytes by the salting-out method (Miller et al., 1988). We selected TGFB1 exon 1 (rs1800471 (Arg25Pro) and rs1800470 (Pro10Leu), and promoter rs1800469 (−509C>T) and rs1800468 (−800G>A) SNPs, using SNPbrowser 4.2 (Applied Biosystems, Foster City, CA, USA). TGFB1 genotyping was done by the allelic discrimination method, using VIC- and FAM-labeled primers (obtained from Applied Biosystems). The reactions were performed in 10 μl volume on StepOne Plus, according to manufacturer’s instructions (Applied Biosystems). We did blinded replicates on 47 cases (15.5%) and 48 controls (12.9%) to assess reproducibility of the genotyping procedure: concordance was >99% (no discrepancy).

Statistical analysis

Data were expressed as mean ± SD (continuous variables) or as a percentage of the total (categorical variables). Intergroup significance was determined by Student’s t-test (continuous variables) and Pearson χ² test (categorical variables). Allele frequencies were calculated by the gene-counting method, and each SNP was tested for the Hardy–Weinberg equilibrium using the χ² goodness-of-fit test from SNPStats (http://bioinfo.iconcologia.net/snpstats). The association of TGFB1 SNPs genotypes with RPL was assessed under additive, dominant and recessive genetic models using SNPStats. Pairwise linkage disequilibrium (LD) values were calculated with Haploview 4.2 (http://www.broadinstitute.org/haploview/haploview), which also calculated haplotype frequency by the expectation maximization method. Logistic regression analysis was performed to calculate...
specific P-values, odds ratios (ORs) and 95% confidence intervals (CIs) after controlling for age, BMI and smoking. The Bonferroni multiple-comparison correction method was employed to calculate the corrected P as per: \( P_c = 1 - (1 - P)^n \), where \( n \) is the number of comparisons. Statistical significance was set at \( P < 0.05 \).

**Results**

**Study subjects**

The demographics and clinical characteristics of RPL cases and control women are shown in Table I. Cases were matched to controls with respect to age (\( P = 0.45 \)), BMI (\( P = 0.720 \)), obesity (\( P = 0.527 \)) and previous oral contraceptive use (\( P = 0.43 \)). A higher frequency of smokers was found in cases than in the control women (\( P = 0.004 \)).

**Association studies**

Table II depicts the association between TGF\( \beta \)1 SNPs and RPL. Genotype distributions of rs1800471 (\( P = 0.169 \)), rs1800469 (\( P = 0.399 \)) and rs1800468 (\( P = 1.00 \)), but not rs1800470 (\( P = 0.048 \)) were in the Hardy–Weinberg equilibrium among control women. Higher minor allele frequency (MAF) for rs1800471 (\( P < 0.001 \)) was found in RPL cases but not for rs1800470 (\( P = 0.307 \)), rs1800469 (\( P = 0.318 \)) or rs1800468 (\( P = 0.403 \)). This association remained significant after applying the Bonferroni correction for multiple testing.

**Discussion**

It has been suggested that a defective maternal immune tolerance to fetal antigens contributes to repeated miscarriages, with altered TGF-\( \beta \)1 production proposed as a key player (Veenstra van Nieuwenhoven et al., 2003). Depending on the target cell and growth factor/cytokine milieu, TGF-\( \beta \)1 may support or alternatively inhibit cellular growth (Sporn and Roberts, 1988; Fontana et al., 2010). Central to pregnancy is the reported capacity of TGF-\( \beta \)1 to regulate the cytokine network that controls trophoblast growth and uterine invasion, as shown elsewhere (Tse et al., 2002; Lash et al., 2010). The current
study is the first to look at a North African population (Tunisian women), and data have shown that the +915G/C TGFB1 polymorphism increased RPL susceptibility; while the remaining three SNPs tested (−509 C/T, −800 G/A, +869T/C) did not influence this adverse pregnancy event.

The contribution of TGFB1 to RPL pathogenesis is not clear, and conflicting results have been reported, linked to the experimental setup and ethnic background of the subjects under study. An earlier study on a limited number of subjects documented higher plasma TGFB1 levels in pregnant women with miscarriage, compared with gestational week-matched pregnant control women, and plasma TGFB1 was increased in three out of eight non-pregnant women with a past history of RPL (Ogasawara et al., 2000). These results should be interpreted with caution, since only total but not active TGFB1 was measured, large variations in plasma level were observed and the population studied was poorly defined. Furthermore, since the plasma TGFB1 measurements were taken after the miscarriage, the values could reflect a consequence of fetal death rather than a causative process. Lastly, as TGFB1 exerts local placental control, the relevance of plasma analysis may be low. Similarly, it could be noted that the production of TGFB1 by mitogen-stimulated peripheral blood leukocytes has been found to be comparable between non-pregnant women with a history of RPL, compared with women with an uneventful obstetric history (Daher et al., 2004; Hossein et al., 2004).

Other studies have examined TGFB1 expression in the placenta of RPL cases, often with conflicting findings, highlighted by either decreased expression (Cheng and Cao, 2005; Giannubilo et al., 2012), or unmodified protein levels (Ball et al., 2007). Once again, since these analyses were done post-fetal death, no causal relationship can be confirmed.

Despite the difficult interpretation of these and related studies, altered TGFB1 expression is probably still involved in RPL pathogenesis. In this study, we chose to assess the contribution of TGFB1 to RPL by analyzing the association of variants in the promoter and exon 1 of TGFB1 (−509 C/T, −800 G/A, +869T/C, +915G/C); our working hypothesis being that altered expression of TGFB1 gene might influence the risk of RPL.

### Table III: RPL association for TGFB1 SNPs.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Controls:cases</th>
<th>Additive P-value</th>
<th>aOR (95% CI)b</th>
<th>Dominanta P-value</th>
<th>aOR (95% CI)</th>
<th>Recessivea P-value</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800471</td>
<td>G/G</td>
<td>330:240</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>G/C</td>
<td>40:57</td>
<td>0.001</td>
<td>2.58 (1.52–4.37)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>1:7</td>
<td>4.63 (0.40–53.71)</td>
<td></td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td>rs1800470</td>
<td>T/T</td>
<td>110:14</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>165:137</td>
<td>0.67</td>
<td>0.93 (0.61–1.42)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>76:53</td>
<td>0.78 (0.45–1.36)</td>
<td></td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td>rs1800469</td>
<td>C/C</td>
<td>157:123</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>168:133</td>
<td>0.56</td>
<td>1.04 (0.69–1.57)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>46:48</td>
<td>1.37 (0.77–2.46)</td>
<td></td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td>rs1800468</td>
<td>G/G</td>
<td>303:256</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>64:45</td>
<td>0.14</td>
<td>0.57 (0.32–1.01)</td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>4:3</td>
<td>1.09 (0.17–6.79)</td>
<td></td>
<td>&lt;0.001</td>
<td>2.63 (1.57–4.43)</td>
<td>0.26</td>
<td>11.33 (0.34–45.03)</td>
</tr>
</tbody>
</table>

aThe reference group (OR = 1.00) is listed first, test group is listed second.
bPc, corrected P-value according to the Bonferroni correction.

### Table IV: Haplotype frequencies across TGFB1 SNPs analyzed.

<table>
<thead>
<tr>
<th>Haplotypea</th>
<th>Frequency</th>
<th>Case:control frequencies</th>
<th>Pcb</th>
<th>aOR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G T C G</td>
<td>0.457</td>
<td>0.451; 0.459</td>
<td>1.00</td>
<td>(Reference)</td>
</tr>
<tr>
<td>G C T G</td>
<td>0.308</td>
<td>0.294; 0.319</td>
<td>0.74</td>
<td>(Reference)</td>
</tr>
<tr>
<td>G T C A</td>
<td>0.077</td>
<td>0.073; 0.086</td>
<td>0.29</td>
<td>(Reference)</td>
</tr>
<tr>
<td>C C G C G</td>
<td>0.052</td>
<td>0.052; 0.048</td>
<td>0.19</td>
<td>(Reference)</td>
</tr>
<tr>
<td>G C C G</td>
<td>0.035</td>
<td>0.018; 0.049</td>
<td>0.01</td>
<td>(Reference)</td>
</tr>
<tr>
<td>G T T G</td>
<td>0.031</td>
<td>0.043; 0.019</td>
<td>0.02</td>
<td>(Reference)</td>
</tr>
<tr>
<td>C C T G</td>
<td>0.017</td>
<td>0.034; 0.003</td>
<td>0.02</td>
<td>(Reference)</td>
</tr>
<tr>
<td>C T C G</td>
<td>0.012</td>
<td>0.021; 0.003</td>
<td>0.02</td>
<td>(Reference)</td>
</tr>
</tbody>
</table>

Bold and underlined characters indicate minor allele.

aPc, corrected P-value according to the Bonferroni correction.
bPc, corrected P-value according to the Bonferroni correction. 

References:

1. Daher et al., 2004
2. Hossein et al., 2004
3. Ball et al., 2007
Significantly, higher +915C allele frequency was seen in our RPL cases. This is the first report describing a significant association between this TGFβ1 variant and RPL, in disagreement with three previous studies (Prigoshin et al., 2004; Amani et al., 2005; Von Linsingen et al., 2005). This discrepancy can be explained by the smaller size of population studied in these previous publications: 38 patients (Prigoshin et al., 2004), 54 patients (Von Linsingen et al., 2005) and 111 patients (Amani et al., 2005), bias in patient-control matching (especially BMI) and differences in ethnic background. Conversely, one previous study did report results comparable to ours, with a significant association of +915C allele associated with a rare IL10 haplotype in 69 Cypriot patients with RPL (Costeas et al., 2004). It is worth noting that in a recent study on kidney transplantation, an increased risk for chronic renal allograft dysfunction in patients having the +915C allele was identified (Jiménez-Souza et al., 2012). This suggests that TGF-β1 is probably one of the cellular pathways involved both in allograft rejection and feto-maternal tolerance failure such as RPL.

It has been suggested that TGFβ1 genotype might interact with smoking in increasing the risk of myocardial infarction (Chen et al., 2012). Smoking is a well-known risk factor for RPL and indeed, 22.2% of our cases are smokers when compared with only 15.3% of our control women (P = 0.004) (Table I). Nevertheless, correction for smoking did not decrease the association between the +915C allele and RPL in our population. In addition, we did not observe an interaction between the TGFβ1 polymorphisms and smoking on the risk of RPL (data not shown).

In agreement with previous studies, we found no significant association of the +867T>C (Leu10Pro) variant with RPL (Costeas et al., 2004; Prigoshin et al., 2004; Von Linsingen et al., 2005; Amani et al., 2005). Lastly, neither of the two promoter variants (−509C>T, −800G>A) was significantly associated with RPL in our Tunisian cohort, in agreement with a recent Iranian study (Amani et al., 2004).

The four tested TGFβ1 polymorphisms were in LD, thereby allowing for construction of four-locus haplotypes (rs1800471/rs1800470/rs1800469/rs1800468). This was based on the notion that interaction of these variants within haplotypes can be more informative than single-variants in determining RPL susceptibility. This analysis demonstrated extensive diversity in the distribution of TGFβ1 haplotypes, with most of the diversity captured by eight common haplotypes. Three haplotypes, namely GTTG, CCTG and CTCG were positively associated with RPL, while the GCCG haplotype was negatively associated with RPL, after adjusting for age, BMI and smoking. At present, we cannot dismiss the possibility that this is a true haplotype effect, or alternatively due to LD between the different polymorphisms. The RPL-associated haplotypes identified in our study (in particular CCTG and GCCG, respectively), even if close, did not precisely correspond to TGFβ1 low-producer CTCA and high-producer GCTG haplotypes deduced from published in vitro studies. In addition, the impact of these haplotypes on TGFβ1 production might be cell type-dependent (Healy et al., 2009). Furthermore, numerous other genes regulate TGFβ1 production. Therefore, without better knowledge of all the factors regulating TGFβ1 expression, it is difficult to make simple correlations between TGFβ1 haplotypes and TGFβ1 production or activity. Along these lines, our present data do not allow us to conclude that the implication of the TGFβ1 genotype in RPL is ascribable to low TGFβ1 production or activity, although this mechanism is still the most likely.

In conclusion, our study reinforces the hypothesis that TGF-β1 is implicated in RPL, and has shown for the first time that the TGFβ1 genotype participates in this pathology, thereby necessitating similar large studies in different ethnic groups to exclude chance findings. Further studies are also needed to estimate the weight of this factor among the other susceptibility determinants for RPL (Daher et al., 2012).

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors’ roles

K.M. contributed to the original data collection, was responsible for the genetic analysis and interpretation of the data and wrote the first draft of the manuscript. V.G.H. participated in setting up and conducting the genetic analysis. S.H. and S.M. contributed to collection, analysis and interpretation of the data. W.Y.A. was responsible for statistical analysis and interpretation of the data. T.M. initiated and designed the study and is in charge of the center where the study was conducted. R.T. contributed to all the genetic analysis, supervised the data analysis and heads the department where the molecular study was performed. All the authors have read and corrected the manuscript, agree as to its intellectual content and that the work is ready for submission and jointly accept responsibility for its content.

Funding

This study was supported by grants from the Ministry of Education and Scientific Research in Tunisia and by the Genetic Department of the CHU de Saint Etienne.

Conflict of interest

None declared.

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