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Research Article

Emerging Genetic Alterations Linked to Male Infertility: X-Chromosome Copy Number Variation and Spermatogenesis Regulatory Genes' Expression

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Abstract

The etiopathogenesis of primary testicular failure remains undefined in 50% of cases. Most of these idiopathic cases probably result from genetic mutations/anomalies. Novel causes, like Copy Number Variation and gene expression profile, are being explored thanks to recent advances in the field of genetics. Our aim was to study Copy Number Variation (CNV) 67, a patient-specific CNV related to spermatogenic anomaly and evaluate the expression of regulatory genes *AKAP4*, responsible for sperm fibrous sheet assembly, and *STAG3*, essential for sister chromatid cohesion during meiosis. One hundred infertile men were tested for CNV67 with quantitative PCR (qPCR). Quantitative real-time PCR was performed to evaluate gene expression patterns of the two mentioned genes in testicular biopsies from 22 idiopathic infertile patients.

CNV67 deletion was found in 2% of patients, with the same semen phenotype described in previous studies. Expression levels of *AKAP4* and *STAG3* were down regulated in infertile patients when compared to control group ($p<0.05$).

Resulting data reinforce the role of CNV67 in male infertility etiology. Its frequency is significantly higher in oligo/azoospermic men and evidence indicates consistency of phenotype. Down regulation of *AKAP4* and *STAG3* cellular transcript levels was observed in the testicular biopsies, suggesting that the gene expression is altered, contributing to unsuccessful sperm production.

As one continues to better understand about the genetics of male infertility, there will be undoubtedly a shift towards better diagnosis and treatment for those patients presenting idiopathic infertility.

INTRODUCTION

Background

An estimated 15% of couples are infertile, not achieving a clinical pregnancy after 1 year of unprotected intercourse, with a great impact on the individual, couple and society [1,2]. Male reproductive dysfunction is the sole or contributory cause of infertility in half of the couples [3,4], with abnormalities of sperm number (azoospermia, oligozoospermia), motility (asthenozoospermia) and morphology (terathozoospermia) being frequently diagnosed [5,6]. Male infertility can be clinically divided in three main categories: acquired, congenital and idiopathic, when no cause is identified [6,7]. The idiopathic group still represents 50% of the cases of primary spermatogenic failure in humans [2,8] and presently, due to the lack of pathophysiological understanding, no specific treatment is offered [9]. Most of the underlying causes are thought to be genetic [6,10,11], mainly due to spermatogenesis defects [3,12], correlated, or not, with environmental factors. Spermatogenesis is a highly complex process controlled by several regulatory genes which assure the correct maturation steps, from spermatogonia to sperm [3]. These men are otherwise usually healthy, suggesting that any genes involved must either be only expressed or be functionally required for spermatogenesis [3].

During the last years, novel tests and diagnostic tools have been employed to identify rare genetic mutations and polymorphism with putative direct or indirect effects on spermatogenesis. The declining cost and increased power of whole-genome sequencing studies, including evaluation of the increasingly important inter genic regions of the genome, is leading to nascent paths of research and likely indicate that, in the future, such studies will be used on daily-basis [13]. Likewise, genetic testing of Copy Number Variation and spermatogenesis's regulatory genes expression may reveal the etiology of idiopathic patients and, consequently, increase the likelihood of successful paternity and reduce potential risks to the progeny [13,14]. Copy Number Variation

(CNV) has raised a considerable interest among scientific and medical communities. CNV is conventionally defined as a DNA segment, 1 kb or longer, that is present in a variable number of copies in the genome, between individuals [15]. Since the first comprehensive CNV map of the human genome, in 2006, several diseases have been linked to CNVs, mainly due to disruption of functional elements (either genes or regulatory elements). In fact, it is well established that Y chromosome CNVs in the AZF region are linked to spermatogenic impairment and are routinely analysed for genetic male infertility diagnosis [9,16]. These unbalanced quantitative variants can be classified into gains (increased number of DNA copies compared to reference genome) and losses (reduction or deletion compared to reference genome) [15]. Recently, high-resolution X-chromosome specific array-comparative genomic hybridization (aCGH) identified CNVs which could be related with male infertility [17]. X chromosome genes are particularly tempting because men are hemizygous for the X-genes. Since compensation by a normal allele is impossible, it is more likely that a mutation may affect the fertility of an individual [6,10]. From the reported CNVs, CNV67 was one of the most promising candidates, resembling AZF deletions of the Y chromosome [16,17]. CNV67 deletion was exclusively found in infertile patients at a frequency of 1.1% ($p<0.01$), ranging patient's phenotypes from azoospermia due to Sertoli-Cell-Only Syndrome (SCOS) to oligozoospermia. It is localized in Xq28 and is likely to be maternally inherited [16]. It has been suggested that CNV67 deletion linked to spermatogenic failure may be related to highly duplicated genes of X-Cancer Testis Antigen (CTA) family, the most represented X-linked testis specific family. In fact, X-CTA genes comprise 10% of all X-linked genes and are expressed specifically in testis [18]. In particular, CNV67 deletion may remove the melanoma antigen family A, 9B *(MAGEA9B)*, expression level in spermatocytes and in some tumour cell lines. It may also affect chromosome X open reading frame *(CXorf40A)*, situated at < 1Mb from the deletion and regulation elements of Heat Shock Transcription Factor Family, X-Linked 1/2 *(HSFX1/2)* [16,17].

Gene expression profile can be used as a basis for identification of candidate genes that contribute to male infertility [19-21]. To date, genetic studies in mice have identified more than 200 genes that are specifically or preferentially involved in the complex regulation of fertility and some are specifically expressed in the germ line [6,10,11,19]. *AKAP4* and *STAG3* are strong candidate genes for male infertility [7,22]. The A-kinase anchor protein 4 (*AKAP4*), an X-linked member of the *AKAP* gene family, encodes the most abundant protein of the spermatozoon's fibrous sheet, a cyto skeletal structure surrounding the region of the principal piece of sperm flagellum [23]. *AKAP4* anchors cAMP-dependent protein kinase A (PKA) to the sperm fibrous sheet, which is essential for sperm capacitation, playing a central role in the regulation of normal sperm motility [11,24,25]. In fact, studies have shown that in *AKAP4*-deficient mice, though sperm count was not reduced, they were immotile, resulting in male infertility [6,11]. Furthermore, another study verified no detection of *AKAP4* immunolabeling in man with 0% sperm mobility [19]. Therefore, *AKAP4* is likely required for the structural and functional integrity of the fibrous sheath [25]. Stromalin 3 (*STAG3*) is a component of all meiosis-specific cohesion complexes, a large ring-shaped proteinaceous structure which tethers sister chromatids, providing cohesion to the structure [22,26]. Its deletion has been related to a Premature Ovary Failure (POF). Interestingly, *STAG3*-deficient male mice display a severe defect in synapses and premature loss of centromeric cohesion during the early stages of prophase I, which causes an arrest during the zygotene-like stage, leading to infertility [22, 27]. The aim of this study is to explore these emerging genetic alterations by quantifying the copy number variation of CNV67 in a group of infertile men and consolidate the pathophysiology which links CNV67 to male infertility. In addition, the expression of spermatogenesis regulatory genes *AKAP4* and *STAG3* will be evaluated in infertile men testicular biopsies and correlated with the (in)fertility status.

MATERIALS AND METHODS

This study includes two distinct analyses – CNV67 screening and expression profile of *AKAP4* and *STAG3.* Each analysis design will be explained separately.

CNV67 Screening Analysis

Patient samples: Peripheral blood samples were collected from 100 Portuguese idiopathic infertile men, with different grades of spermatogenic impairment - 44 azoospermic (AZO), 47 severe oligozoospermic (SOZ), 4 oligozoospermic (OZ) and 5 normozoospermic (N) men (Table 1). Infertile patients were selected on the basis of a comprehensive andrological examination including medical history, semen analysis, scrotal ultrasound, and hormonal and genetic analysis. Patients with abnormal karyotype or Y chromosome microdeletion were excluded. Normal controls were fertile normozoospermic volunteers.

Genomic DNA (gDNA) Extraction: Peripheral blood (3–5 mL) was collected through vein puncture from all participants. High molecular weight DNA was isolated using a salting out method.

Quantitative PCR (qPCR): The number of copies of CNV67 on each sample was determined by Quantitative PCR (qPCR). TaqMan® probes were designed by the manufacturer (Applied Biosystems, Foster City, USA) and were chosen to target specific regions. Hs03323870_cn was selected for the target CNV67 (labeled with FAM) and Hs03323870 was selected for RNase P (labeled with VIC) and used as the reference gene. Reactions were performed in triplicate in a final volume of 20 µL according to the manufacturer's instructions. Briefly, the components of the reaction mix were: 4 µL genomic DNA, 10 µL 2X TaqMan® Genotyping Master Mix, 1 µL 20X TaqMan® Copy Number Assay, 1 µL 20X TaqMan® Copy Number Reference Assay (RNase P) and 4 µL nuclease-free water. qPCR was carried out on a Step One Plus™ Real-Time PCR System (Applied Biosystems). The thermal

Table 1: Clinical description of the study population.

Patient's semen phenotype	(n=100)
Azoospermic	44
Severe oligozoospermic (<5x10^6/mL)	47
Oligozoospermic (5-15x10^6/mL)	4
Normal	5

cycling conditions were as follows: Initial enzyme activation for 10 minutes at 95˚C, 40 cycles were performed, each one consisting of 15 seconds at 95˚C and 60 seconds at 60˚C.

Data Analysis

Applied Biosystems Copy Caller™ Software v2.0 was used to determine the copy number status of each target region, and calculations were performed according to the maximum-likelihood algorithm of the software. Raw copy value (RCV) represents a non-integer number of copy calculated, whereas predicted copy number (PCN) is defined as an integer number of copy determined by the algorithm (0, 1, 2, or 3+). As CNV67 is located on X-chromosome, normal females will display PCN of 2 and normal males PCN equal to 1. In the case of male alteration, Copy Number (CN) gain is defined as PCN higher than 1, and PCN of 0 is regarded as CN loss.

AKAP4 and STAG3 Expression

Patient samples: Testicular samples were collected from 22 idiopathic infertile men with AZS (used as cases). Seven men with secondary infertility were used as controls. Testicular biopsies were obtained to confirm the clinical diagnosis (diagnostic biopsy) or for sperm retrieval (Testicular Sperm Extraction: TESE) to intracytoplasmic sperm injection (treatment biopsy). Patients with abnormal karyotype or Y chromosome micro deletion were excluded. Clinical information of each sample is shown in Table (2).

Each sample was divided into three aliquots: one was reserved for histological analysis, the second (100-200mg) was processed for sperm extraction and the third (10mg) was immediately transferred to a 1.5mL tube with mRNA later® solution (Ambion®, Foster City, USA) and stored at -80ºC for further gene expression studies.

RNA isolation and reverse transcription (RT) reaction: After thawing the frozen pellets, cells were lysed on ice with 1000µL of TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, USA) and passed several times through a syringe and needle. The total mRNA was then extracted according to the associated protocol. At the end, RNA pellet was resuspended in 50µL of diethylpyro carbonate (DEPC)-treated RNase-free water (Promega, Wisconsin, USA) and incubated for 1h on ice. RNA was then quantified in a Biotech Photometer UV 1101 (WPA, Cambridge, UK). 1µg of mRNA in a total volume of 10µL was reverse transcribed to complementary DNA (cDNA) using qScript™ cDNA SuperMix (Quanta, Biosciences™, Gaithersburg, USA), with random hexamers as the priming method and according to the manufacturer's instructions.

Gene Expression Analysis by Quantitative Real-Time PCR (Qrt-PCR): TaqMan® Gene Expression Assays were used for both targeted experimental genes (*AKAP4* - Hs00275849_m1 and *STAG3* - Hs00429370_m1). All TaqMan® probes were labeled with FAM dye and were purchased from Applied Biosystems. RNA 18S Ribosomal (*18S*) was used as the housekeeping gene and TaqMan® Gene Expression Assay was also utilized.

RNA expression levels were analysed by qRT-PCR on a StepOnePlus™ Real- Time PCR System (Applied Biosystems).

Table 2: Clinical description of the study population.

Patients (n=29)	ID	Diagnosis	TESE	Clinical history
Cases (n=22)	w1	AZS	SCOS	Normal
	w77	AZS	MA	Normal
	w113	AZS	MA	Hypospadias, Orchitis (28yo, 29yo)
	w119	AZS	MA	Left testicular torsion
	w131	AZS	HP	Normal
	w140	AZS	MA	Normal
	w147	AZS	MA	Normal
	w149	AZS	SCOS	Stroke
	w152b	AZS	SCOS	Parotiditis
	w154	AZS	SCOS	Inguinal Hernia (6yo, 7yo)
	w160	AZS	HP	Normal
	w162	AZS	SCOS	Normal
	w165	AZS	HP	Normal
	w166	AZS	HP	Epilepsy
	w175	AZS	MA	Normal
	w176	AZS	SCOS	Normal
	w183	AZS	HP	Leftscrotalhydrocele
	w186	AZS	SCOS	Normal
	w187	AZS	MA	Normal
	w195	AZS	SCOS	Left varicocele, Orchitis (24yo)
	w220	AZS	SCOS	Normal
	w227	AZS	HP	Hepatitis C, ex-alcoholic
Control (n=7)	w58	ANE-JACUL	HP	Paraplegy
	w90	ANE-JACUL	HP	Diabetes Mellitus
	w103	ANE-JACUL	HP	Psycologicanejaculation
	w106	ANE-JACUL	SCOS	Paraplegy
	w116	ANE-JACUL	HP	Paraplegy
	w128	OLIGO-ASTHE	HP	Normal
	w164	OLIGO-ASTHE	HP	Normal

ANEJACUL: Anejaculation; AZS: Azoospermia; HP- Hypospermatogenesis; MA: Maturation arrest; OLIGO-ASTHE: Oligo-asthenozoospermia; SCOS: Sertoli-Cell-Only Syndrome; Shaded samples had no expression for at least one of the studied genes.

qRT-PCR was performed in a volume of 10µL, using 2µL of cDNA, 2.5µL of Nuclease Free-water, 5µL of 2xKAPA probe Master Mix (Kappa Biosystems, Boston, Massachusetts, USA) and 0,5µL of 20X TaqMan® Gene Expression Assay for each gene, using a Fast Protocol according to manufacturer instructions. Briefly, after initial enzyme activation for 2 minutes at 50˚C and 20 seconds at 95˚C, 40 cycles were performed, each one consisting of 3 seconds at 95˚C and 20 seconds at 60˚C. Standard curves were performed with five points, in duplicates. Each PCR for relative quantification was run in triplicate (technical replicates) and all genes were run together with a negative control.

Data Analysis and Statistics

Data was analyzed using REST 2009 (Relative Expression Software Tool), which is a standalone software tool that estimates up and down regulation for gene expression studies (http://www.qiagen.com/rest)

The purpose of this software is to determine whether there are significant differences between samples and controls, while taking in account issues of reaction efficiency and reference gene normalization. The obtained hypothesis test P(H1) represents the probability of the alternate hypothesis that the difference between the sample and control groups is due only to chance. Real time PCR-negativity was defined by the absence of amplified product after 40 cycles and because REST software uses Ct values and reaction efficiency for calculations instead of relative expressions values, we proposed that the value of the last cycle of amplification (Ct = 40 cycles) should correspond to the value of absence of relative expression. Wilcoxon Signed Rank Test was used for the statistical analysis (StatView for Windows) with the significance level set at $p < 0.05$.

RESULTS AND DISCUSSION

CNV67 Screening Analysis

In order to screen CNV67 deletion, 100 samples were studied from infertile men with different sperm phenotypes and concentration, as previously described, by RT-PCR (Table 1). Two individuals – Y3790 and Y3803 – were found to have deletion (0 copies) of CNV67 (2%). Y3790 was azoospermic, diagnosed with SCOS after biopsy and Y3803 presented a severe oligozoospermia (2 x10^6 sperm/mL) (Figure 1). Our data supports the sperm phenotypes related to CNV67 deletion (azoospermia in a clinical context of SCOS or oligozoospermia). Moreover, the findings indicate a significantly higher frequency in our Portuguese population, even though our sample was quite small when compared to previous studies [16, 17]. Whether the observed deletion is directly responsible for the altered sperm phenotype (either affecting gene expression or regulatory elements) or is related to increased genomic instability remains uncertain [17].

AKAP4 and STAG3 Expression

Quantification of testicular mRNA levels of genes expression was carried out by qRT-PCR in individuals showing spermatogenic failure. Two spermatogenesis related genes - *AKAP4* and *STAG3* – were analyzed in 20 and 22 testicular biopsies samples, respectively. Clinical and pathologic information on the cases and controls are presented in Table (2).

Analysis of the qRT-PCR results was completed by using REST 2009 software. The findings are summarized in Table 3. For reference gene normalization, 18S housekeeping gene was used. Sample expression ratios were calculated with REST software using the following formula:

Relative Expression = Concentration of Gene of interest ÷ Geometric mean (concentration of reference gene 1, concentration of reference gene 2,…)

With the use of this software, the up or down regulation for each gene expression was estimated comparing cases with controls. Results indicated that the *AKAP4* and *STAG3* were down regulated with statistical significance ($p<0.05$) in the case group compared to the control group (Table 3, Figure 2).

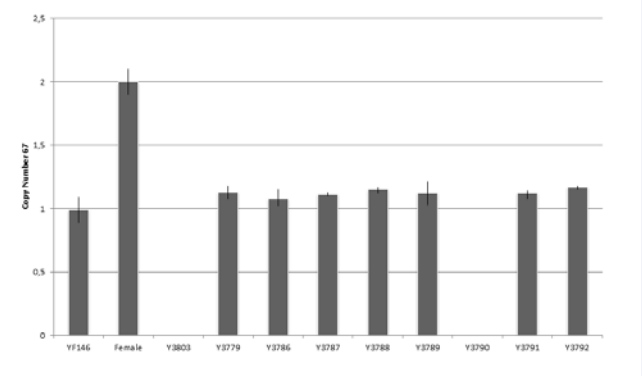

Figure 1 Copy Number Variation 67 deletion of infertile man Y3790 and Y3803. Whiskers refer to maximum and minimum of copies calculated. As CNV67 is located on X-chromosome, normal females will display Predicted Number of Copies (PCN) of 2 and normal males PCN equal to 1. In the case of male alteration, Copy Number (CN) gain is defined as PCN higher than 1, and PCN of 0 is regarded as CN loss. YF154 – Fertile man control.

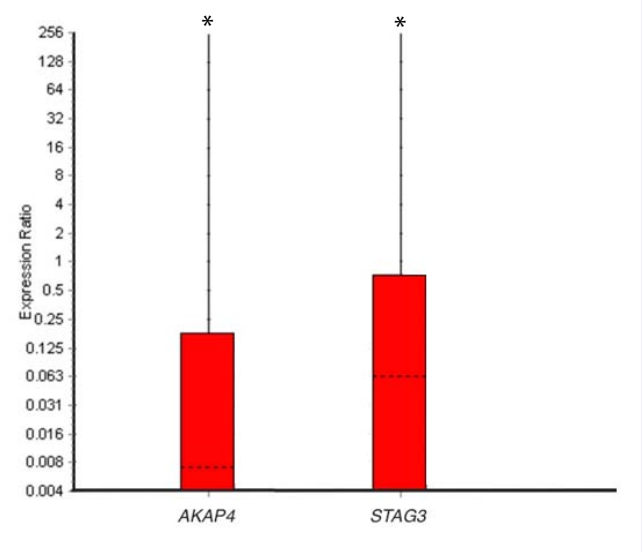

Figure 2 Expresion levels of *AKAP4* and *STAG3* in testicular samples. cDNA expression was normalized using a housekeeping gene (*18S*). Boxes represents the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Significance differences between groups are represented as: * $p<0.05$. The data was analysed by Mann-Whitney-Wilcoxon test.

Interestingly, 6 cases (w147, w149, w154, w176, w186, w195) did not express *AKAP4* and w220 patient did not express *STAG3* (see Table 2). It has been demonstrated that the reduction of gene expression in spermatogenic failure patients could not be exclusively attributed to a decreased number of germ cells, but the contribution of the reduced cellular expression should be also taken in account [19].

Table 3 : *AKAP4*and *STAG3* expression results.

Gene	Type	RxnEffic.	Expression	Std. Error	95% C.I.	P(H1)	Result
18S	REF	0.9558	1.000				
AKAP4	TRG	0.9346	0.011	0.000 - 0.645	0.0-62.299	**0.007**	DOWN
STAG3	TRG	0.9829	0.050	0.001 - 2.076	0.0-114.426	**0.038**	DOWN

REF: Reference; TRG:Target. RxnEffic: Reaction efficiency; Std. Error: Standard Error; 95% C.I. – 95% confidence interval; P(H1): Probability of alternative hypothesis that difference between sample and control groups is due only to chance

AKAP4 encodes a protein involved in fibrous sheet assembly and its regulation [28], and is exclusively expressed in germ cells, during the post-meiotic phase of spermatogenesis [25,29]. The findings of this study on *AKAP4* expression exhibit a statistically significant difference with down regulation in the case group. Interestingly, no expression was detected in several SCOS patients. However, the 2 oligo-asthenozoospermic However, the 2 oligo-asthenozospermic patients used as controls expressed AKAP4, contradicting the data published in a previous study [22]. patients used as controls expressed *AKAP4,* contradicting the data published in a previous study. Similarly, down regulation of *STAG3* was found in the case group. The *STAG3* encodes a predominant STAG protein component of cohesin complexes in primary spermatocytes, participating in the telomere attachment to the nuclear periphery, telomere maintenance, chromosome pairing, chromosome synapses and maintenance of sister chromatid cohesion [27]. This protein is exclusively expressed in meiosis. Gene expression profiles can be used as a basis for identification of candidate genes that contribute to spermatogenic impairment. One must emphasize that an inherent problem in investigating testicular expression changes is the cellular complexity of the organ [21]. Here we analyzed the transcriptional changes in a complete organ, with distinct germ cell types. One advantage is that we revealed complex transcriptional changes related to the whole testis during germ cell differentiation. The same point has the inherent disadvantage, compared with isolated cell fractions, as we cannot directly identify the locus of expression change. Furthermore, whether the observed differential expression profiles represent the cause or consequence of spermatogenic impairment remains to be elucidated. This data should be useful in delineating the patterns of gene expression involved in male germline, which may contribute to understanding male infertility.

CONCLUSION

Classic male infertility tests, like karyotyping, Y chromosome micro deletions and FISH analysis at somatic and germ cell levels, are no longer sufficient to investigate the potential contribution of genome disorders on male infertility. A wide range of molecular methods are required for better understanding of male infertility causes and, therefore, increase the potential offer for a better treatment for infertile patients [30].

Novel genetic alterations have been identified which may be of potential clinical relevance in the etiology of male infertility in the medium term, like Copy Number Variation (CNV). Of all CNVs related to male infertility, X-CNV67 was one of the most interesting ones [16], with a consistent phenotype and significant frequency. It is likely that rare single nucleotide polymorphisms (SNPs) and CNVs, although rare on an individual basis, collectively they can contribute to explain a significant number of cases of male infertility that are currently classified as idiopathic [13]. The present study also explores gene expression profile as an emerging genetic alteration with implications in male infertility. Therefore we assessed the expression profile of regulatory genes *AKAP4* and *STAG3* on infertile men testicular biopsies. Our data reports altered expression of germ-line regulatory genes, providing an initial glimpse into the complex regulatory network controlling germ line development. Further analyses in larger series are required to better understand the biological implications of these differences. Although the importance of diagnosing genetic factors is fully recognized, the diagnostic workup of infertility in men is still limited to a few genetic tests [17]. Genetic testing allows clarifying an obscure infertility diagnosis and help to prevent miscarriage and iatrogenic transmission of genetic defects to the offspring through Assisted Reproduction Techniques (ART) [3,4]. Therefore, we believe that efforts should be made in order to identify potential genetic causes of infertility and, in this way, aid couples to make informed decisions, optimize genetic testing and provide therapeutic targets [2,31].

In conclusion, our findings merit further investigation in order to elucidate the potential of CNV67 in routine fertility workup and the role of *AKAP4* and *STAG3* in male infertility.

Ethics Approval and Consent to Participate

The local Ethical Committees of the Faculty of Medicine of University of Porto/ Centro Hospitalar S. João approved the study. Informed consent was obtained from the patients before being included in this study during their reproductive medical treatment.

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