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Expression of mRNA for human type-I LHRH receptor transcript forms in human benign prostatic hyperplasia

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Abstract. The presence of four different isoforms of luteinizing hormone-releasing hormones (LHRH) and one LHRH receptor (LHRH-R) has been reported in vertebrates. In the human genome only LHRH-I and LHRH-II genes have been identified. The human LHRH-I gene is composed of four exons separated by three introns. Three LHRH receptor or receptor-like genes have been demonstrated. The well-established type-I LHRH receptor (LHRH-R-I) gene is composed of three exons separated by two introns. In this study we investigated the expression of transcript forms of LHRH-R-I in human benign prostatic hyperplasia (BPH) with reverse transcriptase-polymerase chain reaction (RT-PCR) using gene specific primers. Thirty-five human BPH specimens were obtained at surgery. Normal human pituitaries collected at autopsy served as control. RNA extraction and RT-PCR with gene-specific primers for LHRH-R-I forward (F1)/reverse (R1), LHRH-R-I F2/R3, LHRH-R-I F1'/R2' were carried out to determine the mRNA expression for LHRH-R-I transcript forms. The expected PCR products amplified with gene specific primers were LHRH-R-I F1/R1 with 319 bp, LHRH-R-I F2/R3 with 309 bp and LHRH-R-I F1'/R2' with 219 bp. PCR products for LHRH-R-I F1/R1 were detected in 21 (60%) and for LHRH-R-I F2/R3 in 5 of 35 (14%) BPH samples. No PCR products for LHRH-R-I F1'/R2' were found. In conclusion, we detected mRNA for LHRH-R-I in human BPH specimens. Our results suggest that LHRH-R-I gene may have more than two splice variants or uncharacterised transcript forms of LHRH-R-I. Our findings support the merit of further investigation of the expression of LHRH-R-I and its transcript forms in human BPH.

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Introduction

Luteinizing hormone-releasing hormone-I (LHRH-I), also known as gonadotropin hormone-releasing hormone, (GnRH) is the primary link between the brain and the pituitary in the regulation of gonadal function and plays a pivotal role in vertebrate reproduction (1). The discovery of LHRH has had a major impact on medicine and has led to a variety of clinical uses of LHRH analogs in oncology and gynecology (1). Most vertebrates express at least two LHRH isoforms. In the human genome only LHRH-I (mammalian LHRH) and LHRH-II (chicken LHRH-II) genes were identified. LHRH-II has been likewise found in tumors such as breast carcinoma, but the functional receptor for LHRH-II is absent in humans (1). The human LHRH-I gene is composed of four exons separated by three introns and is present as a single gene copy on chromosome 8p11.2-p21 (2,3). Three LHRH receptors or receptor-like genes were demonstrated earlier (2-6). The well-established type-I LHRH receptor (LHRH-R-I) gene has been identified as a single copy on chromosome 4q21.2 (4,5) and is composed of three exons separated by two introns (6). A vast literature indicates that the LHRH-I is expressed not only in the hypothalamus but also in peripherial tissues, both normal and tumoral (7-16). However, only limited findings are available on the presence of full length LHRH-R-I and its splice variants or transcript forms in human malignant and benign tissues (17,18). The LHRH-R-I belongs to the G protein-coupled receptor (GPCRs) family (19,20) with seven transmembrane domains (TMs) connected by extracellular loops (ECLs) and intracellular loops (ICLs).

LHRH-R-I is expressed on the surface of pituitary gonadotrope cells (1,19). This receptor is characterized by the unique absence amongst GPCRs of a carboxyl-terminal tail (21). LHRH-R-I has much higher affinity for LHRH-I than for LHRH-II (22-24). Based on their ligand binding proporties LHRH receptors appear to be quite similar in the human pituitary, human normal extrapituitary tissues and human cancers, but their signalling pathways are different (25,26). In the past 25 years several thousand LHRH analogs have been synthesized. Many analogs were shown to have important clinical applications in gynecology, oncology and urology (1,9,27-29).

Only LHRH-R-I was isolated as a functional receptor in human tissues. Earlier, three different mRNA products for LHRH-R-I have been identified (30). Two splice variants of the LHRH-R-I (LHRH-R-I Sb1), termed Sb2 and Sb3 have been reported (17,18). The shorter transcript Sb3 contains a 220-bp deletion in exon 2, the other splice variant Sb2 carries a shorter deletion of 128 bp. The expression of LHRH-R-I has been demonstrated in normal and malignant tissues by several groups, although some investigators did not use primers designed for the full length LHRH-R-I gene (31-33).

The aim of our work was to investigate the mRNA expression for LHRH-R-I transcript forms in human benign prostatic hyperplasia (BPH) specimens. Our study was carried out with previously published primers as well as with LHRH-R-I specific primers designed by us. LHRH-I receptor transcripts have been already extensively studied in various human tissues, including those of the breast, endometrium, prostate and ovary. In our work, we investigated the mRNA expression for LHRH-R-I transcript forms in human BPH specimens using RT-PCR.

LHRH-R-I F1/R1-(319 bp LHRH-R-I Sb1, LHRH-R-I Sb2, LHRH-R-I Sb3) *(34)*

F1: 5'-gaccttgtctggaaagatcc-3' (EXON 1 1844-1863)

R1: 5'-tgatggtggtgatcagcctg-3' (EXON 1 2143-2162)

LHRH-R-I F2/R3- (309 bp LHRH-R-I Sb1) *(32)*

F2: 5'-agcagacagctctggacagacaaaa-3' (EXON 2 2300-2322)

R3: 5'-tgtctgctggactccctactattgt-3' (EXON 3 2582-2605)

LHRH-R-I F1'/R2'-(219 bp LHRH-R-I Sb1, 91 bp LHRH-R-I Sb2)

(Were designed using Primer3
http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)

F1': 5'-tagtgtctttgcaggaccaca-3' (EXON 1 2262-2273)

R2': 5'-aatcatcttcaccctgacacg-3' (EXON 2 2460-2480)

Figure 1. Nomenclature, sequence and position of forward (F) and reverse (R) oligonucleotide primers with reference to human LHRH-R-I cDNA sequence published previously (19,20).

Materials and methods

Tissue samples. Human BPH specimens from 35 patients (mean age, 67.9 years; range, 55-82 years) were collected at the Department of Urology, University of Debrecen, Debrecen, Hungary. Approximately 8-10 mm^3 of each tissue was used for molecular biology studies. Histopathological examinations of each specimen was undertaken to confirm the presence of benign prostatic hyperplasia (BPH) before the molecular biology studies. Two normal pituitary samples were collected at autopsy from the Department of Pathology, University of Debrecen and were used as positive controls. After removal, all samples were flash frozen and stored at -70˚C. The collection of human tissue specimens and the experimental protocol have been approved by the Institutional Ethics Committee for the current study and the patients gave informed consent.

RNA isolation and RT-PCR. Tissues were homogenized with a Mikro-Dismembrator-U (Sartorius B.Braun Biotech, Melsunge, Germany) and RNA was extracted with Nucleospin Total RNA Isolation Kit (Macherey-Nagel, Germany). RNA concentration and purity were determined using the Nanodrop ND-1000 UV Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Total RNA (100-250 ng) was reverse transcribed to cDNA with MMLV Reverse Transcriptase (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. For amplification from first strand cDNA gene-specific primers for LHRH-R-I (forward) F1/(reverse) R1: F1:5'-GACCTTGTCTGGAAAGATCC-3' (exon 1 1844-1863) and R1: 5'-TGATGGTGGTGATCAGCCTG-3' (exon 1 2143-2162), LHRH-R-I F2/R3: F2: 5'-AGCAGACAGCTCTGGACAGACAAAA-3' (exon 2 2300-2322) and R3: 5'-TGTCTGCTGGACTCCCTACTATGT-3' (exon 3 2582-2605), and beta actin (bACT) housekeeping gene F3/R4: F3: 5'-GGCATCCTCACCCTGAAGTA-3' (exon 3) R4: 5'-GGGGTGTTGAAGGTCTCAAA-3' (exon 4) were described earlier (32,34-36) (Fig. 1). Primers for LHRH-R-I F1'/R2': F1': 5'-TAGTGTCTTTGCAGGACCACA-3' (exon 1 2262-2273) and R2': 5'-AATCATCTTCACCCTGACACG-3' (exon 2 2460-2480) were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For LHRH-R-I F1/R1, LHRH-R-I F1'/R2' and bACT, the PCR reaction mixture contained 2 μl cDNA, 1.5 mM $MgCl_2$, 0.5 μM of each primer (Invitrogen), 1X PCR buffer, 200 μM of each dNTP, and 1 U Taq Polymerase (Invitrogen) in a final volume of 25 μl. After denaturation (3 min at 94˚C) cDNA was amplified for 45 cycles (45 sec at 94˚C; 30 sec at 60˚C; and 90 sec at 72˚C), bACT was amplified with 30 cycles, then a final elongation step at 72˚C for 10 min was applied.

For LHRH-R-I F2/R3 the PCR reaction mixture contained 2 μl cDNA, 3 mM $MgCl_2$, 0.5 μM of each primer (Invitrogen), 1X PCR buffer, 200 μM of each dNTP, and 1 U Taq Polymerase (Invitrogen) in a final volume of 25 μl. After denaturation (3 min at 94˚C) cDNA was amplified for 45 cycles (45 sec at 94˚C; 30 sec at 66˚C; and 90 sec at 72˚C) and then a final elongation step at 72˚C for 10 min was applied. PCR products were separated electrophoretically on 1.5% agarose gel and stained with ethidium bromide.

Results

Expression of mRNA for human type-I LHRH receptors in human benign prostatic hyperplasia. RT-PCR analysis was carried out to investigate the expression of different LHRH-R transcript forms in human BPH tissue samples. We used LHRH-R-I F1/R1 primers encompassing the open reading frame (ORF) from exon 1 to exon 1, LHRH-R-I F2/R3 from exon 2 to exon 3 and LHRH-R-I F1'/R2' primers from exon 1

Gene	GNRHR	Variant 1
DNA:	15.61 Kb	NT 022778
mRNA:	2738 bp	NM 000406
CDS:	987 bp	NP 000397

NM_000406

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1741 cctgggaaaa tatggcaaac agtgcctctc ctgaacagaa tcaaaatcac tgttcagcca
1801 tcaacaacag catcccactg atgcagggca acctccccac tctgaccttg tctggaaaga
1861 tccgagtgac ggttactttc ttccttttc tgctctctgc gacctttaat gcttctttct
1921 tgttgaaact tcagaagtgg acacagaaga aagagaaagg gaaaaagctc tcaagaatga
1981 agctgctctt aaaacatctg accttagcca acctgttgga gactctgatt gtcatgccac
2041 tggatgggat gtggaacatt acagtccaat ggtatgctgg agagttactc tgcaaagttc
2101 tcagttatct aaagcttttc tccatgtatg ccccagcctt catgatggtg gtgatcagcc
2161 tggaccgctc cctggctatc acgaggcccc tagctttgaa aagcaacagc aaagtcggac
2221 agtccatggt tggcctggcc tggatcctca gtagtgtctt tgcaggacca cagttataca
2281 tcttcaggat gattcatcta gcagacagct ctggacagac aaaagttttc tctcaatgtg
2341 taacacactg cagtttttca caatggtggc atcaagcatt ttataacttt ttcaccttca
2401 gctgcctctt catcatccct cttttcatca tgctgatctg caatgcaaaa atcatcttca
2471 ccctgacacg ggtccttcat caggaccccc acgaactaca actgaatcag tccaagaaca
2521 atataccaag agcacggctg aagactctaa aaatgacggt tgcatttgcc acttcattta
2581 ctgtctgctg gactccctac tatgtcctag gaatttggta ttggtttgat cctgaaatgt
2641 taaacaggtt gtcagaccca gtaaatcact tcttctttct ctttgccttt ttaaacccat
2701 gctttgatcc acttatctat ggatattttt ctctgtga
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Figure 2. Coding nucleotide sequence (CDS:1752-2738) of the human LHRH-R-I gene. The start and the stop codons are marked with red lower-case letters. Gene specific primers for LHRH-R-I F1/R1, LHRH-R-I F2/R3 and LHRH-R-I F1'/R2' are marked with blue, green and yellow, respectively. The nucleotide sequences of the human type-I LHRH receptor (19) were used to query the human genome sequence using the BLAST search programs of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

to exon 2 (Figs. 1 and 2). The predicted size of the PCR-amplified LHRH-R F1/R1 cDNA was 319 bp for the full-length transcript (sb1), for the partial exon 2-deleted variant (sb2) and for the exon 2-lacking (sb3). The predicted size of the PCR-amplified LHRH-R F2/R3 cDNA was 309 bp for the full-length transcript (sb1). The predicted size of the PCR-amplified LHRH-R F1'/R2' cDNA was 219 bp for the full-length transcript (sb1) and 91 bp for the partial exon 2-deleted variant (sb2) (Fig. 3). PCR products for LHRH-R-I F1/R1 were detected in 21 of 35 (60%) human BPH specimens. PCR products for LHRH-R-I F2/R3 were detected in 5 of 35 (14%) BPH specimens. We were not able to demonstrate PCR products for LHRH-R-I F1'/R2' in any of the BPH samples investigated (Tables I and II). Although LHRH-R-I transcripts have been previously detected in extensive series of human tissues (18), in our study we were not able to find the mRNA for the full length LHRH-R-I by RT-PCR. PCR amplification with human bACT gene-specific primers generated a single product of the expected size of 203 bp in all samples investigated.

Expression of mRNA for human type-I LHRH receptors in human pituitary tissue. In human pituitary tissue samples, used as a positive control, we were able to detect the mRNA for LHRH-R-I transcript forms with all the three primer sets applied in our study (Fig. 3). We could not detect the partial exon 2-deleted variant (sb2) using LHRHR-I F1'/R2' primers in the human pituitary samples investigated. We did not use specific primers to detect the LHRH-R-I Sb3 that is also a variant lacking exon 2.

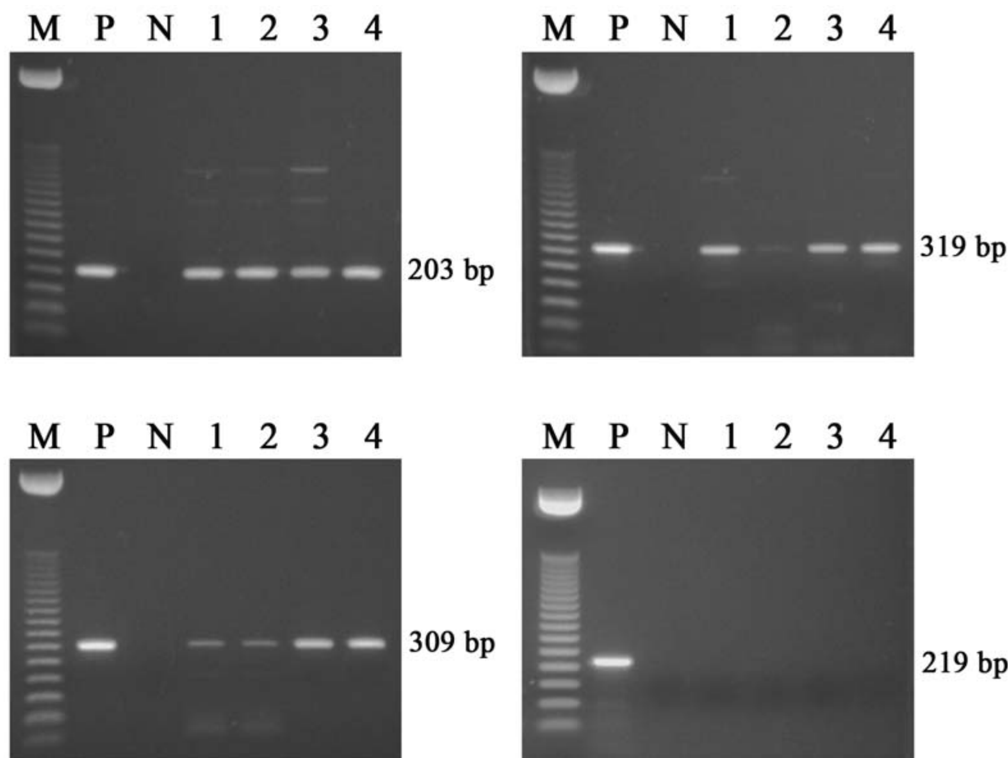

Figure 3. Representative RT-PCR analysis of mRNA for bACT (panel 1), LHRH-R-I F1/R1 (panel 2), LHRH-R-I F2/R3 (panel 3), LHRH-R-I F1'/R2' (panel 4) in human BPH specimens and in a human pituitary sample used as positive control. PCR products were separated electrophoretically on 1.5% agarose gel and stained with ethidium bromide. Lane M, molecular DNA marker (50 bp); lanes 1-4, BPH samples; lane N, no template control; lane P, positive control (human pituitary tissue).

Table I. Clinicopathological findings and mRNA expression pattern of LHRH-R-I F1/R1, LHRH-R-I F2/R3 and LHRH-R-I F1'/R2' in human benign prostatic hyperplasia specimens.

Patient no.	Patient age (years)	PSA-value (ng/ml)	bACT	LHRH-R-I F1/R1	LHRH-R-I F2/R3	LHRH-R-I F1'/R2'
1.	81	-	+	+	+	-
2.	55	0.45	+	+	-	-
3.	82	Normal	+	+	-	-
4.	70	3.52	+	+	+	-
5.	71	6.62	+	+	-	-
6.	65	Increased	+	+	+	-
7.	73	9.45	+	+	-	-
8.	66	NA	+	+	-	-
9.	62	NA	+	+	-	-
10.	70	1.12	+	+	-	-
11.	65	NA	+	+	-	-
12.	70	0.5	+	+	-	-
13.	56	10-12	+	+	-	-
14.	64	2.4	+	+	-	-
15.	73	1.56	+	+	-	-
16.	76	Normal	+	+	-	-
17.	77	0.92	+	+	-	-
18.	55	NA	+	-	-	-
19.	67	3.21	+	-	-	-
20.	59	NA	+	-	-	-
21.	74	NA	+	-	-	-
22.	61	NA	+	-	-	-
23.	75	1.57	+	+	-	-
24.	74	3.76	+	+	-	-
25.	75	10.84	+	-	-	-
26.	73	NA	+	+	-	-
27.	68	3.84	+	-	-	-
28.	67	NA	+	-	-	-
29.	69	NA	+	-	+	-
30.	75	NA	+	-	+	-
31.	62	NA	+	+	-	-
32.	67	0.3	+	-	-	-
33.	58	NA	+	-	-	-
34.	76	2.26	+	-	-	-
35.	55	NA	+	-	-	-

NA, not available.

Table II. Expression of mRNA for the bACT, LHRH-R-I F1/R1, LHRH-R-I F2/R3, and LHRH-R-I F1'/R2' in human benign prostatic hyperplasia samples.

Gene	Positive/total sample size	% Positive
bACT	35/35	100
LHRH-R-I F1/R1	21/35	60
LHRH-R-I F2/R3	5/35	14
LHRH-R-I F1'/R2'	0/35	0

Discussion

LHRH agonists have been used extensively for the treatment of various diseases and medical conditions, in which the suppression of gonadotropin and/or gonadal steroid hormone secretion is desired. The possible usefulness of LHRH analogs in treatment of human benign prostatic hyperplasia based in part on direct effects is also suggested by numerous reports (1,9). Experimental and clinical evidence indicates the existence of a functional LHRH loop, and the presence of specific LHRH receptors in human BPH (1,9,34). In the present study, we examined the expression of LHRH-R-I

gene in 35 human BPH specimens. Our work represents the first demonstration of the presence of LHRH-R-I transcripts in human BPH tissue samples obtained after radical prostatectomy. We were able to detect that the transcripts of LHRH-R-I were widely distributed in human BPH samples. In our study, sequences of LHRH-R-I from exon 1 to exon 1 were amplified in 60% of the specimens and sequences from exon 2 to exon 3 were detected in 14% of the human BPH tissue samples investigated. However, we could not find sequences from exon 1 to exon 2 that correspond to the full-length LHRH-R-I sequences. Our results could be explained by the fact that LHRH-R-I gene may have more than two splice variants or other, uncharacterised transcript forms. We also confirmed the expression of the LHRH-R-I gene transcripts in human pituitary samples. By alternative splicing, multiple transcripts can be generated from a single gene. This represents an important/key molecular mechanism of gene regulation in physiological and pathophysiological processes. It is important to note that different transcript forms of mRNA for LHRH-R-I were expressed in human BPH specimens, but these transcripts are not the same variants lacking exon 2. Finch *et al* (37) suggests that LHRH-R-I splicing may be regulated in a tissue-specific manner, or under specific hormonal or metabolic conditions. Generation of protein isoforms by alternative splicing is known to occur in numerous members of the G-protein-coupled receptors family (38-40), correlating with differences in affinity, potency, coupling efficiency, specificity and sensitivity to desensitization of the receptors. Differential splicing could also have a physiological significance, as production of a short transcript was shown to downregulate the level of a wild-type (WT) transcript (41). Splice variants of peptide hormone receptors can differ fundamentally from their WT receptor counterparts in pharmacological and functional characteristics, in their distribution in normal and malignant human tissues, and in their potential use for clinical applications. It is well-established that regulation of splicing may provide another control mechanism in the expression of the LHRH-R-I gene in the human pituitary and extrapituitary tissues. Although LHRH-I receptor transcripts were detected in an extensive series of human tissues, including those of the breast, uterus, endometrium, prostate and ovary (42-52), we have been unable, so far, to find full length LHRH-R-I using RT-PCR analyses. Although pituitary and extrapituitary LHRH-R-I transcripts appear identical, their functional characteristics may differ. In the pituitary, LHRH-R-I are coupled via $G_{q/11}$ to phospho-lipase C (PLC), causing an IP_3-mediated mobilization of Ca^{2+} and a protein kinase C-mediated activation of MAP kinases. However, there is little evidence for PLC activation by endogenous extrapituitary LHRH-R-I (44,48). Instead, G_i-mediated activation of protein phosphatase and inhibition of MAP kinase activity may be the basis of some of the anti-proliferative effects mediated by LHRH-R-I (44,46,52,53). Investigation of the expression of extrapituitary LHRH-R-I revealed major functional differences between LHRH-R-I in human pituitary and extrapituitary sites, in spite of the expression of identical receptor transcripts (19). In radioligand binding studies (26), pituitary LHRH-R-I displayed high affinity for agonists such as buserelin [dissociation constant (K_d) values being in the nM range], whereas some extrapituitary tissues showed lower affinity (K_d values being in the μM range). In other reports, it was speculated that LHRH-R-I activation inhibits proliferation (or causes cell death) and the identical effects of agonists and antagonists would apply. Such agonist/antagonist dichotomy can be defined mainly with pituitary LHRH-R-I, but not with receptors expressed in extrapituitary sites (26,47,48). The low proportion, conformation and the numbers/levels of LHRH-R-I in cell membranes could be important parameters for better understanding of the pathophysiological function of LHRH-R-I in extrapituitary sites (37). Although we failed to detect the full length LHRH-R-I in human BPH tissues, Kottler *et al* (18) demonstrated the presence of different LHRH-R-I transcripts in a variable percentage of human tumors and non-tumor tissues. The high incidence of specific, high affinity receptors for LHRH and the full-length LHRH-R-I mRNA in human prostate cancers and benign prostatic tissues was also described earlier (34). At present it is not clear whether alternative splicing of LHRH-R-I transcripts could play a physiological role. Alternatively spliced receptor isoforms have been implicated in altered receptor functions. In the human pituitary and extrapituitary tissues, including human BPH, the regulation of splicing might provide another control mechanism in the expression of the LHRH-R-I gene.

Our findings support the merit of further investigation of the expression of LHRH-R-I and its transcript forms in human malignancies as well as the application of LHRH analogues for receptor-based diagnosis and treatment of human benign prostatic hyperplasia.

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