Classic Bladder Exstrophy: Frequent 22q11.21 Duplications and Definition of a 414 kb Phenocritical Region

Markus Draaken*[1,2], Friederike Baudisch[1,3], Bernd Timmermann[4], Heiner Kuhl[4], Martin Kerick[4], Judith Proske[5], Lars Wittler[5], Tracie Pennimpede[5], Anne-Karoline Ebert[6], Wolfgang Rösch[7], Raimund Stein[8], Enrika Bartels[1], Catharina von Lowtzow[1,2], Thomas M. Boemers[9], Stefan Herms[1,2,10], John P. Gearhart[11], Yegappan Lakshmanan[12], Christina Clementsson Kockum[13], Gundela Holmdahl[14], Göran Läckgren[15], Agnetha Nordenskjöld[16,17], Simeon A. Boyadjiev[18], Bernhard G. Herrmann[5], Markus M. Nöthen[1,2], Michael Ludwig[3], and Heiko Reutter[1,19]

Background: Classic bladder exstrophy (CBE) is the most common form of the bladder exstrophy and epispadias complex. Previously, we and others have identified four patients with a duplication of 22q11.21 among a total of 96 unrelated CBE patients. **Methods:** Here, we investigated whether this chromosomal aberration was commonly associated with CBE/bladder exstrophy and epispadias complex in an extended case-control sample. Multiplex ligation-dependent probe amplification and microarray-based analysis were used to identify 22q11.21 duplications in 244 unrelated bladder exstrophy and epispadias complex patients (including 217 CBE patients) and 665 healthy controls. **Results:** New duplications of variable size were identified in four CBE patients and one control. Pooling of our previous and present data (eight duplications in 313 CBE patients) yielded a combined odds ratio of 31.86 (95% confidence interval, 4.24–1407.97). Array-based sequence capture and high-throughput targeted re-sequencing established that all breakpoints resided within the low-copy repeats 22A to 22D. Comparison of the eight duplications revealed a 414 kb phenocritical region harboring 12 validated RefSeq genes. Characterization of these 12 candidate genes through whole-mount in situ hybridization of mouse embryos at embryonic day 9.5 suggested that CRKL, THAP7, and LZTR1 are CBE candidate genes. **Conclusion:** Our data suggest that duplication of 22q11.21 increases CBE risk and implicate a phenocritical region in disease formation.

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Key words: chromosome 22q11.2; duplication; bladder exstrophy and epispadias complex (BEEC); classic bladder exstrophy (CBE); copy number variation

[1]Institute of Human Genetics, University of Bonn, Bonn, Germany
[2]Department of Genomics, Life & Brain Center, University of Bonn, Bonn, Germany
[3]Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany
[4]Next Generation Sequencing Group, Max Planck Institute for Molecular Genetics, Berlin, Germany
[5]Department of Developmental Genetics, Max Planck Institute for Molecular Genetics, Berlin, Germany
[6]Department of Urology and Pediatric Urology, University of Ulm, Ulm, Germany
[7]Department of Pediatric Urology, St. Hedwig Hospital Barmherzige Brμder, Regensburg, Germany
[8]Department of Urology, University of Mainz, Mainz, Germany
[9]Department of Pediatric Surgery and Pediatric Urology, Children's Hospital Cologne, Cologne, Germany
[10]Division of Medical Genetics and Department of Biomedicine, University of Basel, Basel, Switzerland
[11]Division of Urology, The James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore
[12]Children's Hospital of Michigan, Department of Pediatric Urology, Detroit, Michigan
[13]Department of Pediatric Surgery, University Hospital Lund, Lund, Sweden
[14]Department of Pediatric Surgery, Queen Silvia Children's Hospital, Gothenburg, Sweden
[15]Section of Urology, Uppsala Academic Children Hospital, Uppsala, Sweden
[16]Department of Women's and Children's Health, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden
[17]Department of Pediatric Surgery, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden
[18]Section of Genetics, Department of Pediatrics, University of California Davis, Sacramento, California
[19]Department of Neonatology, Children's Hospital, University of Bonn, Bonn, Germany

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*Correspondence to: Markus Draaken, Institute of Human Genetics, University of Bonn and Department of Genomics, Life & Brain Center, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany. E-mail: mdraaken@uni-bonn.de

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Introduction

The bladder exstrophy and epispadias complex (BEEC; MIM #600057) spectrum comprises epispadias (E), classic bladder exstrophy (CBE), and cloacal exstrophy (CE) (Carey, 2001; Gearhart, 2002). The most common form of BEEC is CBE, with a reported incidence of 1 in 37,000 live births and a male-to-female ratio of 2.4:1 (Ebert et al., 2009).

Although no unequivocal cause for BEEC has yet been identified, several lines of evidence implicate genetic factors: (i) approximately 30 multiplex families have been reported, (ii) CBE offspring display a 400-fold increase in recurrence risk (Ebert et al., 2009), (iii) concordance rates are higher in monozygotic compared with dizygotic twins (62% vs. 11%) (Ebert et al., 2009), and (iv) research has implicated *p63* in BEEC etiology (Wilkins et al., 2012; Qi et al., 2013). Reports of numeric and structural chromosomal aberrations in BEEC patients provide further support for a genetic background (Ebert et al., 2009). In two recent studies of a total of 102 CBE cases, we identified four patients with duplication of 22q11.21 (Lundin et al., 2010; Draaken et al., 2010). A further CBE patient with duplication of 22q11.21 was described in a subsequent, independent publication (Pierquin and Uwineza, 2012). These reports render 22q11.21 duplication the most frequent single cause of CBE identified to date. Of interest, two of our CBE patients with 22q11.21 duplication also displayed hearing impairment, and one of these individuals also presented with a mild neuropsychiatric disorder not further specified by the authors (Lundin et al., 2010). The CBE patient with 22q11.21 duplication reported subsequently (Pierquin and Uwineza, 2012) also presented with delayed psychomotor development and short stature. To our knowledge, no other reports to date have described an association between the BEEC and chromosomal region 22q11.21.

The susceptibility of chromosomal region 22q11.21 to misalignments is attributable to nonallelic homologous recombination, a process which is mediated by means of region-specific low-copy repeats (LCRs) (Edelmann et al., 1999; Shaikh et al., 2007). The most common rearrangements are deletions, with an incidence of 1 in every 4,000 live births (Shaffer and Lupski, 2000). Clinically, the 22q11.21 deletion presents as the velocardiofacial syndrome (MIM #192430), the DiGeorge syndrome (MIM #188400), the 22q11.2 proximal deletion syndrome (MIM #611867) (Shprintzen et al., 1978; Kelley et al., 1982), or distal microdeletions (Ben-Shachar et al., 2008; Verhoeven et al., 2011). The reciprocal duplication leads to the 22q11.2 duplication syndrome (MIM #608363) (Portnoï, 2009). The clinical presentation of patients with a 22q11.2 duplication is extremely variable and includes features of the various 22q11.2 deletion syndromes, such as cardiovascular malformations, oro-facial clefts, non-BEEC urogenital malformations, anorectal abnormalities, endocrine disorders, and cognitive and mental impairment (ranging from intellectual disability to mild learning difficulties). Carriers of the same deletion or duplication may present with all of the associated phenotypic features, or may appear completely normal, and broad inter- and intrafamilial variability has been reported (Shprintzen et al., 1978; Kelley et al., 1982; Wu et al., 2002; Portnoï, 2009).

The aims of the present study were to: (i) estimate the frequency of the 22q11.21 duplication in CBE patients compared with healthy controls by screening the largest CBE sample to date ($n = 217$); (ii) delineate the spectrum of associated anomalies in patients with CBE and a 22q11.21 duplication through the addition of new, clinically well characterized cases; and (iii) characterize candidate genes from a phenocritical region in terms of their potential relevance to BEEC development. For the latter investigations, whole-mount in situ hybridization (WISH) was performed in mouse embryos at embryonic day E9.5, the time-point corresponding to the postulated timeframe for BEEC organogenesis in humans.

To investigate associations between the 22q11.21 duplication and other BEEC phenotypes, screening was also performed in patients with E ($n = 9$) and CE ($n = 18$). Given the small sample sizes, these analyses were performed on the understanding that meaningful conclusions would only be possible if some E or CE cases were identified as carriers of a 22q11.21 duplication.

Subjects and Methods

PATIENTS

The sample used to investigate aims 1 and 2 of the study included a total of 217 previously unreported CBE patients (77 females, 140 males). In addition, 9 E patients (4 females, 5 males) and 18 CE patients were investigated (7 females, 11 males). The next generation sequencing analysis included four CBE patients with a 22q11.21 duplication from previous studies (Lundin et al., 2010; Draaken et al., 2010). Written informed consent was obtained from each patient or legal guardian before inclusion. The study was approved by the respective local ethics committees. A total of 665 novel unrelated population based controls from the Heinz Nixdorf Recall Study were screened using Illumina's HumanOmni1-v1-Quad BeadChip (San Diego, CA). A further 554 previously reported controls were also included (Lundin et al., 2010; Draaken et al., 2010). Of these, 383 controls of the Heinz Nixdorf Recall Studies had been screened for the presence of a 22q11.21 duplication using Illumina's HumanHap550-v3 BeadChip (San Diego, CA) (Draaken et al., 2010). Screening of the remaining 171 healthy controls was performed using multiplex ligation-dependent probe amplification, as described elsewhere (Lundin et al., 2010) (Table 1).

COPY NUMBER VARIATION ANALYSIS

Multiplex ligation-dependent probe amplification was performed in accordance with the manufacturer's recommendations, as described previously (Draaken et al., 2010).

TABLE 1. *Patient and Control Cohort Studied for Chromosome 22q11.21 Aberrations*

	CBE patients		E and CE patients		Controls	
Study	**n.a.**	**Dup22**	**n.a.**	**Dup22**	**n.a.**	**Dup22**
Draaken et al., 2010	58	2	6	0	383	0
Lundin et al., 2010	34	2	0	0	170	1
This study	213	4	27	0	665	0
Total	305	8	33	0	1218	1

n.a., no aberration detected; Dup22, duplication at 22q11.21.

Molecular karyotyping was carried out to confirm multiplex ligation-dependent probe amplification findings, narrow down the breakpoints, and exclude the presence of additional copy number variations. This procedure was performed using the Human660W-Quad-v1 (657,366 markers; median marker spacing 2.3 kilobase [kb]), and HumanOmni1-v1-Quad (1,140,419 markers; median marker spacing 1.2 kb) BeadChips (Illumina, San Diego, CA). For copy number variation analysis, the marker fluorescence data of each individual were analyzed using QuantiSNP (v2.2, www.well.ox.ac.uk/QuantiSNP/) and checked visually in GenomeStudio (v2011.1, Illumina, San Diego, CA). For paternity testing, the PowerPlex® 16 System (Promega, Madison, WI) was applied in accordance with the manufacturer's specifications.

NEXT GENERATION SEQUENCING

For next generation sequencing, samples were processed according to the GS-FLX-Titanium-Rapid-Library-Preparation-Method-Manual (Roche; October 2009 [Rev. Jan 2010]). Following nebulization and adaptor ligation, the sequencing library fragments were size-selected using an SPRI-T robot (Beckman-Coulter). Sequencing libraries were hybridized with NimbleGen Sequence Capture Arrays designed to capture at chromosome 22 the genomic region from 18,369,954 to 22,135,032 (hg19 coordinates) in accordance with the manufacturer's protocol. Following hybridization, size distribution was re-checked. The average length was ~507 base pair (bp). Sequencing was performed according to the GS-FLX-Titanium-Sequencing-Method-Manual (Roche; October 2009 [Rev. Jan 2010]), and each sample was sequenced in half of a picotiter plate. Derived sequences were mapped against the human genome (hg18) using Roche/454 Newbler version 2.6 (default parameters). Here, split mappings of long reads enabled detection of both SNPs and larger variants. SNP calls covered by more than nine uniquely mapped reads were extracted and converted to hg19 coordinates (UCSC genome browser liftover tool). To visualize coverage over the enriched region, Newbler BAM files were converted to bedgraph format (BEDtools).

WISH

Candidate genes within the phenocritical region were evaluated for murine orthologs using WISH and mouse embryos at E9.5. This time-point corresponds to human gestational week 4. The E9.5 time-point and gestational week 4 represent the critical timeframe for the initial stages of external genital formation in mouse and human, respectively (Pennimpede et al., 2012). In addition, expression data sets available at the Mouse Genome Informatics Databases of the Jackson Laboratories (MGI; http://www.informatics.jax.org) were used to evaluate expression in mouse embryos at later gestational stages.

STATISTICAL ANALYSIS

Fisher's exact test was used to calculate odds ratios and 95% confidence intervals (CIs).

Results

SCREENING FOR 22Q11.21 DUPLICATIONS

MPLA revealed genomic gains within the 22q11.21 region in four of the 244 previously unreported and unrelated male BEEC patients. All four patients are presenting an isolated CBE. Screening of the novel 665 controls revealed no duplication in this region. Hence, the overall incidence of 22q11.21 duplications in our total BEEC cohort of 346 patients (Table 1), including the previously reported BEEC patients (Lundin et al., 2010; Draaken et al., 2010), compared with the total number of 1219 controls was 2.3% (8 of 346), or 2.6% if only CBE patients (8 of 313) were considered (odds ratio, 31.86; 95% CI, 4.24–1407.97; $p = 2.09 \times 10^{-5}$).

CHARACTERIZATION OF THE DUPLICATIONS

Duplication size ranged from ~0.4 Mb to ~2.5 Mb. The duplications involved LCRs A-D (patients 3–6 in Fig. 1 and Table 2), comprising 12 to 66 genes (RefSeq 2013-12-04). All duplications were verified using microarrays. No other copy number variation was detected at other human genome locations in these four duplication carriers. All four BEEC patients with a newly identified duplication of 22q11.21 had isolated CBE with no other physical or mental abnormality. Parental testing revealed de novo occurrences in patients 3, 5, and 6. In patient 4, the duplication had been inherited from an unaffected father (paternity testing revealed no incompatibilities, data not shown).

BREAKPOINT ANALYSIS

To identify breakpoints in the four newly and the four previously identified duplications, we used array-based sequence capture of a 3.7 Mb region overlapping the most proximal and the most distal marker of the eight duplications, followed by high-throughput sequencing. Coverage (minimum 10×) varied between 58.4% and 73.8% (mean: 69.4%). Here, read depth analyses defined the breakpoints down to the respective LCRs (Fig. 1; Table 2).

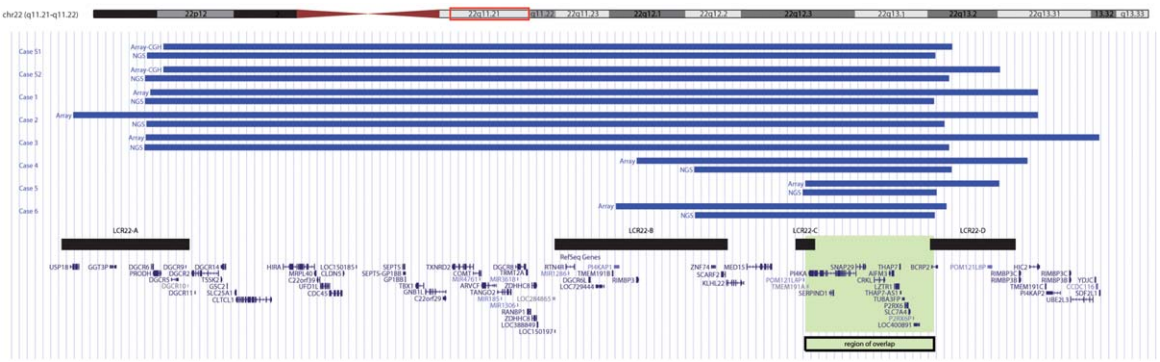

FIGURE 1. *Results of molecular karyotyping.* **(Top)** *Chromosome 22q11.21 duplications observed in the present study (Patients 3–6) and those described in two earlier reports (Patient S1 and Patient S2 [Lundin et al., 2010], Patient 1 and Patient 2 [Draaken et al., 2010]); the figure is compiled from data derived from the results of microarray analysis and next generation sequencing. The maximum region of overlap (chromosome 22:21,050,613-21,464,371; hg19) is indicated in green.* **(Middle)** *Low-copy repeats A–D are represented by black rectangles (Edelmann et al., 1999; Shaikh et al., 2007).* **(Bottom)** *RefSeq genes (according to hg19) located in the duplicated region.*

EXPRESSION PATTERN OF THE GENES LOCATED IN THE 414 KB PHENOCRITICAL REGION

Comparison of all eight 22q11.21 duplications enabled definition of a 414 kb phenocritical region (chr22:21, 050,613-21,464,371; hg 19; Fig. 1). This region harbored 12 RefSeq genes: phosphatidylinositol 4-kinase, catalytic, alpha (*PI4KA*); serpin peptidase inhibitor, clade D (heparin cofactor) member 1 (*SERPIND1*); synaptosomal-associated protein, 29kDa (*SNAP29*); v-crk sarcoma virus CT10 oncogene homolog (avian)-like (*CRKL*); apoptosis-inducing factor, mitochondrion-associated, 3 (*AIFM3*); leucine-zipper-like transcription regulator 1 (*LZTR1*); *THAP domain containing 7* (*THAP7*); THAP7 antisense RNA 1 (*THAP7-AS1*); tubulin, alpha 3f, pseudogene (*TUBA3FP*); purinergic receptor P2X, ligand-gated ion channel, 6 (*P2RX6*); solute carrier family 7 (orphan transporter) member 4 (*SLC7A4*); and breakpoint cluster region pseudogene 2 (*BCRP2*). The expression pattern of nine of these 12 CBE candidate genes was evaluated, with particular emphasis on the region of the ventrolateral trunk and the genital tubercle. The human *BCRP2*, *TUBA3FP*, and *THAP7-AS1* genes in this region have no mouse orthologs, and thus were not included in WISH analyses. No expression at E9.5 was found for *SERPIND1*, *AIFM3*, *THAP7*, *P2RX6*, or *SLC7A4*. In contrast, *PI4KA*, *SNAP29*, *CRKL*, and *LZTR1* showed ubiquitous expression in the embryo at this time-point (Table 3).

TABLE 2. *Characterization of the 22q11.21 Duplication Observed in 9 CBE Patients*

Reference	Patient/sex	Ethnicity	Size (Mb) of dup22q11.21[a]		LCR22 Regions	De novo/ Unaffected parental carrier	Additional clinical features
			Array	NGS data			
6	Patient S1 female	Northern European	2.54–3.2	2.48–2.54	A–D	De novo	Hearing impairment, scoliosis
6	Patient S2 female	Northern European	2.7–3.3	2.52–2.59	A–D	Mother	Hearing impairment, mild neuropsychiatric disorder
7	Patient 1 male	Central European	2.51–2.86	2.52–2.54	A–D	Mother	-
7	Patient 2 female	Southern European	2.53–3.11	2.55–2.57	A–D	De novo	-
8	Patient male	Asian Indian	~2.4	Not known	A–D	Not specified in publication	Delayed psychomotor development, short stature
Present study	Patient 3 male	Central European	2.65–3.07	2.52–2.59	A–D	De novo	-
Present study	Patient 4 male	Southern European	0.67–1.26	0.75–0.83	B–D	Father	-
Present study	Patient 5 male	European origin	0.35–0.62	0.40–0.43	C–D	De novo	-
Present study	Patient 6 male	European origin	0.65–1.06	0.69–0.77	B–D	De novo	-

[a]Size of duplication as estimated from QuantiSNP (array data) or next generation sequencing.

TABLE 3. *Expression by WISH and Transcriptome Analyses in Mice for Candidate Genes in the Smallest Region of Overlap on Chromosome 22q11.21*

Gene	Mouse ortholog	Transcript present E9.5 (cDNA)	Expression E9.5 (WISH)	Expression data from other resources
PI4KA	Pi4ka	yes	ubiquitous	E14.5: ubiquitous (Visel et al., 2004)
SERPIND1	Serpind1	yes	not expressed	E14.5: ubiquitous, stronger signal in liver and epidermis (Visel et al., 2004)
SNAP29	Snap29	yes	ubiquitous	E14.5: cranial and spinal ganglia, central nervous system, epidermis (Visel et al., 2004)
CRKL	Crkl	yes	ubiquitous	E14.5: ubiquitous (McMahon et al., 2008); kidney (Magdaleno et al., 2006)
AIFM3	Aifm3	not amplified	not expressed	no expression data available
LZTR1	Lztr1	yes	ubiquitous	E14.5: ubiquitous, cranial ganglia, follicles of vibrissae (Visel et al., 2004)
THAP7	Thap7	yes	not expressed	E10.5: ubiquitous, stronger signal in limb buds pharyngeal arches, forebrain (Gray et al., 2004); unspecific signal in genitourinary tract (Magdaleno et al., 2006)
THAP7-AS1	not annotated	not done	not done	no expression data available
TUBA3FP	not annotated	not done	not done	no expression data available
P2RX6	P2rx6	not amplified	not expressed	no expression data available
SLC7A4	Slc7a4	yes	not expressed	E14.5: low expression in central nervous system (Visel et al., 2004)
BCRP2	not annotated	not done	not done	no expression data available

Discussion

The present study confirmed an association between CBE and duplication of 22q11.21 in an extended sample of 313 CBE patients and defined a 414 kb phenocritical region comprising 12 RefSeq genes. The four CBE patients with a newly identified 22q11.21 duplication showed no additional clinical features. Therefore, our two previously reported patients—one of whom had hearing impairment and a mild neuropsychiatric disorder not further specified by the authors and the other hearing impairment only (Lundin et al., 2010)—together with the subsequently described patient with delayed psychomotor development and short stature (Pierquin and Uwineza, 2012), remain the only reported CBE patients with 22q11.21 duplication and additional clinical features (Table 2). Three duplications were transmitted from an unaffected parent, which reflects the incomplete penetrance observed in carriers of 22q11.21 duplications (Portnoï, 2009). Combined analyses of patients from the present study and the two previously reported studies (Table 2) resulted in an odds ratio of 31.86 (95% CI, 4.24–1407.97) for CBE patients. However, the very large CI suggests a less precise estimate reflecting the small sample size. The fact that no 22q11.21 duplications were detected among patients with E or CE does not rule out an association with these phenotypes, because the small sample sizes resulted in limited power.

The mechanisms underlying the pleiotropic spectrum of anomalies caused by duplications at 22q11.21 remain elusive (Firth, 2013). Because breakpoints might lead to interruptions of a specific gene and breakpoints may vary between patients, we performed a detailed breakpoint analysis. However, determination of junction sequences revealed that all breakpoints resided within the noncoding regions of LCR22A - LCR22D (Fig. 1) (Edelmann et al., 1999; Shaikh et al., 2007).

WISH analyses in the developing mouse revealed ubiquitous expression of *PI4KA*, *SNAP29*, *CRKL*, and *LZTR1* at E9.5. Of interest, the LZTR1 protein belongs to the kelch-repeat superfamily, a distinct and evolutionarily widespread family of β-propeller domain-containing proteins which have been highly conserved throughout evolution. Furthermore, Morcel et al. (2011) reported a girl with the BEEC related phenotype of utero-vaginal aplasia (Mayer-Rokitansky-Kuster-Hauser syndrome) and a small deletion of chromosomal region 22q11.21, for which *LZTR1* was the most distal deletion flanking gene. This finding may implicate *LZTR1* in urogenital malformation (Morcel et al., 2011). Data from GUDMAP (GenitoUrinary Development Molecular Anatomy Project; http://www.gudmap.org/) reported expression of *CRKL* in mouse kidney at E14.5 and expression of *THAP7* in the genitourinary tract at E10.5, and thus both of these genes also seem likely candidates for CBE and warrant follow-up. The present analyses could not exclude the involvement in CBE of the three genes that lacked mouse orthologs, or regulatory elements within the phenocritical region.

In conclusion, our data suggest that 22q11.21 duplications increase the CBE risk with incomplete penetrance. Our WISH analyses and previous mice expression data suggest that *CRKL*, *THAP7*, and *LZTR1* are possible CBE

candidate genes. Further studies are warranted to elucidate the involvement of the phenocritical region in the formation of CBE.

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