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Complex X-Chromosomal Rearrangements in Two Women with Ovarian Dysfunction: Implications of Chromothripsis/Chromoanasynthesis-Dependent and -Independent Origins of Complex Genomic Alterations

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Keywords

Chromothripsis · Genomic rearrangement · Isochromosome · Turner syndrome · X inactivation

Abstract

Our current understanding of the phenotypic consequences and the molecular basis of germline complex chromosomal rearrangements remains fragmentary. Here, we report the clinical and molecular characteristics of 2 women with germline complex X-chromosomal rearrangements. Patient 1 presented with nonsyndromic ovarian dysfunction and hyperthyroidism; patient 2 exhibited various Turner syndrome-associated symptoms including ovarian dysfunction, short stature, and autoimmune hypothyroidism. The genomic abnormalities of the patients were characterized by array-based comparative genomic hybridization, high-resolution karyotyping, microsatellite genotyping, X-inactivation analysis, and bisulfite sequencing. Patient 1 carried a rearrangement of unknown parental origin with a 46,X,der(X)(pter→p22.1::p11.23→q24::q21.3→q24::p11.4→pter) karyotype, indicative of a catastrophic chromosomal reconstruction due to chromothripsis/chromoanasynthesis. Patient 2 had a paternally derived isochromosome with a 46,X,der(X)(pter→p22.31::q22.1→q10::q10→q22.1::p22.31→pter) karyotype, which likely resulted from 2 independent, sequential events. Both patients showed completely skewed X inactivation. CpG sites at Xp22.3 were hypermethylated in patient 2. The results indicate that germline complex X-chromosomal rearrangements underlie nonsyndromic ovarian dysfunction and Turner syndrome. Disease-causative mechanisms of these rearrangements likely include aberrant DNA methylation, in addition to X-chromosomal mispairing and haplo-

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insufficiency of genes escaping X inactivation. Notably, our data imply that germline complex X-chromosomal rearrangements are created through both chromothripsis/chromoanasynthesis-dependent and -independent processes.

Complex chromosomal rearrangements are common in cancer genomes and can also appear in the germline [Liu et al., 2011; Kloosterman and Cuppen, 2013]. To date, germline complex rearrangements have been identified in a small number of individuals [Liu et al., 2011; Ochalski et al., 2011; Auger et al., 2013; Kloosterman and Cuppen, 2013; Plaisancié et al., 2014]. Of these, complex autosomal rearrangements were often associated with congenital malformations and mental retardation, which probably reflect dysfunction or dysregulation of multiple genes on the affected chromosome [Liu et al., 2011; Kloosterman and Cuppen, 2013; Plaisancié et al., 2014]. In contrast, complex X-chromosomal rearrangements were detected primarily in women with nonsyndromic ovarian dysfunction and were occasionally associated with other clinical features such as short stature, muscular hypotonia, and an unmasked X-linked recessive disorder [Ochalski et al., 2011; Auger et al., 2013]. The lack of severe developmental defects in women with complex X-chromosomal rearrangements is consistent with prior observations that structurally abnormal X chromosomes, except for X;autosome translocations, frequently undergo selective X inactivation [Heard et al., 1997]. The clinical features of these women, such as ovarian dysfunction and short stature, are ascribable to X-chromosomal mispairing and haploinsufficiency of genes that escape X inactivation [Zhong and Layman, 2012]. Mutations in *BMP15* at Xp11.22, *POF1B* at Xq21.1, *DIAPH2* at Xq21.33, or *PGRMC1* at Xq24 have been shown to lead to ovarian dysfunction, while mutations in *SHOX* at Xp22.33 impair skeletal growth [Bione et al., 1998; Bione and Toniolo, 2000; Mansouri et al., 2008; Zhong and Layman, 2012]. However, considering the limited number of reported cases, further studies are necessary to clarify the phenotypic characteristics of germline complex X-chromosomal rearrangements. Furthermore, it remains uncertain whether such rearrangements perturb DNA methylation of the affected X chromosomes.

Recent studies revealed that complex genomic rearrangements are caused by catastrophic cellular events referred to as chromothripsis and chromoanasynthesis [Liu et al., 2011; Pellestor, 2014; Leibowitz et al., 2015; Zhang et al., 2015]. Chromothripsis is characterized by massive DNA breaks in a single or a few chromosomes followed by random reassembly of the DNA fragments [Liu et al., 2011; Pellestor, 2014; Zhang et al., 2015]. Chromothripsis is predicted to arise from micronucleus-mediated DNA breakage of mis-segregated chromosomes, although several other mechanisms such as telomere erosion, p53 inactivation, and abortive apoptosis have also been implicated [Liu et al., 2011; Pellestor, 2014; Zhang et al., 2015]. Chromothripsis typically results in copy-number-neutral translocations/inversions or rearrangements with copy number loss; however, in some cases, genomic rearrangements with copy number gain have also been linked to chromothripsis [Liu et al., 2011; Pellestor, 2014]. Copy number gains in these cases are ascribed to replication-based errors during chromosomal reassembly [Liu et al., 2011]. Chromoanasynthesis is proposed to arise from serial template switching during DNA replication [Leibowitz et al., 2015]. Chromoanasynthesis has been reported as a cause of complex rearrangements with duplications and triplications [Leibowitz et al., 2015]. To date, the clinical significance of germline chromothripsis/chromoanasynthesis has not been fully determined. In particular, it remains unknown whether these catastrophic events account for all cases of complex rearrangements in the germline. Here, we report the clinical and molecular characteristics of 2 women with complex X-chromosomal rearrangements.

Patients and Methods

Patients

Patients 1 and 2 were unrelated Japanese women. Patient 1 was hitherto unreported, while patient 2 was previously reported as a female with Turner syndrome [Uehara et al., 2001]. Both patients underwent G-banding analysis in endocrine clinics and were found to have X-chromosomal rearrangements. Thus, they were referred to our institute for further investigation.

Molecular Analysis

Copy number alterations in the genomes were analyzed by comparative genomic hybridization using catalog human arrays (2x400K or 4x180K formats; Agilent Technologies, Palo Alto, CA, USA). We referred to the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) to exclude benign copy number polymorphisms. Then, we genotyped 15 microsatellite loci on the X chromosome. Each locus was PCR-amplified using fluorescently labeled forward primers and unlabeled reverse primers. Primer sequences are available from the authors upon request. We also examined the X inactivation status by performing methylation analysis of CpG sites and microsatellite assays of a polymorphic CAG repeat tract in the androgen receptor (*AR*) gene. The methods were described previously [Muroya et al., 1999]. Furthermore, to clarify whether the genomic rearrangements in the patients affect the

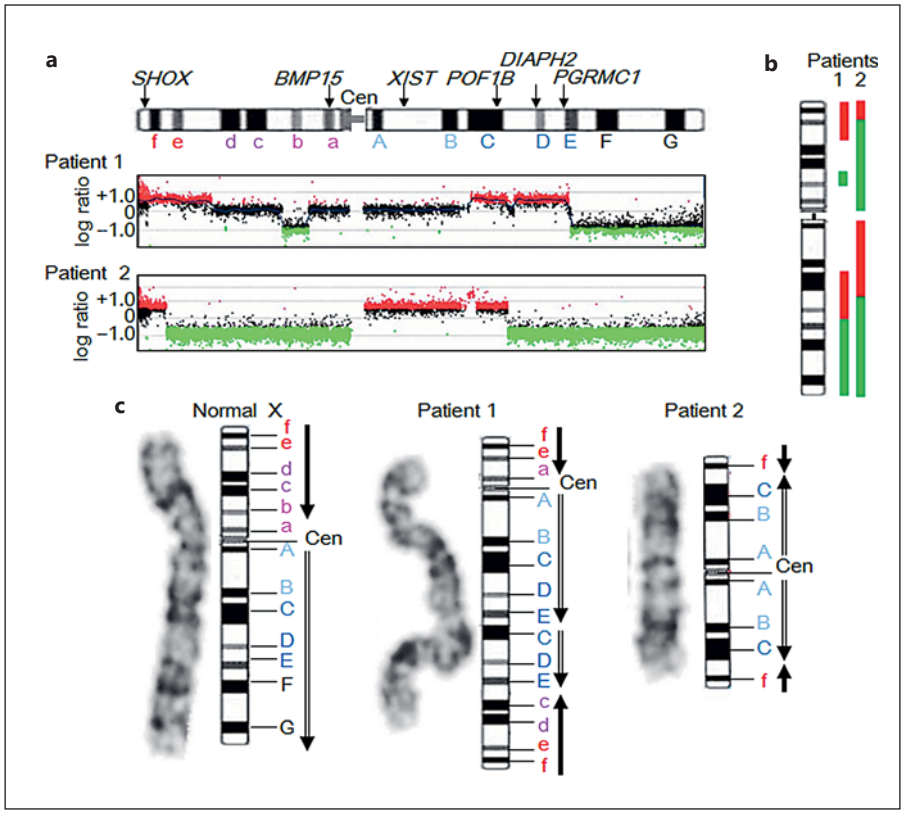

Fig. 1. a Array-based comparative genomic hybridization of the patients' X chromosomes. The black, red, and green dots denote normal, increased (log ratio higher than +0.4), and decreased (log ratio lower than –0.8) copy numbers, respectively. The upper panel shows the structure of the X chromosome and the positions of *SHOX*, *BMP15*, *XIST*, *POF1B*, *DIAPH2*, and *PGRMC1*. Cen, centromere. **b** Summary of copy number alterations in patients 1 and 2. The red and green lines depict duplicated and deleted regions, respectively. **c** High-resolution banding of a normal and the rearranged X chromosomes. The black and double-line arrows indicate the orientation of the X chromosome segments (from pter to the centromere and from the centromere to qter, respectively).

DNA methylation of X-chromosomal genes, we performed bisulfite sequencing for CpG sites in the upstream region of *SHOX*. In this experiment, genomic DNA samples were treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). A DNA fragment (chrX:580,597–580,771, hg19, build 37) containing 12 *SHOX*-flanking CpG sites was PCR-amplified using a primer set that hybridizes with both the methylated and unmethylated clones. The PCR products were subcloned with the TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, USA) and subjected to direct sequencing.

Results

Clinical Manifestations of Patients 1 and 2

Patient 1 was born to phenotypically normal nonconsanguineous parents. This patient showed normal growth during childhood. At 12 years of age, she developed goiter. She was diagnosed with hyperthyroidism and was treated with propylthiouracil for 13 years. This patient exhibited age-appropriate sexual development and experienced menarche at 12 years of age (mean menarcheal age in the Japanese population: 12.3 years). However, her menstrual cycles were irregular and ceased at 15 years of age. Blood examinations at 26 years of age revealed markedly increased gonadotropin levels. She received estrogen and progesterone supplementation and had periodic withdrawal bleeding. She was otherwise healthy and had no Turner stigmata. Her mental development was normal. Her adult height was within the normal range (151.0 cm, –1.3 SD).

Patient 2 was previously reported as a female with Turner syndrome [Uehara et al., 2001]. At 16 years of age, she presented with short neck, shield chest, and cubitus valgus. She also exhibited hypertension, diabetes mellitus, and autoimmune hypothyroidism. In addition, she showed severe short stature (138 cm, –3.8 SD) despite being treated with growth hormone from 8 years of age. She lacked spontaneous pubertal development and was diagnosed with hypogonadism. Her mental development was normal.

Characterization of Genomic Rearrangements

Patient 1 had a 46,X,der(X)(pter→p22.1::p11.23→q24::q21.3→q24::p11.4→pter) karyotype (Fig. 1). The rearranged X chromosome involved at least 5 breakpoints and showed copy number gain of ~20-Mb and ~27-Mb regions at Xp and Xq, respectively, and copy number loss of ~7-Mb and ~36-Mb regions at Xp and Xq, respective-

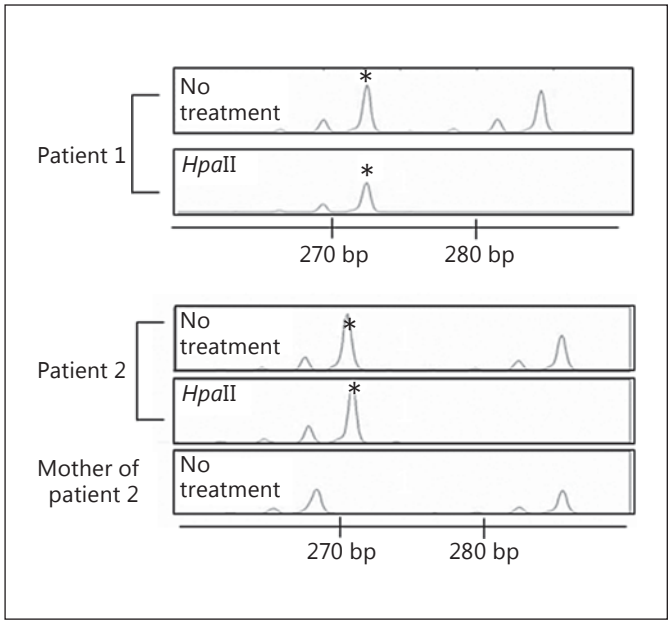

Fig. 2. X-inactivation analysis of *AR*. Microsatellite analysis was performed for polymorphic dinucleotide repeats before and after digestion with the methylation-sensitive enzyme *Hpa*II. In patient 1, the 274-bp peak (indicated by an asterisk) represents the PCR products amplified from the inactive X chromosome, while the 283-bp peak indicates the products amplified from the active X chromosome. In patient 2, the 271-bp peak (asterisk) represents the PCR products amplified from the inactive rearranged X chromosome, while the 286-bp peak depicts the products amplified from the maternally transmitted normal X chromosome. These data suggest that the rearranged X chromosome of patient 2 was of paternal origin.

Table 1. Representative results of the microsatellite analysis in patient 2 and her mother

Locus	Chromosomal position[a]	Copy number in the genome of patient 2	PCR products, bp	
			patient 2	mother
SHOX (CA)	Xp22.33	3	142/150	132/142
DXYS233	Xp22.33	3	277	277
DXYS85	Xp22.33	3	200/204	204
DXS1449	Xp22.33	3	116	116
DXS85	Xp22.2	3	174/232	174/232
DXS8025	Xp11.4	1	186	180/186
DXS1069	Xp11.4	1	256	256
DXS1068	Xp11.4	1	254	250/254
ALAS2	Xp11.21	1	155	155/157
AR	Xq12	3	271/286	268/286
DXS8020	Xq22.1	3	194/196	194/196
HPRT1	Xq26.2–26.3	1	290	282/290
DXS8377	Xq28	1	233	229/233
DXS7423	Xq28	1	187	183/187
DXS15	Xq28	1	148	146/148

[a] Based on Ensembl Genome Browser (http://www.ensembl.org).

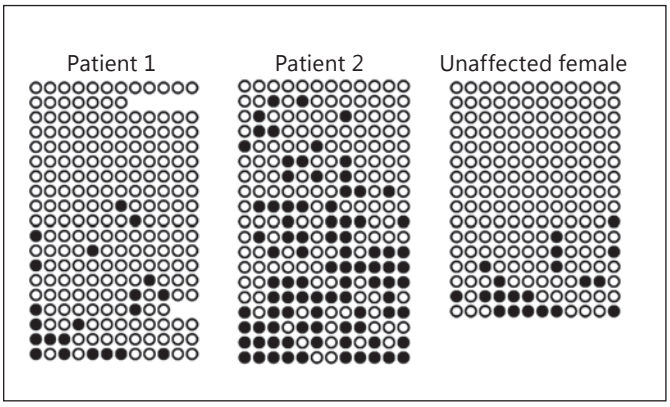

Fig. 3. Methylation analysis of *SHOX*-flanking CpG sites. Each horizontal line indicates the results of 1 clone. Filled and open circles indicate methylated and unmethylated cytosines in the CpG dinucleotides, respectively.

ly. This rearrangement caused overdosage of *SHOX*, *POF1B*, *DIAPH2*, and *PGRMC1* but did not affect the copy number of *BMP15* or *XIST* (X inactive specific transcript). X-inactivation analysis confirmed completely skewed inactivation (Fig. 2). *SHOX*-flanking CpG sites were barely methylated both in patient 1 and in an unaffected control individual (Fig. 3).

Patient 2 had a 46,X,der(X)(pter→p22.31::q22.1→q10::q10→q22.1::p22.31→pter) karyotype (Fig. 1). The rearranged X chromosome comprised at least 3 breakpoints and showed copy number gain of an ~8-Mb region at Xp and an ~40-Mb region at Xq and copy number loss of an ~53-Mb region at Xp and an ~54-Mb region at Xq. *SHOX*, *XIST*, and *POF1B* were duplicated, while *BMP15*, *DIAPH2*, and *PGRMC1* were deleted. There were no copy-number-neutral regions on this X chromosome. Microsatellite analysis suggested that this chromosome consisted of 2 identical arms ("isochromosome") of paternal origin (Table 1). The rearranged X chromosome was selectively inactivated (Fig. 2). *SHOX*-flanking CpG islands in patient 2 were hypermethylated (Fig. 3).

Discussion

We characterized complex germline X-chromosomal rearrangements in 2 patients. The clinical manifestations of the patients are consistent with the genomic structure. First, both patients manifested ovarian dysfunction. This feature is attributable to X-chromosomal mispairing, as suggested in cases of Turner syndrome due to X mono-

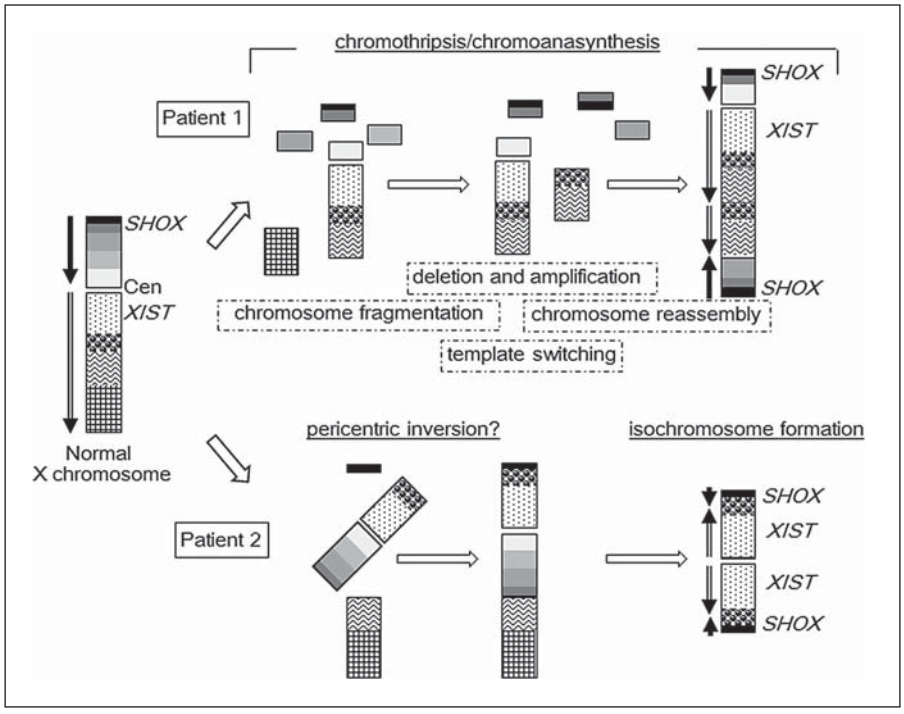

Fig. 4. Predicted mechanisms of the chromosomal rearrangements. The black and double-line arrows indicate the orientation of X chromosome segments (from pter to the centromere and from the centromere to qter, respectively). The rearranged X in patient 1 is consistent with a catastrophic reconstruction due to chromothripsis/chromoanasynthesis, while that in patient 2 likely results from 2 independent sequential events. It remains to be clarified whether the father of patient 2 carries a pericentric inversion.

somy [Ogata and Matsuo, 1995]. Furthermore, patient 2 lacked *BMP15*, *DIAPH2*, and *PGRMC1*, which have been implicated in ovarian function [Bione et al., 1998; Bione and Toniolo, 2000; Mansouri et al., 2008]. Copy number changes of other genes might also have contributed to the ovarian dysfunction in patients 1 and 2, because multiple X-chromosomal loci have been linked to this phenotype [Zhong and Layman, 2012]. Second, Turner stigmata such as short neck, shield chest, and cubitus valgus were observed in patient 2 but not in patient 1. These results support the previously proposed notion that a lymphogenic gene responsible for Turner stigmata resides at Xp11.2 [Ogata et al., 2001a], a genomic region deleted in patient 2 and preserved in patient 1. Third, both patients manifested thyroid disorders. Notably, isochromosome Xq is known to be associated with a high risk of autoimmune thyroid disorders [Elsheikh et al., 2001]. Indeed, the hypothyroidism of patient 2 may have resulted from copy number gain of *GPR174* at Xq21.1, because increased expression of *GPR174* has been linked to the risk of an autoimmune thyroid disorder [Chu et al., 2013]. However, the copy number of *GPR174* remained intact in patient 1. Thus, the genomic interval at Xq21.32q22.1> Xq21.32-q22.1, duplicated in both patients, may contain a hitherto uncharacterized gene associated with autoimmune thyroid disorders. Lastly, patient 1 had a normal stature, and patient 2 showed severe short stature, although both patients carried 3 copies of *SHOX*. This is inconsistent with previous findings that trisomy of the Xp22.3 region encompassing *SHOX* leads to tall stature [Ogata et al., 2001b]. In patients 1 and 2, positive effects of *SHOX* overdosage on skeletal growth may be balanced by negative effects of X-chromosomal mispairing and copy number alterations of minor growth genes on the X chromosome. Furthermore, short stature in patient 2 may be associated with *SHOX* dysregulation, because *SHOX*-flanking CpG islands were hypermethylated in this individual. These sites were barely methylated in the control individual, which is in agreement with the fact that *SHOX* escapes X inactivation [Rao et al., 1997]. It has been shown that in patients with X;autosome translocations, aberrant DNA methylation can spread to regions larger than 1 Mb of the autosomal segments [Cotton et al., 2014]. Hypermethylation of the *SHOX*-flanking CpG sites in patient 2 may reflect decreased physical distance between *SHOX* and *XIST* and/or copy number gain of *XIST*.

The genomic rearrangements in patients 1 and 2 appear to have been formed through different mechanisms (Fig. 4). The rearrangement in patient 1 is consistent with catastrophic reconstruction due to chromothripsis/chromoanasynthesis [Liu et al., 2011; Leibowitz et al., 2015].

This case provides further evidence that X-chromosomal chromothripsis/chromoanasynthesis accounts for a small portion of cases with nonsyndromic ovarian dysfunction. In contrast, the rearrangement in patient 2 is inconsistent with the "all-at-once" nature of chromothripsis/chromoanasynthesis [Liu et al., 2011; Hatch and Hetzer, 2015]. The rearranged chromosome of this patient had 2 identical arms consisting of Xp and Xq material, indicating that this chromosome arose by 2 independent sequential events, namely, a fusion between the Xp22.31 and Xq22.1 segments followed by isochromosome formation. Notably, the rearrangement occurred in the paternally inherited X chromosome. Thus, although the Xp22.31;Xq22.1 translocation is the simplest explanation of this rearrangement, it is implausible in this case, because X;X translocation rarely occurs during male meiosis. The results of patient 2 can be explained by assuming that the phenotypically normal father carried a pericentric inversion, inv(X)(p22.31q22.1), which was subjected to meiotic or postzygotic isochromosome formation (Fig. 4). However, since a paternal DNA sample was not available for genetic testing, we cannot exclude the possibility that this rearrangement was formed via other rare processes.

In conclusion, the results indicate that complex X-chromosomal rearrangements in the germline lead to ovarian dysfunction with and without other Turner syndrome-associated features. Clinical outcomes of such rearrangements likely reflect X-chromosomal mispairing, haploinsufficiency of genes escaping X inactivation, and/or perturbed DNA methylation. Most importantly, our findings imply that germline complex X-chromosomal rearrangements are created through both chromothripsis/chromoanasynthesis-dependent and -independent processes.

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Statement of Ethics

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent.

Disclosure Statement

The authors have no competing interests to declare.

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