Hidden mosaicism in patients with Klinefelter’s syndrome: implications for genetic reproductive counselling†

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BACKGROUND: Most individuals with Klinefelter’s syndrome (KS) are azoospermic but residual foci of spermatogenesis have been observed in some patients. However, no consistent predictive factors for testicular sperm extraction success have been established and mosaicism could be a factor to investigate. In this study, we have assessed the degree of mosaicism in somatic and germinal tissues in KS, the meiotic competence of 47,XXY germ cells and the aneuploidy rate of post-reductional cells.

METHODS: Five patients with KS previously diagnosed as pure 47,XXY have been studied. Samples from four donors were processed as controls. The chromosome constitution of lymphocytes, buccal mucosa and testicular tissue was assessed by interphase fluorescence in situ hybridization for chromosomes X, Y and 18. In meiotic figures, sex chromosome number and pairing was confirmed.

RESULTS: 46,XY cell lines were observed in all patients and tissues analysed. The degree of mosaicism (mean ± SD) differed among tissues (lowest in lymphocytes: 4.8 ± 2.5%; highest in Sertoli cells: 42.3 ± 11.1%). Meiotic figures were found in three cases (KS1, KS2 and KS5), all of them showed an XY complement. Hyperhaploid post-reductional cells were found in all patients (range: 3.3–36.4%) and increased rates versus controls (P < 0.05) were observed.

CONCLUSIONS: Diagnosis of homogeneous KS based on lymphocyte karyotyping should be contrasted in other tissues. Mucosa cells could help to better approximate the degree of germ cell mosaicism. Our results indicate that 47,XXY germ cells are not meiotically competent. Increased post-reductional aneuploidy rate is related to meiotic errors in 46,XY cells. Appropriate genetic counselling is recommended in KS.

Key words: aneuploidy / cytogenetics / Klinefelter’s syndrome / meiosis / mosaicism

Introduction

Klinefelter’s syndrome (KS) is the most common chromosome abnormality, with an incidence of 3% in infertile males, and it represents 9–11% of azoospermic patients (De Braekeleer and Dao, 1991; Mau-Holzmann, 2005). Genetic diagnosis is usually ascertained by cytogenetic studies from blood lymphocytes through the evaluation of 20–50 metaphases (Hook, 1977). More than two-thirds of all cases are reported as homogeneous (47,XXX) and the remaining as mosaic (46,XY/47,XXX) or higher grade chromosome aneuploidies (reviewed by Lanfranco et al., 2004).

As mosaicism can be the source of the well-known clinical variations reported in KS individuals, and as karyotype analysis can overlook low mosaicism degree, several authors recommend incorporating interphase fluorescence in situ hybridization (FISH) analysis as a complement to conventional cytogenetics for a more specific and sensitive detection of mosaicism (Okada et al., 2001; Abdelmoula et al., 2004; Lenz et al., 2005). Moreover, the study of mucosa cells has also been proposed as a quick and reliable screening test for a better ascertainment of the chromosome constitution of patients with KS (Kruse et al., 1998; Westlander et al., 2001).

Individuals with KS are traditionally described as being infertile, however, residual foci of spermatogenesis have been observed in both non-mosaic and mosaic individuals (reviewed by Lanfranco et al., 2004). The reported presence of spermatozoa in the testicular tissue of patients with KS has led to offering testicular sperm...
Clinical and experimental approaches are being used to determine if spermatogenic patches are individuals at risk in these patients. From mosaicism data; (iii) to analyse the meiotic competence of KS individuals previously diagnosed as being pure 47,XXY; (ii) to demonstrate that the mosaicism level in the testicular tissue, not only concerning germ cells but also for somatic cells, could be of relevance for the final outcome of spermatogenesis in KS and could have an influence on the probability of successful testicular sperm recovery as well as the quality of the spermatozoa retrieved. Five azoospermic individuals (KS1–KS5) aged between 29 and 35 years, diagnosed as homogeneous 47,XXY by standard cytogenetic procedures (analysis of 20 G-banded metaphases from a peripheral blood lymphocyte culture) were the subjects of our study. Plasma hormone levels were as follows: 13–47.7 IU/l for FSH, 6.7–32.8 IU/l for LH and 4.5–16.2 nmol/l for testosterone. All patients had small testis volume (5–10 ml) and were enrolled as candidates for TESE. For every patient, a new sample of peripheral blood, a buccal swab and a sample of the testicular tissue obtained for sperm retrieval were analysed. Four cell types have been assessed: lymphocytes from peripheral blood, epithelial cells from buccal mucosa and Sertoli and germ cells (pre- and post-reductional cells and meiotic figures) obtained from testicular biopsies.

Four testicular samples from adult organ donors aged between 20 and 33 years have been used as the control population. Furthermore, control samples from buccal mucosa and lymphocytes were obtained from four healthy men (mean age: 31 ± 9.5 years).

Informed consents were obtained from participants and protocols were approved by the ethics committees of the centres involved.

**Materials and Methods**

**Study populations**

Five azoospermic individuals (KS1–KSS) aged between 29 and 35 years, diagnosed as homogeneous 47,XXY by standard cytogenetic procedures (Evans et al., 1964). Before FISH, samples were stained with Leishman (20%) for 8–10 min. Prophase I, metaphase I and metaphase II figures were captured and coordinates were recorded to facilitate location and analysis after FISH. Preparations were de-stained in an ethanol series (70, 80 and 100%) before FISH. The chromosome constitution of metaphases II, which is distinctive in patients with KS (Mroz et al., 1999).

The alteration of the testicular environment has been related to abnormal hormone levels and to the dysfunction of testicular somatic cells (reviewed by Radicioni et al., 2010). The mosaicism level in the testicular tissue, not only concerning germ cells but also for somatic cells, could be of relevance for the final outcome of spermatogenesis in KS and could have practical implications for the clinical management of these individuals.

The aims of the present study were: (i) to assess the occurrence of mosaicism in different tissues and cell types (somatic and germinal) in KS individuals previously diagnosed as being pure 47,XXY; (ii) to evaluate if any predictive value for successful TESE can be inferred from mosaicism data; (iii) to analyse the meiotic competence of 47,XXY spermatocytes and (iv) to evaluate the genetic reproductive risk in these patients.

**Lymphocyte and buccal mucosa cells**

Heparinized peripheral blood from patients KS1 to KS4 was obtained and cultured in 4% phytohaemagglutinin-supplemented medium (PHA M; GibCo, Invitrogen; Paisley, UK) at 37°C for up to 72 h. Cytogenetic preparations were obtained following standard procedures after 25 min incubation in Colcemid (0.16 μg/ml; GibCo, Invitrogen), hypotonic treatment (0.075M KCl) for 20 min at 37°C and fixation in Carnoy’s solution.

Lymphocyte preparations were obtained by scraping the inner cheek and were processed for cytogenetic analysis. Buccal mucosa cells were incubated with hypotonic solution (0.035 M KCl) for 30 min at 37°C. Cells were washed twice with Carnoy’s fixative solution before spreading. Slides were treated with acetic acid solution (50% in H2O) for at least 30 min at 40°C to permeabilize the cells prior to FISH processing. A triple-colour FISH was performed in both lymphocytes and buccal mucosa cells using centromeric DNA probes for chromosomes X, Y and 18 (CEP Y, Spectrum Orange; CEP X, Spectrum Green; CEP 18, Spectrum Aqua) as described by the manufacturer (Aneuvisyon Assay Kit, Abbott Molecular, Abbott Park, IL, USA).

Control samples were processed following the same protocols described for KS individuals.

**Testicular tissue**

Testicular biopsies were obtained under local anaesthesia with the aim of freezing tissue for future ICSI. All testicular samples were sent to the laboratory at 4°C in isotonc solution and were processed for cytogenetic studies with the conventional method of Evans et al. (1964).

A sequential FISH with Whole-Chromosome Painting probes (WCP) for the sex chromosomes (X-XP 23-FITC, Y-XP 24-TexasRed; MetaSystems GmbH; Altuslense, Germany) was performed to evaluate the sex chromosome pairing at pachytene and metaphase I and to confirm the sex chromosome constitution of metaphases II.

Control testicular samples were processed following the same protocols described above.
Microscope analysis and evaluation criteria

All evaluations were carried out using an Olympus BX-60 fluorescent microscope (Olympus Barcelona, Spain) equipped with specific filters for FITC, Cy3, Aqua and a multiband pass filter (DAPI/FITC/Texas Red).

A minimum of 500 lymphocytes (except for KS), 200 buccal mucosa cells and 1000 interphase nuclei (germ cells and Sertoli cells), and all of the meiotic figures observed were analysed for each individual.

FISH analysis in interphase cells was performed in accordance with the criteria described by Blanco et al. (2001). Briefly, germ cells were classified according to the number of sex chromosome hybridization signals and chromosome 18 was used as ploidy control. Strict scoring criteria for signal evaluation were used: signals must be of the same size and intensity and the distance between signals must be at least the same as the diameter of the signal. Overlapped nuclei were discarded from the analysis.

Sertoli cells exhibit a characteristic morphology, clearly distinct from interphase germ cells, showing a large fusiform nucleus with a prominent nucleolus and diffuse chromatin.

Meiotic figures were classified taking into account the distribution of hybridization signals from the two rounds of FISH. In pachytene cells, the presence of sex chromosome-specific centromeric signals plus one single WCP domain for the X and Y chromosomes (the sex vesicle), and a single centromeric signal for chromosome 18 (assuming pairing) was considered as being normal in 46,XY cells.

A cut-off level for false-negative FISH results was established by the analysis of 45,X and 45,Y interphase nuclei in testicular cells (Sertoli cells and pre-reductional cells), buccal mucosa cells and lymphocytes from control samples.

Statistical analysis

The X-bearing/Y-bearing ratio was performed with McNemar test (Statistical Package for the Social Sciences 15.0.1 for windows). All other comparisons were performed with Fisher’s exact test (GraphPad InStat, version 3.05, 32bit for Windows 95/NT). Statistical significance was set at P < 0.05. Data are presented as mean ± SD values.

Results

Successful FISH results were obtained in the three tissues analysed (Fig. 1). Control preparations were used to establish cut-off levels for possible FISH false negatives in the different cell types analysed. Values for 1712 lymphocyte nuclei, 663 buccal mucosa cells, 620 Sertoli cells and 1113 pre-reductional cells were recorded. The mean (± SD) percentages of 45,X and 45,Y nuclei in the different cell types analysed were as follows: 0.5% (± 0.9%) for lymphocytes, 0% for buccal mucosa and Sertoli cells, and 0.8% (± 0.5%) for pre-reductional germ cells. The results obtained were in accordance with the sensitivity and specificity expected for the DNA probes used and are consistent with the figures obtained in the standard internal controls for FISH studies currently performed in our laboratory.

Cells with the XY complement (Figs 1 and 2) were observed in all five KS individuals at different percentages, among the tissues analysed (Fig. 3 and Table I). Nuclei corresponding to Sertoli cells were clearly identified in the testicular cell suspension (Fig. 2). The lowest percentage of 46,XY cells corresponds to the lymphocytes (4.8 ± 2.5%), while the highest corresponds to the Sertoli cells (42.3 ± 11.1%; Table I) except in KS3, who has a lower percentage of Sertoli XY cells than mucosa and pre-reductional germ cells (Fig. 3). Furthermore, the cytogenetic analysis of mucosa cells shows that the percentages observed in this tissue (21.9 ± 10.9%) are closer to the degree of mosaicism in testicular tissue in the five patients.

Table II summarizes the number of mitotic and meiotic figures analysed. Eight spermatogonial metaphases corresponding to the 46,XY line were found. Meiotic figures were seen in three of the five cases. All 758 prophases observed had the XY complement (Table II). Concerning the pachytene cells, in all cases chromosomes X and Y were seen forming the sex vesicle (Fig. 4). Nine metaphases I, having the XY complement, and I I metaphases II, with either the X chromosome or Y chromosome, were observed.

Post-reductional germ cells were observed in all patients (Table III). The ratio between X-bearing/Y-bearing post-reductional cells was equivalent to 1:1 for all patients. Cases K1–K4 showed a significant increase of aneuploidy rates (24,XY) versus controls (P < 0.05; Table III). Higher percentages of 25,XXXY cells were observed in three of the five patients compared with controls (P < 0.05; Table III). Spermatozoa were not found in the samples studied.

Discussion

Karyotyping from lymphocyte culture of peripheral blood is the standard diagnostic technique used to determine chromosomal anomalies.

Figure 1  Triple-colour FISH with centromeric probes for chromosome X (green), Y (red) and 18 (blue) in patients with KS. (A) Peripheral blood lymphocytes: 46,XY cell on the right and a 47,XXY cell on the left. (B) Buccal mucosa cells, both showing an XXY complement. (C) Testicular tissue cells: on the left, a 46,XY Sertoli cell nucleus and on the right, a pachytene nucleus with a single 18-chromosome signal (homologue chromosomes paired) and the sex vesicle showing a single signal for both X and Y chromosomes. Scale bar represents 10 μm.
However, there is a high heterogeneity in the methods used to search for, establish and confirm mosaicism. Although the study of 50 metaphases is recommended to exclude <10% of mosaicism with a 0.99 confidence level (Hook, 1977) most routine karyotyping is based on the study of 20 metaphases. The patients analysed in our study would be a clear example of undetected mosaicism, since all have

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age (years)</th>
<th>Blood</th>
<th>Buccal mucosa</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-reductional</td>
<td>Sertoli</td>
</tr>
<tr>
<td>KS1</td>
<td>31</td>
<td>14/527 (2.7%)</td>
<td>37/203 (18.2%)</td>
<td>95/210 (45.2%)</td>
</tr>
<tr>
<td>KS2</td>
<td>29</td>
<td>16/530 (3%)</td>
<td>44/132 (33.3%)</td>
<td>355/836 (42.5%)</td>
</tr>
<tr>
<td>KS3</td>
<td>35</td>
<td>41/510 (8%)</td>
<td>97/292 (33.2%)</td>
<td>287/714 (40.2%)</td>
</tr>
<tr>
<td>KS4</td>
<td>31</td>
<td>28/509 (5.5%)</td>
<td>31/192 (16.1%)</td>
<td>225/866 (26%)</td>
</tr>
<tr>
<td>KS5</td>
<td>34</td>
<td>18/206 (8.7%)</td>
<td>353/834 (42.8%)</td>
<td>40/86 (46.5%)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>32 ± 2.4</td>
<td>4.8% ± 2.5%</td>
<td>21.9% ± 10.9%</td>
<td>36.4% ± 8.7%</td>
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\(^{a,b}\)Indicate significant differences within columns (Fisher’s exact test; \(P < 0.05\)).
previously been diagnosed as pure 47,XXY. However, upon amplifying
the study by interphase FISH, the presence of XY lymphocytes was
seen with variable percentages (2.7–8%). These figures are clearly
over the cut-off level for false-negative FISH results established in
our laboratory for control individuals (0.5 ± 0.9%). Thus, for the diag-
nosis of ‘pure KS’, it is advisable to increase the number of cells ana-
lysed, either by karyotyping or by FISH in interphase nuclei.

The observation of different percentages of 46,XY cells in the ana-
lysed tissues indicates that the degree of mosaicism is not uniform.
Our data demonstrate that even in cases of a low percentage of
mosaicism in peripheral blood (<10%), the patients present degrees
of germinal mosaicism >36% (Table I). Our results confirm that the
analysis of lymphocytes is not a good indicator of the testicular
status and are in good agreement with what other authors suggest
(Bielanska et al., 2000; Lanfranco et al., 2004). It has been described
that the analysis of buccal mucosa is a rapid method to confirm and
increase the accuracy of the diagnosis of patients with KS (Kamischke
et al., 2003). In our study, the percentages of observed buccal mucosa
46,XY cells (21.9 ± 10.9%) are closer to the mosaicism observed in
the germinal line (36.4 ± 8.7%), although its predictive value for

| Table II | Mitotic and meiotic figures analysed in patients with KS. |
|----------------|
| Spermatogonial | Leptotene | Zygote | Pachytene | Metaphase I | Metaphase II | Total |
| metaphases   |           |         |           |             |            |       |
| KS1          | 48        | 32      | 27        | 107         |             |       |
| KS2          | 5         | 60      | 188       | 166         | 7           | 10    | 436   |
| KS5          | 3         | 29      | 122       | 86          | 2           | 1     | 243   |
| Total        | 8         | 137     | 342       | 279         | 9           | 11    | 786   |

| Figure 4 | Sequential cytogenetic analysis in pachytene-stage spermatocyte in patient with KS. (A) Leishman stain. Arrows point to the sex vesicle. (B) Triple-colour FISH with centromeric DNA probes for chromosomes X (green), Y (red) and 18 (blue). A single signal for each sex chromosome (XY) is identified inside the sex vesicle. (C) WCP for the sex chromosomes (X-green and Y-red) confirming the presence of a single X chromosome plus a Y chromosome forming the sex vesicle. Scale bar represents 10 μm. |

| Table III | Chromosome constitution of post-reductional cells in patients with KS and in controls. |
|----------------|
| 23, X | 23, Y | 24, XY | 25, XXY | Others |
| KS1 | 6/14 (42.9%) | 4/14 (28.6%) | 4/14 (28.6%) |             |
| KS2 | 14/34 (41.2%) | 9/34 (26.5%) | 7/34 (20.6%) | 4/34 (11.8%) |
| KS3 | 12/38 (31.6%) | 12/38 (31.6%) | 11/38 (28.9%) | 3/38 (7.9%) |
| KS4 | 9/33 (27.3%) | 5/33 (15.2%) | 12/33 (36.4%) | 7/33 (21.2%) |
| KS5 | 27/61 (44.3%) | 27/61 (44.3%) | 5/61 (8.2%) | 2/61 (3.3%) |
| Control mean (n = 4) | 667/1454 (45.9%) | 687/1454 (47.3%) | 87/1454 (6%) | 1/1454 (0.1%) | 12/1454 (0.8%) |

No significant differences 23,X versus 23,Y in the five patients (McNemar test; P > 0.05). Others group includes: 22,X; 24,YY; 24,XX; 26,XXYY.
*Significant differences versus controls (Fisher’s exact test; P < 0.05).
successful sperm recovery could not be confirmed owing to the fact that spermatozoa were not observed in any of the five patients with KS.

Although the clinical significance of the mosaicism level in KS remains to be elucidated, the knowledge of the degree of mosaicism can help us to understand the reason for the variable response that some patients with KS show when faced with different therapies (treatments with aromatase inhibitors, hCG or clomiphene citrate). It has been reported that 77% of the patients who show blood concentrations \( \geq 250 \text{ ng/dl} \) testosterone after treatment have successful sperm retrieval (Ramasamy et al., 2009). It could be hypothesized that the Sertoli 46,XY cells would be those which would respond to the testosterone levels, recovering the functions of nursing. In fact, in our series, the two patients observed who do not show meiotic phases (KS3 and KS4) are those who have the lowest percentages of Sertoli 46,XY cells. Furthermore, patient KS5 with a high percentage of euploid Sertoli cells (46.5%), and despite having a low percentage of pre-reductional XY cells (28.2%), has meiotic phases. Thus, taken together, results suggest that the progression of spermatogenesis would depend on the percentage of Sertoli XY cells which colonize the seminiferous tubules. However, further studies would be of interest to evaluate the value of mosaicism in predicting successful sperm retrieval when pharmacologic therapeutic treatments are used.

The origin of mosaicism in germinal cells has been attributed to the occurrence of 'correcting mitotic errors' associated with the mitotic proliferation of the primordial germinal cells in the fetal testicle (Levron et al., 2000) and also of the spermatagonia in adult tissue (Sciurano et al., 2009), giving rise to isolated zones of euploid cells. These corrective processes would also have occurred in the other cell types, causing different degrees of mosaicism in each one. On the other hand, it has been described that the lack of inactivation of the X chromosome would preferentially affect the XXY cells, raising the relative proportion of the XY cells as well as somatic cells at different levels (Aksgaæde et al., 2006). In the mouse model, Sertoli XXY cells show low levels of expression of androgen receptor (AR) (Lue et al., 2005). In human males, a delay in disappearance of anti-Müllerian hormone expression coupled with the up-regulation of AR has been described, and it is known that both are required for the last step of Sertoli maturation during puberty (Wikstrom et al., 2007). This, together with an altered activation pattern of apoptosis because of the hypergonadotrophic hypogonadism (Aksgaæde et al., 2006), suggests that the degeneration of the Sertoli cells would preferentially affect the XXY cells, raising the relative proportion of the XY cells. Thus, the maintenance of the mosaicism or degeneration of the aneuploid line would be determined by the presence of two functional X chromosomes.

Mosaicism has been observed at the testicular level in all of the patients studied (mean of 36.4 ± 8.7%). All prophases and metaphases I (Table II) show the XY complement, even in the patient where the aneuploid line represents almost 55% of the cells (KS2, Table I). These results agree with those previously reported by our group (Blanco et al., 2001) and with recent studies (reviewed by Tuttelmann and Gromoll, 2010) and confirm the meiotic incompetence of the 47,XXY spermatocytes. Furthermore, neither the deviation from the 1:1 ratio nor the equivalent proportions of hyperhaploidies (XX and XY) in post-reductional cells described by other authors (Foresta et al., 1999; Yamamoto et al., 2002; Ferlin et al., 2005) have been observed in our study. What is more, the few spermatagonia metaphases observed were also 46,XY, indicating that this line would be more proliferative than the 47,XXY germ line.

The meiotic incompetence of the 47,XXY line has been related, as in the mouse model (Hunt et al., 1998), to the presence of the second X chromosome and the insufficient inactivation of the genes associated with the extra chromosome, which could cause the degeneration of the 47,XXY germinal cells (Aksgaæde et al., 2006). On the other hand, given that it has been reported that anomalies in the sex vesicle formation lead to apoptotic processes (Burgoyne et al., 2009), it can be inferred that the anomalies of synopsis of the XXY complement lead to cell degeneration of any 47,XXY spermatocytes. Thus, the results of our study are in agreement with the descriptions made in the mouse model (Hunt et al., 1998) and support the hypothesis that the spermatogenic patches are populated by 46,XY cells.

The occasional presence of spermatozoa in testicular tissue has been described in certain KS individuals (Foresta et al., 1999; Rives et al., 2000; Blanco et al., 2001; Bergere et al., 2002; Lanfranco et al., 2004; Sciurano et al., 2009; Fullerton et al., 2010) and confirms that the process of spermatogenesis can be accomplished in some of the spermatogenic patches.

Our findings, where a significant incidence of aneuploidies in post-reductional cells was observed, clearly give support to the hypothesis that an altered testicular environment compromises the meiotic progression of the 46,XY cells (Mroz et al., 1999). Furthermore, the evidence from this study and others (reviewed by Templado et al., 2011) of increased chromosomal abnormalities in mosaic, as well as non-mosaic, patients with KS, deserves to be taken into account when considering offering assisted reproduction techniques to these individuals. The offer of TESE and ICSI to KS men has resulted in the reported births of more than one hundred children (Fullerton et al., 2010) and has resulted in the placing of a ‘scientific question mark’ on the infertility definition classically attributed to KS individuals; nevertheless, the label of ‘patients at genetic risk’ still merits being maintained and highlighted.

In conclusion, homogeneous KS diagnosis based on lymphocyte karyotyping should be contrasted in other tissues. FISH analysis on mucosa cells could help to ascertain the degree of germ cell mosaicism, however, further studies would be of interest to evaluate the predictive value of the degree of mosaicism for successful sperm retrieval. Spermatogenesis foci are populated by 46,XY cells and our results support the hypothesis that 47,XXY germ cells are not meiotically competent. The post-reductional aneuploidy rate in KS is higher than in controls and is related to meiotic errors in 46,XY cell lines. KS individuals should be considered as being patients with a reproductive risk, and appropriate genetic counselling is recommended.

Authors’ roles

L.G.-Q. was involved in experimental procedures, data collection and assembly, data analysis and interpretation, manuscript writing and final approval. J.B. was involved in study conception and design, data analysis and interpretation, manuscript writing and final approval. Z.S. was involved in data analysis and interpretation, and final approval of the manuscript. V.C. was involved in data collection and final approval of the manuscript. L.B. was the consultant andrologist, contributed in clinical assessment, tissue sampling, data collection and final approval of the manuscript. F.V. was involved in study conception.
and design, data collection and assembly, data analysis and interpretation, manuscript writing and final approval.

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