N- and KRAS Mutations in Primary Testicular Germ Cell Tumors: Incidence and Possible Biological Implications


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Recently, conflicting results have been reported on the incidence of RAS mutations in primary testicular germ cell tumors of adults (TGCTs). In four studies a low incidence of mutations (less than 15%) in a variety of TGCTs or derived cell lines was found, whereas in two other studies a high incidence of N- or KRAS mutations (over 40%) was shown. A total of 62 testicular seminomas (SE) and 34 nonseminomatous TGCTs (NS) were studied thus far. The largest series consisted of 42 TGCTs, studied on paraffin embedded tissue. We present the results of analysis for the presence of N- and KRAS mutations, in codons 12, 13, and 61, in snap frozen samples of 100 primary TGCTs, comprising 40 SE and 60 NS. Using the polymerase chain reaction (PCR) and allele specific oligonucleotide hybridization (ASO), mutations were found in five SE (three in NRAS and two in KRAS, all codon 12), and in one NS (KRAS, codon 61). To exclude underestimation of the incidence of RAS mutations in TGCTs due to the presence of an excess of wild type alleles in the analyzed sample, a PCR technique preferentially amplifying KRAS alleles with a mutation in codon 12 was applied to all SE. This approach, allowing a 250 times more sensitive assay, resulted in the detection of only one additional SE with a mutation. Based on a critical analysis of published data and on our results from the largest series of frozen samples investigated thus far, we conclude that N- or KRAS mutations are rare and apparently not essential for initiation or progression of TGCTs. Genes Chromosom Cancer 12:110-116 (1995). © 1995 Wiley-Liss, Inc.

INTRODUCTION

The p21 proteins encoded by N-, H- and KRAS are involved in signal transduction from activated receptors in the cellular membrane to the nucleus. Mutations in these genes, particularly those in codons 12, 13, and 61, cause a decrease in intrinsic GTPase activity, leading to constitutive activation of the encoded protein (Rodenhuis, 1992; McCormick, 1993). RAS mutations have been detected in a wide variety of human neoplasms, although with varying incidence (Rodenhuis, 1992), and are considered to represent only one of many events in the multistep process of transformation (Kumar et al., 1990).

A low incidence of KRAS codon 12 mutations (less than 15%) has been found in all studies on human primary testicular germ cell tumors (TGCTs) (Mulder et al., 1989; Ganguly et al., 1990; Moul et al., 1992; Ridanpää et al., 1993). In contrast, conflicting data have been reported for NRAS. One study showed mutations at codon 12 or 61 in 13 of 20 seminomas (SE) and two of three nonseminomatous TGCTs (NS) (Ganguly et al., 1990). Another study, comprising only SE, revealed NRAS codon 61 mutations in four of 14 tumors (Mulder et al., 1989). No mutations were found in NRAS codons 12 or 61 in two studies, comprising 28 SE and 31 NS (Moul et al., 1992; Ridanpää et al., 1993). A consistent finding is the absence of mutations in N-RAS codon 13; HRAS codon 12, 13, and 61; and KRAS codons 13 and 61 in TGCTs and derived cell lines (Mulder et al., 1989; Dmitrovsky et al., 1990; Ganguly et al., 1990; Tesch et al., 1990; Moul et al., 1992; Ridanpää et al., 1993).

Several factors may explain the varying findings on the incidence of RAS mutations in TGCTs. Besides possible geographical influences (Rodenhuis, 1992), technical factors related to the method of fixation of the tissue samples, frozen (Mulder et al., 1989; Ganguly et al., 1990; Ridanpää et al., 1993) versus paraffin embedded (Moul et al., 1992), might be involved. In addition, the applied polymerase chain reaction and allele specific oligonucleotide hybridization (PCR/ASO) might underestimate the incidence of mutations when an excess of wild type alleles is present, since it allows detection of one mutant among 40 wild type alleles (Slebos et al., 1992). This could especially be important for SE, which may contain a high percentage (over 80%) of nonmalignant cells (Mostof et al., 1995).
Tumor Samples

Each sample three 10 μm thick sections were collected (Mostofi et al., 1987) was based on histology. An alternative is the use of a PCR technique which preferentially amplifies mutant alleles (mutant-enriched PCR, MEPCR). It has been shown by Kahn et al. (1991) that this approach allows detection of one mutant among 10,000 wild type alleles using ASO (MEPCR/ASO).

We have studied snap frozen samples of 100 primary TGCTs, comprising 40 SE and 60 NS, using PCR/ASO for detection of N- and KRAS codons 12, 13, and 61 mutations. In addition, MEPCR/ASO for KRAS codon 12 was used on all SE.

MATERIALS AND METHODS

Tumor Samples

One hundred primary TGCTs and adjacent parenchyma (when present) were collected at the operating theatres of collaborating hospitals. After frozen section diagnosis (using a haematoxylin and eosin stained tissue section), representative parts of tumor and adjacent parenchyma were snap frozen in liquid N\textsubscript{2}. Typing according to the WHO classification (Mostofi et al., 1987) was based on histology and immunohistochemical analyses of expression of placental-like alkaline phosphatase (PLAP), alpha fetoprotein (AFP), human chorionic gonadotropin (hCG) (Dako, Glostrup, DK), and cytokeratins 8 and 18 (Beckton Dickinson, San Jose, CA) using representative paraffin and frozen tissue sections, as described (Oosterhuis et al., 1989).

Frozen samples destined for DNA extraction were microscopically checked for the presence of tumor cells. In addition, suspensions of cell nuclei were prepared from 25 NS samples. These were stained with propidium iodide and analysed by flow cytometry to determine the fractions of aneuploid and diploid cells as described by Mulder et al. (1989).

DNA Extraction

DNA was extracted from snap frozen tumor samples, as described (Maniatis et al., 1982). From each sample three 10 μm thick sections were collected in 100 μl lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 1% SDS, 10 μg/ml proteinase K (Sigma, St. Louis, MO), pH 8.2) and lysed overnight at 37°C. Subsequently the samples were extracted twice with an equal volume of phenol/chloroform. After precipitation in ethanol/sodium acetate acid, the DNA pellet was dissolved in 10 mM Tris, 0.1 mM EDTA to a final concentration of approximately 0.5 μg/μl. From each sample 0.5 μl (approx. 250 ng DNA) was used for PCR. The agarose gel electrophoresis performed to confirm the presence of PCR products, as described in the next section, was also used to confirm the presence of comparable amounts of DNA in each PCR. Matched, carcinoma-in situ containing parenchyma of tumors with a RAS mutation was also studied, when available (n = 4).

Polymerase Chain Reaction

Amplification was performed in a reaction mixture which consisted of buffer (50 mM KCl, 2.5 mM MgCl\textsubscript{2}, 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.01 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100) (Promega, Madison, WI), 0.2 mM of each nucleotide (dATP, dTTP, dCTP, dGTP) (Pharmacia, Woerden, the Netherlands), 1 U Taq polymerase (Promega), 0.5 μM of both sense and anti-sense primer in a total volume of 50 μl. Four different primer sets, described by Verlaan-de Vries et al. (1986), were used to amplify the KRAS 12 and 13, KRAS 61, NRAS 12 and 13, and NRAS 61 regions. The reaction mixture was covered with paraffin oil. Samples were placed in a Thermal Cycler (Perkin-Elmer, Norwalk, VA) and heated to 94°C for 1 min. The PCR included 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min. Finally, the samples were kept at 72°C for 2 min and subsequently stored at 4°C till further analysis. All samples were tested for the presence (or absence, in the negative control, containing H\textsubscript{2}O instead of DNA) of the desired fragment by agarose gel electrophoresis and ethidium-bromide staining, using 4 μl of the PCR mixture.

Mutant-Enriched Polymerase Chain Reaction for KRAS Codon 12

Amplification was performed in a reaction mixture which consisted of buffer (3.5 mM MgCl\textsubscript{2}, 15 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 60 mM Tris-HCl pH 8.4), 0.2 mM of each nucleotide (dATP, dTTP, dCTP, dGTP), 1.5 U Taq polymerase, 1 μM of both sense and anti-sense primer in a final volume of 50 μl. Using a mismatched sense primer (introducing a BstN1 restriction site in the wild type allele) together with an antisense primer (Kahn et al., 1991), KRAS 12 regions were amplified. The samples were heated to 96°C for 5 min, followed by one cycle at 96°C for 2 min, 55°C for 1 min, and 73°C for 1 min. During 24 cycles, the samples were brought to 96°C for 30 sec, 55°C for 30 sec, and to
73°C for 1 min. Each sample (12.5 μl) was then digested overnight at 37°C to completion with 5 U endonuclease MvaI (Boehringer, Mannheim, Germany), in 26 μl buffer H (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithioerythritol) (Boehringer, Mannheim, Germany). In a second round of PCR, under the same conditions as described above, except for the use of the mismatched sense primer and a nested antisense primer, only the uncleaved (mutated) fragments were amplified. To show the effect of mutant-enrichment, the second PCR was also applied to undigested amplification product of the first PCR.

Allele Specific Oligonucleotide Hybridization

Paraffin oil was removed from the PCR-samples by extraction with diethyl ether. DNA was denatured at 96°C for 3 min and 1 μl of PCR mixture from each sample was dotted on a dry Nytran-N membrane (Schleicher & Schuell, Dassel, Germany), which had previously been soaked in H₂O and 10 × SSC, and air dried. DNA from previously characterized mutation-carrying samples was used as positive control. These controls were available for all KRAS 12 positions (Slbos et al., 1990) and for KRAS 13 position 1 and KRAS 61 position 3 (Oudejans et al., 1991). As negative control a sample of the PCR mixture without DNA was used. DNA was crosslinked to the membrane by exposure to UV light for 2 min.

The N- and KRAS 12 and 13 wild type alleles were detected by specific wild type probes, and identification of KRAS 12 mutations was performed using 6 specific probes after conventional PCR, and 6 after mutant-enriched PCR (Verlaan-De Vries et al., 1986; Kahn et al., 1991). Two mixtures of three probes (codon positions 1 and 2) were used for KRAS 13 and NRAS 12 and 13, while for KRAS 61 and NRAS 61 three mixtures of three mutation specific probes (for positions 1, 2, or 3 of the codons) were applied (Verlaan-De Vries et al., 1986). Five picomoles (1 μl) of probe was endlabeled using 2.5 U T4 polynucleotide kinase (PNK) (Gibco BRL, Breda, the Netherlands) in 4 μl reaction mix (50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine, 10 μCi (1 μl) ³²P–γ-ATP (Amersham, Den Bosch, the Netherlands). After incubation at 37°C for 30 min, 70 μl of 10 mM Tris/0.1 mM EDTA and 1 μl tRNA were added and the mix was centrifuged through a Clontech-100 column (Clontech Laboratories, Palo Alto, CA).

All blots were soaked in aqua dest in separate hybridization tubes. The aqua dest was replaced by 2 ml hybridisation mix (3 M tetramethylammonium chloride, 50 mM Tris pH 7.5, 5 mM EDTA, 1% SDS, 1% (w/v) milk protein (Protifar) (Nutricia, Zoetermeer, the Netherlands). After prehybridization for 60 min at 56°C, 20 μl of labeled probe was added and hybridization was done for 1 hr at 56°C. Blots were washed in hybridization mix without milk protein at 56°C for 30 min, taken from the tubes, rinsed with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), dried, and exposed overnight to an XomatAR film (Eastman Kodak, Rochester, NY) at -70°C. Subsequently, blots were washed at higher stringency in 5 × SSPE (50 mM sodium phosphate, 0.9 M NaCl, 5 mM EDTA)/0.1% SDS, for 30 min, to remove any probe mismatched to the PCR product. In the PCR/ASO, temperature was kept at 57°C for NRAS 61, 59°C for KRAS 61, 61°C for NRAS 12 and 13, and 63°C for KRAS 12 and 13, while the temperature was 61°C in the MEPCR/ASO for KRAS 12. The blots were again rinsed with 2 × SSC, dried, and exposed for 1 to 4 days, as described. Mutations could be detected comparing the dot signals obtained before (to verify the presence of PCR products) and after the stringent wash.

Identification of Mutations

The exact nature of the NRAS 12 mutations was revealed by a cycle sequencing protocol (Cycle Sequencing Kit, Perkin-Elmer Cetus, Norwalk, CT) using a ³²P-labeled sense primer. In brief, the tumor samples were amplified in triplicate using conventional PCR. The PCR products were pooled and subjected to electrophoresis on a 2.5% preparative agarose gel. The fragments were excised from the gel and incubated with 6 U agarase (Boehringer, Mannheim, Germany) for 1 hr at 45°C. The purified DNA fragments were ethanol precipitated at -70°C for 15 min and dissolved in 25 μl of sterile water. Twenty cycles of denaturation at 95°C (1 min), annealing at 63°C (1.5 min), and extension at 72°C (2 min) were performed in a thermal cycler (Hybaid™, Teddington, United Kingdom). The sequencing reactions were analyzed on a denaturing 6% polyacrylamide sequencing gel at 60 W for 2.5 hr. The gel was exposed at -70°C for 16 hr as described above, with an intensifying screen.

RESULTS

Classification of the obtained tumors revealed 40 SE and 60 NS. The group of NS comprised 22 pure tumors (12 embryonal carcinomas [EC]), four yolk sac tumors [YS], five teratomas [T] [both mature
RAS MUTATIONS IN PRIMARY TGCTS

Figure 1. Dot blot signals (after high stringency wash) of all mutant tumors after amplification of the N- or KRAS 12 region, using PCR/ASO or MEPCR/ASO. TL = tumor; P = parenchyma adjacent to the tumor; DOT = signal of the mutant tumor; -T = signal of a representative mutation-negative tumor; +C = signal of the corresponding positive control containing a GGT to GCT transition (for TL614), a GGT to TGT transition (for TL2308), or a GGT to CGT transition (for TL7573 and TL8837); - and + indicate MEPCR/ASO without, respectively, with MvaI digestion of the product from the first PCR, resulting in mutant enrichment for the + condition.

and immature) and one choriocarcinoma (CHI), 35 tumors containing EC mixed with one or more other components (CH, SE, T, YS), two tumors containing CH and T, and one tumor containing T and YS.

A low incidence of codon 12 mutations was found in both N- and KRAS, whereas no mutations in N- or KRAS 13 or 61 were detected in any of the analyzed TGCTs. Three SE (TL1049, TL2191, and TL3544) were found with a mutation in NRAS 12, while two SE (TL7573, TL8837) and one NS (TL2308, containing EC, T, and YS) with a KRAS 12 mutation were detected (Fig. 1).

Using the PCR/ASO approach, mutations in some tumor samples might have remained undetected due to the presence of an excess of wild type alleles. Therefore suspensions of cell nuclei were prepared from 25 frozen NS samples, stained with propidium iodide, and analysed by flow cytometry to determine the fraction of aneuploid (tumor) and diploid (nontumor) cells. The fraction of aneuploid cells varied between 22 and 93%, while 18 tumors contained more than 50% aneuploid cells.

It has previously been shown that in SE samples the fraction of nontumor cells can be greater than 90% (Mulder et al., 1989). We therefore decided to repeat the mutation analysis in our series of SE using MEPCR/ASO for KRAS 12. In this procedure mutant alleles are preferentially amplified, hereby allowing the detection of one mutant among 10,000 wild type alleles (Kahn et al., 1991). The analysis revealed one extra mutant SE (TL614). The previously detected mutations were confirmed, showing an intensified signal after MEPCR/ASO, compared to PCR/ASO and MEPCR/ASO without MvaI digestion (Figs. 1 and 2).

Parenchyma adjacent to a mutant tumor was available for TL614, TL2308, TL3544, TL7573, and TL8837. Only the parenchyma of TL614 did not contain carcinoma in situ (CIS), the precursor of all TGCTs (Skakkebæk et al., 1987) and therefore this sample was not studied further. In the parenchyma of TL7573 and TL8837 a weak signal was found using MEPCR/ASO without MvaI digestion, while no signal was found for TL2308 (Fig. 1 and 2). Using MEPCR/ASO the signals for the parenchyma of TL7573 and TL8837 were intensified, while the mutation in TL2308 was now detectable. Since the parenchyma of TL7573 and TL8837 contained micro-invasive SE cells we cannot determine whether the RAS mutation was only present in these SE cells or also in the CIS component. However, the findings for the parenchyma of TL2308, containing only CIS, indicate that RAS mutations can occur in a noninvasive precursor lesion. No NRAS mutation was found in the parenchyma of TL3544 (not shown). Since only PCR/ASO was used to detect NRAS mutations, the presence of a mutation in the CIS cells cannot be excluded, in analogy to the findings for the parenchyma of TL2308.

The nature of the detected mutations is summarized in Table 1, showing identical base conversions in both the tumor and the adjacent parenchyma.

**DISCUSSION**

Since no HRA mutations have been detected in TGCTs or derived cell lines (Mulder et al.,
Figure 2. Example of dot blot analyses of mutant-enriched polymerase chain reaction products for detection of KRAS 12 mutants. The upper A-D panel represents results using the wild-type specific probe, which detects GGT. The lower A-D panel represents results after hybridization with the probe specific for the CGT sequence. Both blots were exposed after washing at high stringency. The first six positions (A1-A6) represent the positive controls for all possible mutations of the first or second base of codon 12 (A3 corresponding with CGT). The remaining positions (A7, D8) are examples of testicular germ cell tumors, with paired signals from each tumor: without (left dot) and with Mvel digestion (right dot) after the first amplification. For example: C7 (without digestion) and CB (with digestion) = parenchyma of tumor TL7573, note the enhancement in signal intensity after Mvel digestion using the mutant specific probe; 85 and 86 = tumor HT85; D1 and D2 = tumor TL7573; D3 and D4 = parenchyma of tumor TL8837; D5 and D6 = tumor TL8837.

TABLE I. Summary of the Nature of the N- and KRAS Codon 12 Mutations in Testicular Seminomas (SE) and Testicular Nonseminomatous Germ Cell Tumors (NS), and Their Adjacent Carcinoma In Situ Containing Parenchyma (P).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histology</th>
<th>NRAS 12 mutation</th>
<th>KRAS 12 mutation</th>
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<tbody>
<tr>
<td>TL614</td>
<td>SE</td>
<td>GGT→GCT</td>
<td>gly→ala</td>
</tr>
<tr>
<td>TL1049</td>
<td>SE</td>
<td>GGT→GAT</td>
<td>gly→asp</td>
</tr>
<tr>
<td>TL2191</td>
<td>SE</td>
<td>GGT→GAT</td>
<td>gly→asp</td>
</tr>
<tr>
<td>TL2308</td>
<td>NS</td>
<td>GGT→TGT</td>
<td>gly→cys</td>
</tr>
<tr>
<td>TL2308</td>
<td>P</td>
<td>GGT→TGT</td>
<td>gly→cys</td>
</tr>
<tr>
<td>TL3544</td>
<td>SE</td>
<td>GGT→GCT</td>
<td>gly→ala</td>
</tr>
<tr>
<td>TL7573</td>
<td>SE</td>
<td>GGT→CGT</td>
<td>gly→arg</td>
</tr>
<tr>
<td>TL7573</td>
<td>P</td>
<td>GGT→CGT</td>
<td>gly→arg</td>
</tr>
<tr>
<td>TL8837</td>
<td>SE</td>
<td>GGT→CGT</td>
<td>gly→arg</td>
</tr>
<tr>
<td>TL8837</td>
<td>P</td>
<td>GGT→CGT</td>
<td>gly→arg</td>
</tr>
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</table>

Taking into account the sensitivities of the two detection methods used (one mutant among 40 or one mutant among 10,000 wild type alleles for PCR/ASO and MEPCR/ASO, respectively), the minimal percentage of mutant alleles that needs to be present for detection can be calculated. A consistent overrepresentation of (parts of) 12p, on which KRAS is located, is found in TGCTs (De Jong et al., 1990; Oosterhuis et al., 1990; Rodriguez et al., 1993a,b), with a mean copy number of about six. Assuming the presence of one mutant (codon 12) and five wild type KRAS alleles per mutant tumor cell, at least 5.5% of the cells in a sample has to carry the mutant allele to allow detection with PCR/ASO, while 0.02% is needed using MEPCR/ASO. Our comparison of the conventional and mutant-enriched PCR based methods indicates that the low incidence of KRAS 12 mutations in TGCTs must be due to the absence of mutations in the samples studied, and not to the threshold of the PCR/ASO.

The study of flow-sorted SE nuclei by Mulder et al. (1989) showed NRAS mutations in four out of 14 cases. Three of these mutant tumors were studied for heterogeneity for the presence of mutant alleles.
between separate areas within one tumor. Two tumors were shown to be heterogeneous. Of the two KRAβ mutations, coded HT31 and HT85 in the study of Mulder et al. (1989), HT31 was also found to be heterogeneous. We confirmed the data on these KRAβ mutant SE, which were analyzed in addition to the 40 SE in our series, using MEPCR/ASO (Fig. 2, only shown for HT85). Therefore, it cannot be excluded that tumor heterogeneity for RAS mutations leads to underestimation of the incidence of these mutations.

A pathogenetic event can be considered of importance for tumor formation when it can be demonstrated in a high percentage of malignant cells within one tumor and in most independent tumors of the same histology. Taking together the results described here and those of Mulder et al. (1989), Moul et al. (1992), and Ridanpää et al. (1993), a total incidence of RAS mutations in primary TGCTs of 11% is found, with 18% in SE and 4% in NS. Together with the heterogeneity for RAS mutations within a single tumor (Mulder et al., 1989), and the detection of a RAS mutation in parenchyma containing CIS and not in the adjacent TGCTs (Moul et al., 1992), this supports the contention that RAS mutations are not essential in the initiation or progression of TGCTs. However, a role of KRAβ in the development of TGCTs by relative overrepresentation of (parts of) the short arm of chromosome 12 cannot be excluded. The difference between SE and NS concerning the incidence of mutations needs further investigation. The question whether mutations can already be present in noninvasive CIS, or only in the (micro-)invasive tumor, can be answered using microdissection of tissue sections of parenchyma under histological control to isolate CIS cells specifically.

Recently, Arends et al. (1993) reported on the suppression of apoptosis in rat fibroblast cell lines by mutant HRAS. In this context, it is interesting that in vitro culture analysis revealed a consistent difference between 14 SE, without and three with a RAS mutation (Olle et al., in press). Using a coculture system with an embryonal fibroblast feeder, tumors TL1049, TL3544, and TL7573 (all associated with a strong hybridization signal in PCR/ASO) showed enhanced survival and proliferation. TL8837 and TL2191 were not available for in vitro culture. Frisch and Francis (1994) recently reported on the induction, and abrogation by mutant HRAS, of apoptosis by disruption of epithelial cell-matrix interactions. Since we have indications that SE cells also enter apoptosis immediately after isolation from their micro-environment (Olle et al., in preparation), suppression of apoptosis by mutant RAS could explain the enhanced survival and proliferation in vitro of the mutant tumors. It is unlikely that dysfunction of other genes in the RAS pathway, mimicking the effect of RAS mutations, are involved in the development of TGCTs, because of the absence of SE without RAS mutations which could be cultured in vitro. The possible tumor heterogeneity of the KRAβ mutation in TL614, suggested by the presence of less than 5.5% mutant cells, and its relation to in vitro behavior will be studied.

N- and KRAβ mutations indicate an unfavorable prognosis in childhood acute lymphocytic leukemia (Lübbert et al., 1990) and nonsmall cell lung cancer, especially adenocarcinoma (Slebos et al., 1990; Mitsudomi et al., 1991), respectively. In addition, an enhanced in vitro proliferative capacity is reported for adult acute myeloid leukemia with a poor prognosis (Löwenberg et al., 1993). In view of these and our findings we will investigate the prognostic relevance of RAS mutations in TGCTs.

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