Triple-negative breast cancers express receptors for growth hormone-releasing hormone (GHRH) and respond to GHRH antagonists with growth inhibition

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PRECLINICAL STUDY

Triple-negative breast cancers express receptors for growth hormone-releasing hormone (GHRH) and respond to GHRH antagonists with growth inhibition

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Abstract Triple-negative breast cancers do not express receptors for estrogen or progesterone and do not overexpress HER2. These tumors have an unfavorable prognosis and at present chemotherapy is the only treatment option. Because the antagonists of growth hormone-releasing hormone (GHRH) have been shown to inhibit growth of a variety of cancers by endocrine and paracrine/autocrine mechanisms, we evaluated the expression of GHRH receptors in human specimens of triple-negative breast cancers and the response to GHRH by in vitro models. In samples of triple-negative breast cancers we found mRNA expression for the GHRH receptor and its functional splice variant SV1 in 25 and 70% of the cases, respectively and for GHRH in 80% of the samples. Immunoreaction of SV1 was detected in the human triple-negative breast cancer cell line HCC1806 while HCC1937 was negative. The growth of HCC1806 was stimulated by GHRH(1-44)NH_2 and inhibited by GHRH antagonist MZ-J-7-118. In addition, in HCC1806 MAP-kinases ERK-1/2 were activated by GHRH. Our findings suggest the existence of an autocrine loop consisting of GHRH and GHRH receptors in triple-negative breast cancers. Our in vitro studies demonstrate that targeting the GHRH receptor may be a therapeutic option which should be evaluated in studies in vivo.

Keywords Triple-negative · Breast cancer · Growth hormone-releasing hormone · GHRH antagonist · MAP-kinase

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Introduction

Breast cancer is a heterogeneous disease that encompasses several distinct entities with different biological characteristics and clinical behaviour. Currently, breast cancer patients are managed according to different treatment approaches based on various clinical parameters in conjunction with assessment of the status of sex steroid receptor (estrogen and progesterone receptors) and the overexpression of the HER2 receptors. Although effective tailored therapies have been developed for patients with hormone receptor-positive or HER2-positive disease, at present chemotherapy is the only modality of systemic therapy for patients with triple-negative breast cancers.

This definition of triple-negative breast cancer refers to a group of tumors, which do not express receptors for estrogen or progesterone and which do not overexpress

HER2. This subgroup shows distinctive clinical features and accounts for 10–17% of all breast carcinomas [1, 2]. Triple-negative breast cancers tend to affect more frequently younger patients [3], are more prevalent in African Americans [4] and clinically more aggressive than tumors belonging to the other known molecular subgroups [1, 2, 5, 6]. Although triple-negative cancers are sensitive to chemotherapy [1], the prognosis of patients with such tumors is poor. Thus, in patients with triple-negative cancers disease recurrence takes place earlier and most of deaths occur in the first 5 years after diagnosis [2, 7]. These clinical findings underline the paramount importance of the development of the novel targeted therapies for triple-negative breast cancers.

In recent years several series of antagonistic analogs of growth hormone-releasing hormone (GHRH) were synthesized in an endeavour to develop a new class of antineoplastic agents [8–11]. GHRH antagonists inhibit the growth of various experimental human cancers [12], but their effects on triple-negative breast cancers have not been studied. GHRH antagonists suppress tumor growth through indirect and direct pathways. The indirect, endocrine mechanism operates through the suppression of the GH release from the pituitary, and the resulting inhibition of the hepatic production of IGF-I [8, 9]. In addition, it was observed that GHRH antagonists can inhibit the proliferation of diverse cancer lines by direct action in vitro, under conditions in which the contribution of the hypothalamic GHRH/pituitary GH/hepatic IGF-I axis is clearly excluded [8, 13–25]. These studies led to the conclusion that the main mechanism responsible for tumor inhibition could be a direct effect of the antagonists on the tumor tissue [8, 11]. These findings may now be explained by recent discoveries regarding the role of tumoral GHRH and GHRH receptors in the proliferation of cancers. The fact that some cancers produce GHRH has been known for more than two decades [26], but recent studies also suggest that this peptide is an autocrine growth factor for many malignancies. Thus, it was shown that various cancer lines synthesize GHRH and proliferate in response to exogenous GHRH and its agonistic analogs [14, 19–21, 24, 27–30]. Accordingly, the expression of mRNA for GHRH or its peptide product were detected in surgical specimens of human endometrial, ovarian, breast and prostatic cancers [27, 31, 32].

mRNAs encoding four splice variants (SV) of GHRH receptors and specific high affinity binding sites for GHRH and its antagonistic analogs have been identified in diverse cell lines and specimens of human cancers [19, 20, 24, 25, 28, 29, 31, 33–36]. These findings suggest that the direct antiproliferative action of GHRH antagonists could be exerted through the disruption of an autocrine/paracrine stimulatory loop formed by tumoral GHRH and its receptors on tumors [14, 19, 20, 28, 29, 35, 37, 38].

Although the post receptor mechanisms of tumoral GHRH receptors is not fully elucidated, recent studies demonstrate that growth stimulation by GHRH requires the Ras/Raf signaling pathway and the activation of MAP-kinases in estrogen/progesterone negative HER2 over-expressing MDA-MB-231 breast cancer cells [39].

The current study reports for the first time the expression of GHRH-R in specimens of triple-negative human breast cancers. In addition, we evaluated the effects of GHRH and a recently developed GHRH antagonist on human triple-negative breast cancer cell lines.

Materials and methods

Peptides

The 44 amino acids form of human GHRH(1-44)NH_2 was purchased from PeptaNova GmbH (Sandhausen, Germany) and stored at −20°C. The GHRH antagonist MZ-J-7-118 was synthesized in the laboratories of the Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, Miami, FL, USA.

Cell culture

Cell lines derived from patients with triple-negative breast cancer HCC1806 and HCC1937 were obtained from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany). Cells were grown in growth-media RPMI-1640 (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS) (PAA-Laboratories, Cölbe, Germany) in a humidified atmosphere containing 5% CO_2/95% air at 37°C. The media were supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Karlsruhe, Germany).

RNA extraction and reverse transcription (RT)

Total RNA was extracted from frozen tissues by using the Trizol reagent (Invitrogen, Karlsruhe, Germany). Frozen tissue sections of 300 µg were homogenized in 3 ml Trizol reagent using an Ultra-Turrax (IKA, Staufen, Germany) and further purified according to manufacturer's instructions.

DNA contaminations were digested with RNAse-free DNAse and afterwards purified by using the RNeasy-Mini-Kit (both Qiagen, Hilden, Germany). The amount and purity of the RNA was determined spectrophotometrically. One microgram of the RNA was reverse-transcribed with Superscript-III (Invitrogen, Karlsruhe, Germany) using a mixture of oligo-dT and random primers.

Polymerase chain reaction (PCR)

The primers for the amplification of GHRH were (sense: 5′ ATG CAG ATG CCA TCT TCA CCA A 3′; antisense: 5′ TGC TGT CTA CCT GAC GAC CAA 3′), the pituitary GHRH-R (pit-GHRH-R) (sense: 5′ ATG GGC TGC TGT GCT GGC CAA C 3′; antisense: 5′ CAC GTG CCA GTG AAG AGC ACG G 3′) and the SV1 receptor (sense: 5′ TGG GGA GAG GGA AGG AGT TGT 3′; antisense: 5′ GCG AGA ACC AGC CAC CAG AA 3′), as previously published by Havt et al. [40] or Rekasi et al. [33]. PCR was performed by 40 cycles of 15 s at 95°C, 10 s at the annealing temperature of 66°C for pit-GHRH-R and SV1 or 59°C for GHRH followed by 20 s at 72°C. The amplified DNA was electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV-illumination.

In vitro proliferation assay

For in vitro proliferation assays 3,000 cells were seeded on a 96 well microplate with 100 µl growth-media for 24 h. The media were then exchanged with RPMI-1640 without FCS for another 24 h. At that time, 100 µl of test substances were added at a twofold concentration. After another 48 h, an MTT tetrazolium salt assay was performed. The test measures the reduction of the tetrazolium into insoluble colored formazan crystals by the activity of a dehydrogenase within metabolically active cells. The media was withdrawn and 100 µl of a 1:10 mixture of methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma, Deisenhofen, Germany; 5 mg/ml in phosphate buffered saline (PBS), pH 7.4) in phenol red-free DMEM media (Invitrogen, Karlsruhe, Germany) was added to the cells. The cells were incubated under growth conditions until visible precipitates had formed. The assay was stopped by the addition of 100 µl stop solution containing 10% *N*,*N* dimethyl formamide and 40% sodium dodecyl sulphate (SDS). The solubilization of the formazan crystals was performed in the dark over-night. The next day, the intensity of the blue color was quantified in a microplate reader at a wavelength of 560 nm. The measured absorbance is proportionate to the viability of the cells.

Protein extraction from cell cultures

To detect the expression of GHRH-R or its splicevariant SV1 in lysates of the cell lines we prepared crude cell extracts in RIPA-buffer (tris buffered saline (TBS), pH 7.6, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added orthovanadate, 1 mM phenylmethylsulfonylfluoride and 2 µg/ml aprotinin for 40 min on ice. The extracts were homogenised by several passages through a 21 gauge needle and afterwards centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant contained the total cell lysate. The protein concentration was determined using the BCA-protein-assay-kit (Promega, Mannheim, Germany).

Preparing cells for detection of phosphorylated MAP-kinases ERK 1/2

Cells were seeded in growth media on a culture dish. After 24 h the media was replaced by fresh media not containing serum. Another 24 h later, cells were detached with Accutase (PAA-laboratories, Pasching, Austria), washed and diluted in PBS. Fractions of 30,000 cells were treated for 2.5 min with either 100 nM GHRH or left untreated. After treatment, cells were immediately mixed with 1 volume of sample loading buffer (62 mM Tris–HCl, pH 6.8; 2% SDS; 10% glycerol; 0.001% Bromophenol Blue; 10% β-mercaptoethanol) and heated to 95°C for 5 min. The samples were applied to Western blot analysis and analyzed with antibodies against ERK 1/2 phosphorylated at tyrosine residue 204 (p-ERK) (sc-7383) and afterwards with normal ERK 1/2 (ERK) (sc-94, both Santa Cruz Biotechnology, Germany). The relative amount of p-ERK to ERK was compared under the differently treated samples.

Western blot analysis

The protein extracts were separated by SDS polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes (Bio-Rad, Germany). The blots were incubated with a GHRH-R antibody (ab28692, Biozol, Eching, Germany) diluted 1:25,000 in TBST (TBS, 0.1% Tween-20) with 5% nonfat dried milk overnight at 4°C followed by a horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000; GE-Healthcare, München, Germany) for 1 h. Incubations with antibodies were followed by three washing steps in TBST for 10 min. The immunoreaction was detected by Immobilon-Western-chemiluminescent-HRP-substrate (Millipore, Schwalbach, Germany) and autoradiography.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM). The differences between groups in proliferation assay were evaluated by one way ANOVA followed by Dunnet's test, $P < 0.01$ being considered significant.

Results

Expression of mRNA for the pituitary GHRH-R and SV1 receptor and GHRH in specimens of triple-negative human breast cancers

PCR of reverse transcribed mRNA from deep frozen tumor tissues of patients with triple-negative breast cancer

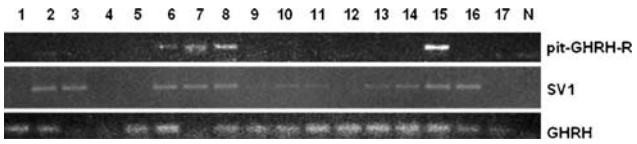

Fig. 1 RT-PCR of mRNA of tumor samples from patients with triple-negative breast cancer. The tumor samples are numbered 1–17 and N is the negative control using mRNA without RT reaction. Bands of the appropriate sizes appear for the pituitary-GHRH-R at 601 bp, for the SV1 receptor at 523 bp and for GHRH at 150 bp

showed that 4 of 17 samples expressed the pituitary GHRH-R (pit-GHRH-R) and 12 of 17 samples expressed the splicevariant SV1 (Fig. 1). Using specific primers for the pituitary-GHRH-R spanning from exon 3 to exon 8 resulted in the amplification of a 601 bp fragment. The 523 bp amplicon for SV1 starting within the intron between exon 3 and 4 of the GHRH-R gene and ending in the exon 7 was found in 12 of 17 samples. The 150 bp amplicon for the mRNA of GHRH was detected in 14 of 17 tumor samples.

Expression of the GHRH receptor protein in human triple-negative breast cancer cell lines HCC1806 and HCC1937

We also determined the GHRH-R expression in HCC1806 and HCC1937 human breast cancer cells by Western blot analysis (Fig. 2). The antibody against a peptide representing both the pituitary GHRH-R and the SV1 receptor detected a 39 kDa band in HCC1806, but not in HCC1937 cells. Because the expected size of the SV1 receptor is 39 kDa, while the pituitary GHRH-R is known to correspond to 47 kDa, we conclude that HCC1806 cells expressed only the SV1 receptor at a detectable level. The mammary carcinoma cell line MDA-MB-231 (MD) was used as positive control while the cervical carcinoma line HeLa was used as negative control showing nonspecific imunoreactions not corresponding with any of the expected sizes for GHRH-R or SV1.

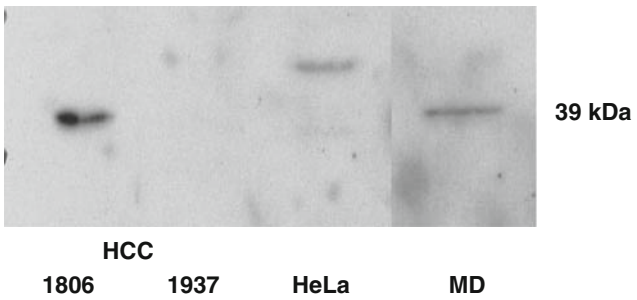

Fig. 2 Western blot analysis using GHRH-R antibody in HCC1806 and HCC1937 triple-negative human breast cancer cell lines. An immunoreactive band of 39 kDa representing the SV1 receptor was detected in HCC1806. A band with equal size was detected in the breast cancer cell line MDA-MB-231 (MD) serving as a positive control for SV1 receptor expression. HCC1937 shows no detectable immunoreaction with the GHRH-R antibody. HeLa serves as a negative control

Proliferative activity of GHRH and antagonistic analog of GHRH

To determine proliferative and inhibitory effects of GHRH and GHRH antagonist MZ-J-7-118, HCC1806 and HCC1937 were subjected to MTT assay after incubation with GHRH, MZ-J-7-118 and a mixture of both substances. After serum deprivation for 24 h GHRH or the GHRH antagonist MZ-J-7-118 were added to the cultures now supplemented with serum replacement SR2. The cell viability after a period of 48 h was measured (Fig. 3). The growth of the SV1 receptor expressing cell line HCC1806 was significantly increased by approximately 20% after treatment with exogenous GHRH at a concentration of 0.1 μM ($P < 0.01$) compared to controls. The GHRH antagonist MZ-J-7-118 at a concentration of 1 μM inhibited the proliferation of HCC1806 cells by 20% ($P < 0.01$) as compared to control cultures. MZ-J-7-118 at 1 μM decreased the proliferation of HCC1806 cells stimulated with 0.1 μM GHRH by 85% ($P < 0.01$). In the GHRH-R negative cell line HCC1937, neither pronounced stimulatory effects of GHRH nor growth inhibition by MZ-J-7-118 were observed at a statistically significant level.

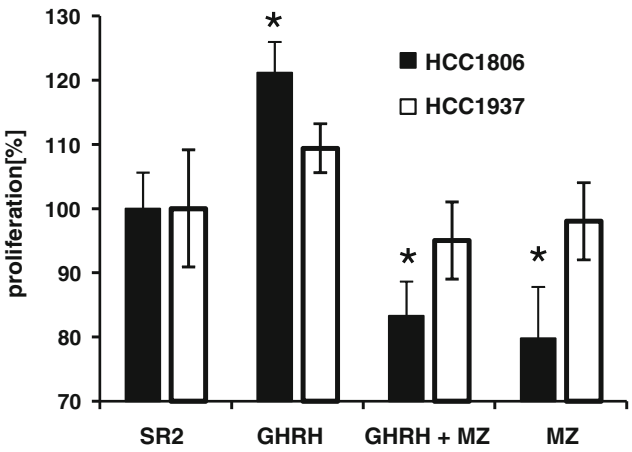

Fig. 3 Influence of GHRH and the GHRH antagonist MZ-J-7-118 (MZ) on the proliferation of HCC1806 (black bars) and HCC1937 (white bars) human breast cancer cell lines. Serum starved cells were incubated for 48 h with serum replacement (SR2) and 0.1 μM GHRH, 1 μM of MZ or the combination of GHRH and MZ were added. Compared to the SR2 control group, the MTT assay resulted in a significant (*$P < 0.01$) growth stimulation by GHRH and significant growth inhibitions by the mixture of MZ and GHRH or MZ only in the SV1 receptor expressing cell line HCC1806

Stimulation of MAP-kinases (ERK 1/2) by GHRH in HCC1806 cells

As the proliferative activity of GHRH is mediated by Ras/Raf kinase pathway and the phosphorylation of MAP-kinases, we determined the phosphorylation of the MAP-kinases ERK 1/2 after incubation with GHRH for 2.5 min. Following treatment HCC1806 cells were directly lysed by adding sample loading buffer and heating. The samples were analyzed in Western blot (Fig. 4a). Compared to the PBS control the proportion of phosphorylated ERK 1/2 and thus activated ERK 1/2 was threefold higher after incubation with 0.1 μM GHRH (Fig. 4b).

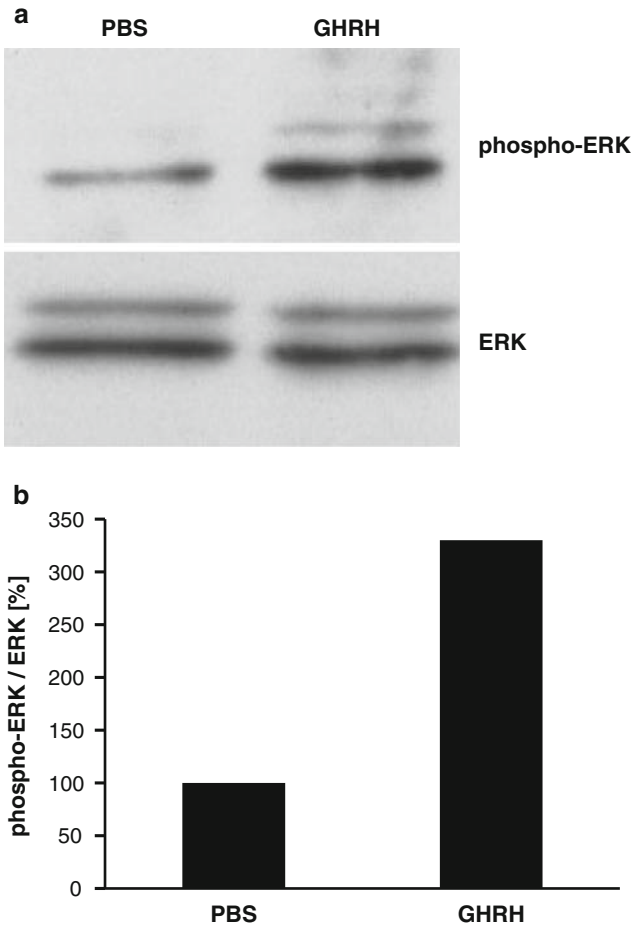

Fig. 4 Activation of the MAP-kinases ERK 1/2 by GHRH in HCC1806 triple-negative breast cancer cells. Serum starved cells were washed and resolved in PBS and treated with 0.1 μM GHRH for 2.5 min before they were immediately homogenized in sample loading buffer at 95°C. The Western blot (**a**) shows the immunoreactivity of phosphorylated ERK 1/2 (phospho-ERK) and the overall expression of ERK 1/2 (ERK). Densitometric analysis of the immunoreactivities (**b**) results in a three-fold increase of the relative amounts of phospho-ERK in GHRH treated cells over the PBS control

Discussion

In recent studies, the presence of an autocrine stimulatory loop, consisting of a tumoral receptor for GHRH, in most cases a functional splice variant (SV1) of the pituitary GHRH receptor, and its ligand GHRH was observed in various tumor entities. These findings prompted us to examine GHRH receptors and GHRH expression in human specimens of triple-negative breast cancers. Using specific primers we found the expression in 25% for the pituitary and the SV1 receptor in 70% of the cases. In addition, 80% of the tumor samples expressed the mRNA for GHRH. Thus, the GHRH receptor may be a suitable target for novel therapeutic approaches in this tumor entity. In an unselected breast cancer population, including sex steroid receptor positive and HER2 overexpressing cancers, Chatzistamou et al. [41] found an expression rate of 40% for the SV1 receptor. While we performed RT-PCR, in that study receptor expression was determined by immunohistochemistry. Nevertheless, both results should not be compared quantitatively.

In addition, we determined the expression of GHRH receptors in two human triple-negative breast cancer cell lines by Western blot analysis, using an antibody targeted against both, the pituitary and the SV1 receptor. While no expression was observed in HCC1937 cells, we detected a protein band of the expected size corresponding to the SV1 receptor in HCC1806 cells. In a previous study, mRNA for the SV1 receptor was also found in human estrogen receptor positive MCF-7-MIII and T47D breast cancer cells as well as in HER2 overexpressing, steroid receptor negative breast cancer cell line MDA-MB-468 [29].

In order to determine the potential relevance of our findings, we treated GHRH receptor positive HCC1806 human breast cancer cell line and HCC1937 as GHRH receptor negative human breast cancer cell line in vitro with GHRH, the GHRH antagonist MZ-J-7-118 and a mixture of both compounds. We found moderate, but significant stimulation of HCC1806 cells by GHRH. As expected, MZ-J-7-118 decreased the proliferation of this cell line. Co-incubation with MZ-J-7-118 and GHRH abolished the proliferative effects of GHRH and led to a moderate inhibition of HCC1806 cells. In GHRH receptor negative HCC1937 cells no significant effect with either compound could be determined. In previous breast cancer studies independent of the receptor status an inhibition of proliferation was observed, for example, in T47D estrogen receptor positive breast cancer cells by a GHRH antagonist in vitro [29] and in MXT mouse mammary cancer in vivo [16]. In a recent study we could show that in human estrogen receptor negative MX-1 breast cancers GHRH antagonists lead to marked inhibition of tumor growth in a nude mice model [42]. It was also demonstrated in this

model, that co-treatment with a GHRH antagonist potentiated the antitumor effect of docetaxel [42], suggesting that GHRH antagonists are suitable partners for conventional chemotherapy.

Results by Siriwardana et al. [39] indicate that GHRH stimulates cell proliferation of MDA-MB-231 breast cancer cells through a pathway that requires MAP-kinase phosphorylation via the Ras/Raf-kinase signal transduction pathway, and in line with these findings, we observed that stimulation with GHRH induced a threefold increase of phosphorylation of the MAP-kinases ERK1/2.

In conclusion, we have shown that triple-negative breast cancers express mRNA for GHRH and its corresponding receptors, the SV1 receptor at a high percentage of cases and the pituitary GHRH-R at a lower percentage. Furthermore, we could show that GHRH can stimulate proliferation in triple-negative breast cancer cells, probably through activation of MAP-kinases. In addition, we demonstrated in an in vitro model that the GHRH-R or its SV1 splice variant are suitable targets for therapy with GHRH antagonists. Given the facts that so far apart from chemotherapy, no targeted treatment has been established for triple-negative breast cancer and GHRH antagonists have been successfully tested in combination with cytostatic agents, our data would be of clinical importance if confirmed by studies in vivo.

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