

Csk homologous kinase inhibits CXCL12-CXCR4 signaling in neuroblastoma

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Abstract. Neuroblastoma is the second most common pediatric malignancy. The clinical course of this disease ranges from spontaneous regression and good survival to highly malignant therapy-resistant tumors. There is a continuous need for genetic and biologic markers for the diverse clinical phenotypes observed in neuroblastoma patients. One of the known markers in neuroblastoma is expression of the CXCR4 chemokine receptor. CXCR4 expression correlates with high-stage disease, and the autocrine stimulation of CXCR4 by its ligand (CXCL12) was shown to be necessary for the survival of some neuroblastoma cells *in vitro*. However, the mechanisms responsible for activation of the CXCL12-CXCR4 autocrine pathway in neuroblastoma remain uncertain. Our previous findings suggest that Csk homologous kinase (CHK) is a physiological inhibitor of CXCR4 expression. Since CHK is highly expressed in neurons, we evaluated changes in CHK expression in human neuroblastoma. CHK protein expression was below detectable levels based on Western blot analyses in 13 out of 16 human neuroblastoma cell lines and in 6 out of 16 primary neuroblastoma tissues. When CHK expression was restored in IMR32 neuroblastoma cells by retrovirus-mediated cDNA transfer, diminished CXCR4 mRNA and protein levels were observed, as assessed by RT-PCR and flow cytometry analyses, respectively. Furthermore, exogenous expression of CHK markedly suppressed the mRNA levels and secretion of the CXCL12 chemokine from IMR32 cells as well as inhibited the growth rate of these cells. Taken together, our data strongly suggest that CHK is capable of inhibiting the CXCL12-CXCR4 pathway in neuroblastoma.

Introduction

Human neuroblastoma is a childhood tumor of the peripheral sympathetic nervous system with a cumulative incidence of

~17 cases per 100,000 children. The course of the disease varies strongly in individual patients. Tumors may regress spontaneously, which is accompanied by apoptosis or differentiation, or they may exhibit extremely malignant behavior with chemotherapy resistance and very low cure rates (1). It is unknown at present whether different tumor stages show consistent genetic differences and whether such variations reflect alterations in signaling pathways that control neuroblastoma proliferation and differentiation.

The spectrum of clinical behavior indicates that genetic, biological and morphological features may be useful markers to identify children with this disease to facilitate the most appropriate treatment. Indeed, significant efforts have been made to elucidate the genes affecting the mechanisms of tumor growth, regression and maturation. Growing evidence has implicated chemokine receptors (especially CXCR4, a receptor for the CXCL12 chemokine), which normally control immune and inflammatory cell migration, as playing important roles in tumor progression (reviewed in ref. 2). A recent report demonstrated that a higher expression of CXCR4 was found in primary neuroblastomas from patients with high-stage disease and in patients with bone and bone marrow metastases. The clinical outcome in patients with tumors expressing high levels of CXCR4 is significantly worse than in patients with low CXCR4 tumor expression (3). Based on the published evidence, CXCR4 may play a role in tumor metastasis (4) and, along with its ligand (CXCL12), can participate in the autocrine survival pathways of neuroblastoma cells (5). Disruption of autocrine CXCL12-CXCR4 signaling was shown to induce the death of neuroblastoma cells *in vitro* (6). However, it is unknown which intracellular factors are responsible for the increased expression of CXCR4 and CXCL12 in certain neuroblastoma tumors. Identification of such factors should allow for the creation of novel antitumor strategies to inhibit CXCR4 signaling in neuroblastoma.

CHK is a cytoplasmic tyrosine kinase belonging to the Csk-family kinases (7-11). CHK is highly expressed in neural tissue, especially in neurons. It was shown that Csk and CHK exhibit opposite temporal expression in the CNS (12,13). Previously, we observed a different pattern of expression for CHK and Csk in brain malignancies (13). While Csk expression is not altered in brain malignancies, CHK expression is significantly downregulated in some brain tumor types (13). The exact role of CHK in the nervous system has not been well elucidated. Although CHK was primarily reported to act

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like Csk kinase and to inhibit activation of Src-family kinases, we showed that CHK, unlike Csk, participates in signaling mediated by the TrkA receptors (14). Accordingly, recent studies suggest CHK's involvement in neuronal differentiation (12,13,15). Importantly, our recent data suggest that CHK could serve as a natural suppressor of CXCR4 expression (16). Therefore, we evaluated the expression of CHK in neuroblastoma as well as the effects of CHK expression on CXCL12-CXCR4 autocrine signaling in neuroblastoma cells *in vitro*.

Materials and methods

Cells. SK-N-SH, SH-SY5Y, IMR32, SK-N-MC, SK-N-DZ, and D341Med cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell lines referred to as 5425, 4030.2, 4181, and 9080 are newly derived neuroblastoma cell lines and were kindly provided along with the Be(2)c cells by Dr Donald Bloch of Massachusetts General Hospital. PVDF membranes with transferred SDS-PAGE-separated protein lysates of the following cell lines: KCN69n, SK-N-FI, SK-N-RA, LA-N-1, LA-N-2, and LA-N-5 were generously provided by Dr Sven Pålman (Lund University, Malmö, Sweden). Cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (all reagents were from Cellgro, Herndon, VA).

Patients. Snap-frozen samples of primary neuroblastoma tumors were obtained from the Children's Oncology Group (COG) Neuroblastoma Tumor Bank. Tumor specimens derived from sixteen patients at the time of diagnosis were included in this analysis. All patients were enrolled in a COG biology study (ANBL00B1, 9047, B973 or B947), and consent was obtained to use the tumor specimens for research purposes.

Western blotting. Cells or primary tissue samples were lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol) containing protease inhibitors (Complete Tablets, Roche, Basel, Switzerland), and 1 mM sodium orthovanadate inhibitor for 45 min at 4°C. Protein concentration was determined using a protein assay (Bio-Rad, Hercules, CA). Seventy µg of total protein extracts was electrophoretically separated on 10% polyacrylamide-SDS gels, transferred to nitrocellulose membrane, and probed with appropriate antibodies. Expression of CHK and Csk was detected using anti-CHK and anti-Csk polyclonal antibodies, respectively (Lsk, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-actin antibody was obtained from Chemicon (Temecula, CA).

Retroviral vectors and generation of semi-stable CHK infectants. The cDNA inserts encoding CHK wild-type (wt) or CHK dead-kinase (dk) were excised from a pcDNA3-based construct (11) by *Bam*HI/*Sal*I digestion and subcloned into the corresponding sites of a pBABE-hygro vector (kindly provided by Dr Scott Lowe). Empty pBABE-hygro vector was used as a control. Retroviruses were packaged in a Phoenix cell line (a generous gift from Dr Piotr Sicinski). The target cells were infected for 4 h in media containing 8 µg/ml polybrene (Sigma,

St. Louis, MO), and subsequently selected for 5 days with hygromycin (Alexis Corporation, San Diego, CA). Expression of CHK protein was evaluated by the Western blotting procedure.

RT-PCR analysis of CXCR4 and CXCL12 expression. RNA from IMR32 cells was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. A QIAshredder spin column and DNase digestion were included in the isolation procedure to limit the possibility of PCR amplification of CXCR4 and CXCL12 from the genomic DNA. cDNA and PCR amplification were performed with the BD Biosciences Titanium One-Step RT-PCR kit using 200 ng of RNA as a template for first-strand synthesis. CXCR4 was amplified using primers: 5'-CGT GGG CAG CCT GTT CCT CA-3' and 5'-CAT GCG GGC TTG GTC TGG-3'. CXCL12 was amplified using: 5'-CCA TGG AGG AAT GCT GGG TG-3' and 5'-TCA GCA ATC AGA GAG GTC TAG-3'. GAPDH was used as a positive control with primers: 5'-CTC ACT GGC ATG GCC TTC CG-3' and 5'-ACC ACC CTG TTG CTG TAG CC-3', which yield a product of 292 bp. The template was first denatured at 94°C for 2 min followed by 35 cycles (94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min), followed by 68°C for 2 min in a MyCycler Personal thermal cycler (Bio-Rad Laboratories, Inc). Aliquots (20 µl) of the PCR products were run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Densitometric analysis on the bands was performed using Scion Image software (NIH, Bethesda, MD).

CXCL12 ELISA. IMR32 cells (pBabehygro-, CHKwt-, and CHKdk-infectants) were grown in 12-well plates until 60% confluent. Then, medium was replaced by 500 µl of fresh culture medium and the cells were grown for an additional 48 h. After that time, 400 µl of the supernatant (in duplicate for each sample) was collected and the level of CXCL12 in the supernatants was measured using a CXCL12 ELISA Quantikine kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Flow cytometry. IMR32 cells were grown on 10-cm Petri dishes until 80% confluent. Then, cells were detached by incubation in 0.5 mM EDTA in cold Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS). Cells were then resuspended in FACS buffer [1% bovine serum albumin (BSA)/PBS] and incubated for 30 min on ice with mouse monoclonal anti-CXCR4 antibody (clone 44717.111, R&D Systems). Following the incubation, cells were washed 3 times with cold FACS buffer and incubated (30 min on ice in the dark) with secondary FITC-conjugated anti-mouse antibody. After the latter incubation, cells were washed and fixed with 1% paraformaldehyde in FACS buffer. Sample acquisition was performed with a FC500 flow cytometry system (Beckman Coulter, Fullerton, CA). Analysis of the acquired data was performed using FCS Express V3 software (De Novo Software, Thornhill, Ontario, Canada).

Growth curve. To assess the growth capabilities of IMR32 cells semi-stably infected with retroviruses encoding CHKwt or CHKdk, 1x10³ cells of each type of infectant were seeded

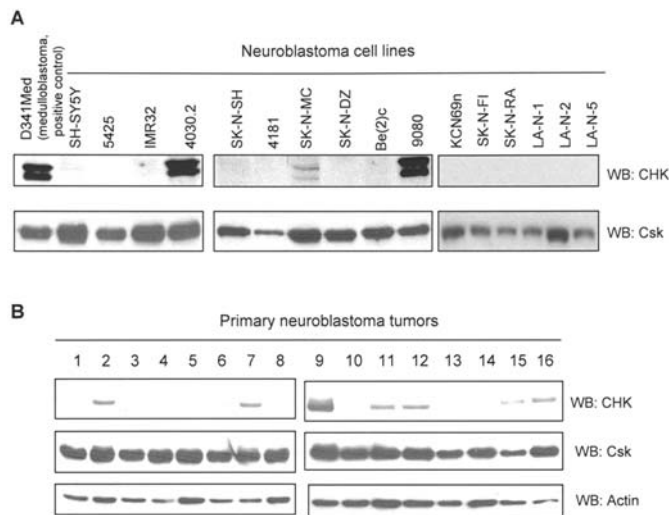


Figure 1. Comparison of the expression levels of CHK versus Csk in human neuroblastoma cell lines (A) and primary neuroblastoma tumor specimens (B) by Western blotting. (A) Be(2)c cells and cell lines referred to as 5425, 4030.2, 4181, and 9080, which are newly derived neuroblastoma cell lines, were kindly provided by Dr Donald Bloch of Mass General Hospital. The six lanes shown in the right panel were blotted onto a PVDF membrane in Dr Sven Pålman's lab (Lund University, Malmö, Sweden) and generously sent to us for probing with the antibodies. Other cell lines were obtained from ATCC. (B) Snap-frozen samples of primary neuroblastoma tumors were obtained from the Neuroblastoma Tumor Bank at the Children's Hospital of Philadelphia, PA. See Materials and methods for the details.

in quadruplicate onto 96-well flat-bottomed microtiter plates. Then, at certain time points (48 and 96 h) following cell seeding, cell viability was measured using a standard MTT assay, as described previously (17). Briefly, 30 μ l of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Sigma) solution (3 mg/ml) was added into each well. Plates were centrifuged 4 h later (200 g/10 min), and the supernatants were carefully removed and replaced with 200 μ l of 2-propanol. After a 15-min incubation at room temperature with shaking (200 rpm), plates were read using a standard ELISA reader with 490/650-nm filters. The viability of the cells was expressed as the optical density (OD 490-650). The experiment was performed twice. The differences in data were calculated by using the Student's t-test. P-values <0.05 were considered to be statistically significant.

Results

Expression of CHK in neuroblastoma cell lines and primary tumor samples. First, we evaluated CHK expression in neuroblastoma cell lines and primary tumors by Western blot analysis. We assessed 16 neuroblastoma cell lines for the expression of CHK and Csk (Fig. 1A). While Csk was easily detectable in all cases, CHK was detected only in three cell lines (high expression levels in 4030.2 and 9080, moderate expression level in SK-N-MC cells). Importantly, one of the CHK-expressing neuroblastoma cell lines (SK-N-MC) is known to express relatively low levels of CXCR4 protein (4). Next, we examined CHK expression in primary neuroblastoma specimens. As shown in Fig. 1B, 6 out of 16 primary neuroblastoma tumors did not express CHK at levels detectable by Western blotting. When a qRT-PCR procedure was applied

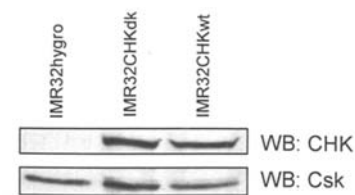


Figure 2. Restoration of CHK expression in IMR32 cells by retrovirus-mediated cDNA transfer. IMR32 cells were infected with CHKdk- or CHKwt-encoding retroviral vectors or empty-pBabehygro vector as a control. Then, infected cells were selected by incubation with hygromycin. Expression of CHK versus Csk was assessed by Western blotting.

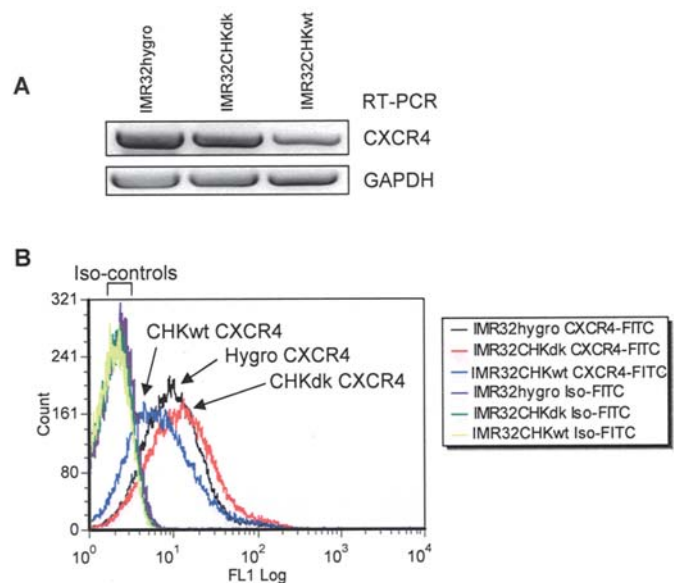


Figure 3. Effects of CHK expression on the mRNA (A) and protein (B) levels of CXCR4 in IMR32 neuroblastoma cells. Semi-stable infectants of IMR32 cells [empty vector (hygro)-, CHKdk-, and CHKwt-infected] were analyzed for expression of the CXCR4 receptor by RT-PCR or immunostaining followed by flow cytometry. GAPDH was used as an internal control for A and Iso-FITC was used as a control for B.

(data not shown), in most cases, differences in the levels of CHK mRNA closely correlated with the levels of CHK protein.

Effects of CHK expression on CXCR4 expression level. To assess the effect of CHK on CXCR4 expression, we chose a model system consisting of the IMR32 neuroblastoma cell line. Notably, IMR32 cells are known to express CXCR4 (18) and CXCL12 (19). Our data (Fig. 1A) show that IMR32 cells do not express detectable levels of CHK. This result was confirmed by RT-PCR analysis (data not shown). To investigate the effects of restoring CHK expression on the expression level of CXCR4, we generated semi-stable retroviral infectant pools of IMR32 cells expressing either the wild-type (wt) or kinase-inactive (dead-kinase, dk) form of human CHK (Fig. 2). Analysis of the expression of CXCR4 in these semi-stable infectants as compared to that in the IMR32 cells infected with empty retroviral vector (IMR32hygro) showed that restoration of CHKwt, but not CHKdk, expression resulted in a decrease in CXCR4 mRNA expression, as assessed by the RT-PCR method (Fig. 3A). Immunofluorescent staining

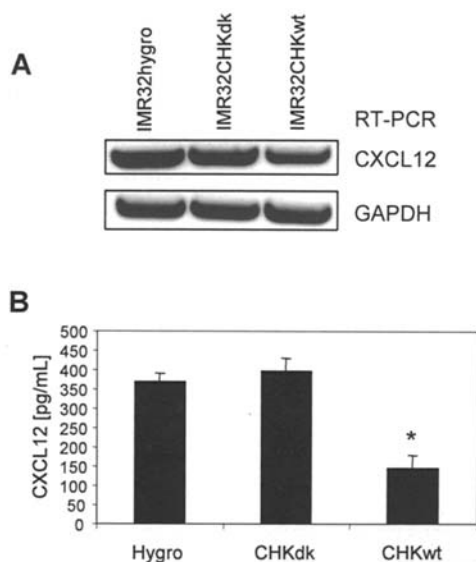


Figure 4. Effects of CHK expression on the mRNA (A) and protein secretion (B) levels of CXCL12 in IMR32 neuroblastoma cells. Semi-stable infectants of IMR32 cells [empty vector (hygro)-, CHKdk-, and CHKwt-infected] were analyzed by RT-PCR or ELISA assay for the production of the CXCL12 chemokine. Bars in B represent the means \pm SD. * $P < 0.05$ versus the IMR32hygro group.

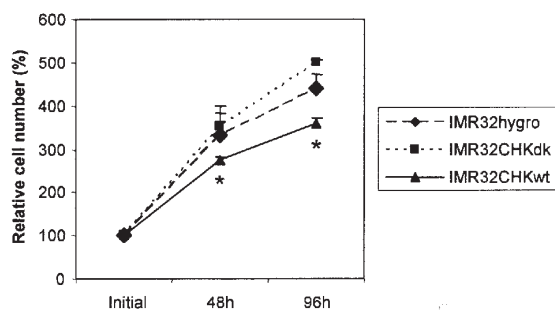


Figure 5. Effects of CHK expression on the growth rate of IMR32 cells. Semi-stable infectants of IMR32 cells [empty vector (hygro)-, CHKdk-, and CHKwt-infected] were grown on 96-well plates and analyzed for cell number 48 and 96 h following seeding by MTT assay. Relative cell number was calculated by dividing the optical density (OD) value for each sample by the initial value for the same group, and is presented as a percentage of the initial value. Data points represent the means \pm SD. * $P < 0.05$ versus the IMR32hygro group.

with anti-CXCR4 antibody followed by FACS analysis (Fig. 3B) also showed a diminished expression of CXCR4 on the protein level in IMR32CHKwt cells as compared to IMR32hygro and IMR32CHKdk cells. These results are in agreement with the RT-PCR data (Fig. 3A).

Effects of CHK expression on CXCL12 production. To identify potential changes in the secretion of the CXCR4 cognate ligand CXCL12, we used both RT-PCR and ELISA methods. Our RT-PCR analysis (Fig. 4A) showed that while the expression of CXCL12 mRNA in IMR32 cells expressing kinase-inactive CHKdk was not significantly modulated as compared to that in cells expressing the control-retrovirus infectants, the CXCL12 mRNA levels in the CHKwt-expressing cells were markedly lower (~50% as assessed

by densitometry, data not shown). To validate these findings on the protein level, we performed an ELISA assay using the supernatants from the IMR32 cells infected with CHK-encoding retroviruses. As shown in Fig. 4B, CHKwt-expressing IMR32 cells secreted significantly lower amounts of CXCL12, while the secretion of CXCL12 from CHKdk-expressing cells did not differ significantly from that of the control-infected cells.

Effects of CHK expression on the growth rate of neuroblastoma cells. Our results showed that the expression of CHKwt induces a significant decrease in the expression of CXCR4 as well as a reduction of CXCL12 production in IMR32 neuroblastoma cells. As autocrine CXCL12-CXCR4 signaling has been shown to be necessary for the survival of neuroblastoma cells *in vitro*, we examined the effects of CHKwt expression on the growth rate of IMR32 cells. As expected, expression of CHKwt significantly inhibited the proliferation of IMR32 cells (Fig. 5). This effect was dependent on the kinase activity of CHK, since the expression of CHKdk had no significant effects on the IMR32 growth rate.

Discussion

The up-regulated expression of CXCR4 has been implicated as a causative factor in neuroblastoma progression, invasion and as a part of the autocrine stimulation pathway utilized by neuroblastoma cells, yet the actual mechanism of this up-regulation is currently poorly understood (20). Although cytokines, including interleukin 2 (21), transforming growth factor β and IFN- γ (22), and G-CSF (23) have been shown to modulate the level of CXCR4 expression, the detailed molecular mechanisms involved in the regulation of CXCR4 expression are unknown. Moreover, all of these studies were performed in hematopoietic cells.

Our previous studies in breast cancer revealed a novel link between CHK and CXCR4 and suggested that CHK tyrosine kinase is a natural factor responsible for the suppression of CXCR4 expression (16). Since neuroblastoma cells are derived from peripheral neuronal tissue and CHK is known to be highly expressed in neurons, we decided to investigate changes in CHK expression in neuroblastoma. We found that CHK is absent in the grand majority of neuroblastoma cell lines and in a high proportion of primary neuroblastoma specimens (Fig. 1). Differences in the frequency of CHK expression between neuroblastoma cell lines and primary neuroblastoma tumors suggest a negative role of CHK in neuroblastoma development by analogy to the situation of the most prominent oncogene in neuroblastoma, namely N-myc. Amplification of N-myc occurs in 20-25% of primary neuroblastoma tumors, while it can be detected in the great majority of neuroblastoma cell lines. The reason for such disparity is that most of the attempts to establish cell lines from low-stage neuroblastoma tumors have failed, and hence the existing cell lines represent the most aggressive pool of neuroblastoma cases. Thus, similar to the results regarding amplified N-myc, our findings showing a different incidence of CHK expression in neuroblastoma cell lines as compared to primary tumors suggest that down-regulation of CHK expression correlates with the more aggressive forms of neuroblastoma.

Next, we examined the effects of restoring CHK expression on CXCR4 levels in neuroblastoma cells. We found that CHK is capable of suppressing CXCR4 on the mRNA and protein levels. This is in agreement with our previous findings in breast cancer cells, where the anti-CXCR4 actions of CHK were transduced via the transcriptional factor YY1 (16). In addition, we report that CHK expression decreases production of the CXCL12 chemokine, the ligand for CXCR4, in neuroblastoma cells and inhibits their growth rate. Taken together, our results strongly suggest that CHK-mediated signaling is a natural inhibitor of the CXCL12-CXCR4 stimulation loop.

Of note, it is not known whether the CHK-mediated anti-CXCL12-CXCR4 effects are dependent on the inhibition of Src-family kinases by CHK. Increased Src activity is frequently associated with the metastasis of cancer cells. Src activation by CXCL12 also contributes to this metastasis (24,25). Src is one of the downstream molecules of CHK, and its kinase activity is often increased significantly in advanced cancer (26). Nevertheless, even though CHK was primarily reported to inhibit the activation of Src-family kinases, studies from our laboratory have highlighted several instances of the Src-independent actions of CHK (14-16). Importantly, suppression of CXCR4 expression by CHK in breast cancer was shown to be Src-independent (16). Therefore, further studies are warranted to identify the molecular mechanisms responsible for these effects in neuroblastoma cells.

In summary, we present novel evidence of inhibition of the CXCL12-CXCR4 stimulatory pathway by CHK kinase in neuroblastoma cells. These findings provide a new avenue for characterizing the mechanism of CXCR4 up-regulation in neuroblastoma.

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