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Original Article

Relationship between CD44^{high}/CD133^{high}/CD117^{high} cancer stem cells phenotype and Cetuximab and Paclitaxel treatment response in head and neck cancer cell lines

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Abstract: Recent evidence suggests that cancer stem cells (CSCs), a small population of cancer cells that are highly tumourigenic, capable of self-renewal and have the ability to differentiate into cells that constitute the tumor, are the "drivers" of local recurrence and metastatic spread and may be associated with resistant to conventional therapy. The objectives of the study are to identify and characterize two head and neck cancer cell lines with regard CD44^{high}/CD133^{high}/CD117^{high} profile (CSCs) and CD44^{low}/CD133^{low}/CD117^{low} profile (Non-CSCs); to investigate the influence of chemotherapy treatment in CSCs and compare with Non-CSCs; to evaluate CD44 and EGFR gene expression in CSCs. Fluorescent-activated cell sorting (FACS) using specific cell surface marker combination (CD44, CD117 and CD133) was performed to isolate CSCs of Non-CSCs from cell lines. The Wound Healing assay was performed to confirm the presence of CSCs. After, the CSCs subpopulation and Non-CSCs were cultured and exposed for 24 h to Cetuximab and Paclitaxel treatment, separately. Cell proliferation was determined by MTS assay. CD44 and EGFR gene expression was quantified by quantitative real time PCR (qPCR) using TaqMan® Assay in both subpopulations. CSCs subpopulation untreated were considered as relative expression control. We firstly characterized CSCs in HN13 and HEP-2 cell lines with CD44, CD133 and CD117 biomarkers. We treated CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel treatment and found that CSCs subpopulations demonstrated more resistance to Paclitaxel chemoterapy, when compared with Non-CSCs subpopulations of oral cancer cell line. These CSCs subpopulations presented up-regulation of *CD44* gene and down-regulation of *EGFR* gene in oral cancer cell line, and down-regulation of *CD44* gene and up-regulation of *EGFR* gene in laryngeal cancer cell line when compared with Non-CSCs subpopulations. We conclude that the combination of CD44, CD133 and CD117 biomarkers have stem cell properties in both cell lines. CSCs has ability to resist to Paclitaxel treatment in oral cancer cell line. CSCs present high expression of CD44 gene and down expression of EGFR gene in oral cancer cell line. CSCs in laryngeal cell line present down expression of *CD44* gene and high expression of *EGFR* gene when compared with cells without characteristics of cancer stem cells.

Keywords: Cancer stem cells, chemotherapy, head and neck neoplasias, gene, expression, CD44, EGFR, cell line

Introduction

Head and neck cancer (HNC) is an aggressive disease that accounts for more than 500,000 cases each year worldwide [1]. The high prevalence of the disease is due to high rates of recurrence and metastasis. Furthermore the rate of success in treatment still remains low [2-4]. The treatment options for HNC depend of tumoral stage and can be surgery, radiotherapy and/or chemoterapy [5]. Treatment for HNC in early stage (stage I and II) generally involves single-modality therapy: Surgery or radiotherapy. However, patients with HNC locally advanced (stage III and IV A/B) are treated with chemoradiotherapy with or without chemotherapy [3, 7].

Chemotherapy treatment has improved in the last years but the supportive care for patients in treatment has increased because still there are many collateral effects as mucositis, skin desquamation, depression, fatigue, nausea, vomiting and others. Furthermore some patients have no answer for chemotherapy treatment compared to other patients with the same tumoral stage and the overall survival rate remains low [2, 8-10]. The fact can be associated with the presence of cancer stem cell (CSC) in tumor [11, 12].

CSC are defined as a small subpopulation of cells located within the tumor mass with high capacity of tumorigenic potential, self-renewal properties and slow growth cycle which is responsible to resistance to therapies that firstly target cancer cells that present faster growth [13-15]. The identification of CSC can provide interesting data regarding new therapeutic approaches in HNC and they may be identified through molecular biomarkers as CD44, CD117 and CD133 [16-18].

In the current study, the aim was to identify and separate cancer stem cells through CD44, CD133 and CD117 biomarkers in two subpopulations of head and neck cancer cell lines (HN13 and HEP-2 cell lines): $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ (CSCs) and $CD44^{low}$/$CD133^{low}$/$CD117^{low}$ (Non-CSCs), to verify if these biomarkers have stem cell properties; to compare effectiveness of Cetuximab and Paclitaxel treatment in CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines, and to evaluate *CD44* and *EGFR* gene expression in the CSCs subpopulations.

Material and methods

Cell line and culture conditions

HN13 (squamous cell carcinoma of oral cancer cell line) and HEP-2 (laryngeal cancer cell line) cells were cultured in D-MEN (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 100 units/mL penicillin, and 100 lg/mL streptomycin (all reagents were from Invitrogen, Grand Island, NY).

Flow cytometry (Indetification and isolation of CSCs)

The trypsinized cells were resuspended, incubated with monoclonal antibodies for 30 min at 4°C, washed twice with phosphate buffered saline (PBS). The antibodies utilized were CD44-phycoerythrin (PE), CD117-fluorescein isothiocyanate (FITC) and CD133-allophycocyanin (APC). Fluorescent-activated cell sorting (FACS) of live cells was used to separate subpopulations of HN13 and HEP-2 subpopulation of cells using specific cell surface biomarkers combinations (CD44/PE, CD117/FITC and CD133/APC) with BD FACSAria Fusion equipament (BD Biosciences).

The subpopulation of sorted cell lines were classified based on the expressions of CD44/CD117/CD133 in combination as: $CD44^{high}$/$CD133^{high}$/$CD117^{high}$: presence of CSCs and $CD44^{low}$/$CD133^{low}$/$CD117^{low}$ profile (Non-CSCs). CSCs and Non-CSCs were resuspended in D-MEN for further experiments.

Wound healing assay

For confirmation of presence of CSCs, the CSCs and Non-CSCs subpopulations cells were plated at a density of 2×10^6 cells/wells and cultured until they reached confluence. A diametric scratch was created using a pipette tip and washed with PBS 3 times. The cells were photographed in microscope (OLYMPUS - CKX61/40 × objective lens) in three pre-marked spots as 0 h. Images were then acquired at 24 h in the same spots for comparison.

Drug sensitivity and MTS assay

CSCs and Non-CSCs subpopulations were plated at a density of 2×10^6 cells/well in six well plates. Cetuximab (CT), Paclitxel (P) chemotherapeutic agents at 0.06 mg/ml and 0.05 mg/ml concentrations, respectively, were added in the CSCs and Non-CSCs subpopulations [19, 20]. The cultures were incubated at 37°C for 24 h. The proliferation of cell lines were measured at OD 490 nm using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI, USA). The experiments were repeated two times. The results were expressed as percentage relative to the control cells. The chemotherapeutics evaluated are widely utilized in patients with oral cancer, so they were included in the study.

Real-time quantitative RT-PCR

RNA isolation was performed using Trizol (Invitrogen) according to manufacturers' manuals. The concentration of RNA utilized was 2 *ug*

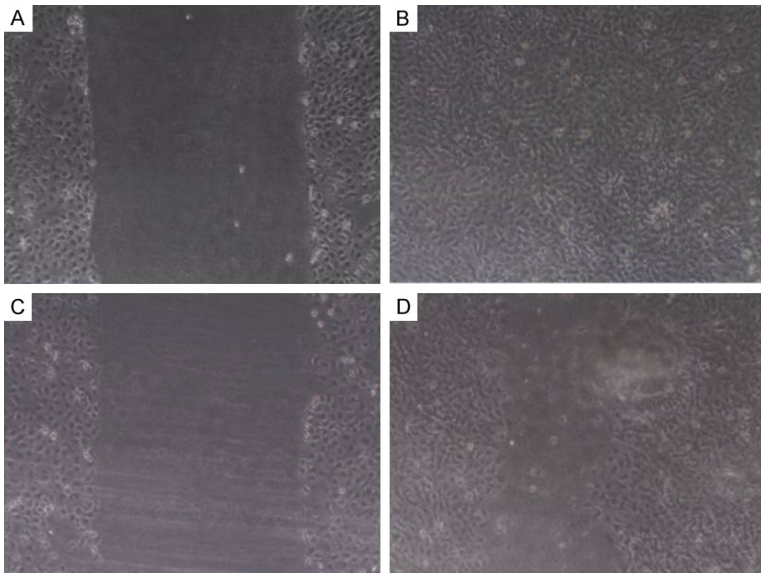

Figure 1. Cell Migration of CSCs and Non-CSCs subpopulations of HN13 cell line seeded in 6-well plates and cultured for 24 h. A. CSCs subpopulations of HN13: A diametric scratch using a pipette tip was made at 0 h; B. CSCs subpopulations of HN13 after 24 h; C. Non-CSCs subpopulations of HN13: A diametric scratch using a pipette tip was made at 0 h; D. Non-CSCs subpopulations of HN13 after 24 h.

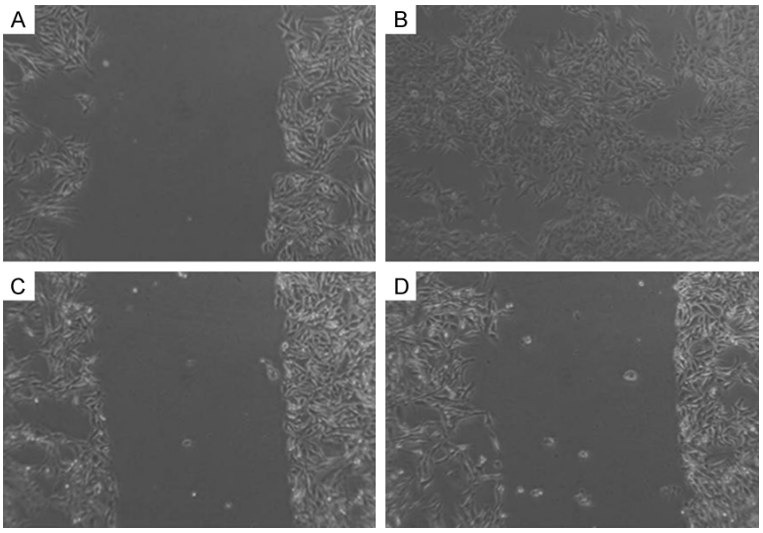

Figure 2. Cell Migration of CSCs and Non-CSCs subpopulations of HEP-2 cell line seeded in 6-well plates and cultured for 24 h. A. CSCs subpopulations of HEP-2: A diametric scratch using a pipette tip was made at 0 h; B. CSCs subpopulations of HEP-2 after 24 h; C. Non-CSCs subpopulations of HEP-2: A diametric scratch using a pipette tip was made at 0 h; D. Non-CSCs subpopulations of HEP-2 after 24 h.

(Picodrop Equipament). For cDNA synthesis, 1 ug RNA was used with primers by High capacity cDNA kit (Applied Biosystem®) according manufacturer's protocol. Genetic expression in all samples was evaluated by quantitative RT-PCR (qRT-PCR) with StepOnePlus™ Equipament (Applied Biosystems).

A polymerase chain reaction (PCR) was realized with 10 µL of Taqman Universal PCR Master Mix (Applied Biosystems), 80 nmol/L of primer, 2 nmol/L probe and 2 µL of cDNA. The cycling conditions were: 95°C for initial denaturation by 20 s, 40 cycles of 95°C for denaturation by 0,3 seconds, 60°C for annealing by 1 min and 72°C for extension by 30 seconds. TaqMan® Gene Expression Assay was pre-optimized PCR primer and probe sets for qRT-PCR formulated at 20 × concentration. Specific primers were utilized for quantification of genes evaluated through TaqMan® Custom Array Plate. Two reference genes (b-actin and Glyceraldehyde-3-phosphate dehydrogenase-GAPDH) and 2 target genes (CD44 and EGFR) were utilized. All reactions were realized in duplicate to better PCR specificity. Gene expression was normalized with β-actin and GAPDH genes. Gene expression of CD44 and EGFR genes were compared in CSCs and Non-CSCs and it was calculated by delta threshold cycle (Ct) method according to mathematical following formula: Expression level of target gene = 2-(Delta Ct) × 1,000 Delta Ct = Ct of target gene - (Mean Ct of β-actin and GAPDH genes).

Results

Identification and isolation of CSCs and Non-CSCs subpopulations in cell lines

The subpopulation of sorted HN13 cell line with $CD44^{high}/CD133^{high}/CD117^{high}$ (CSCs) was detected in 0.7% and isolated of Non-CSCs. The expression of CD44, CD117 and CD133 were 0.1%, 0.4% and 0.2%, respectively.

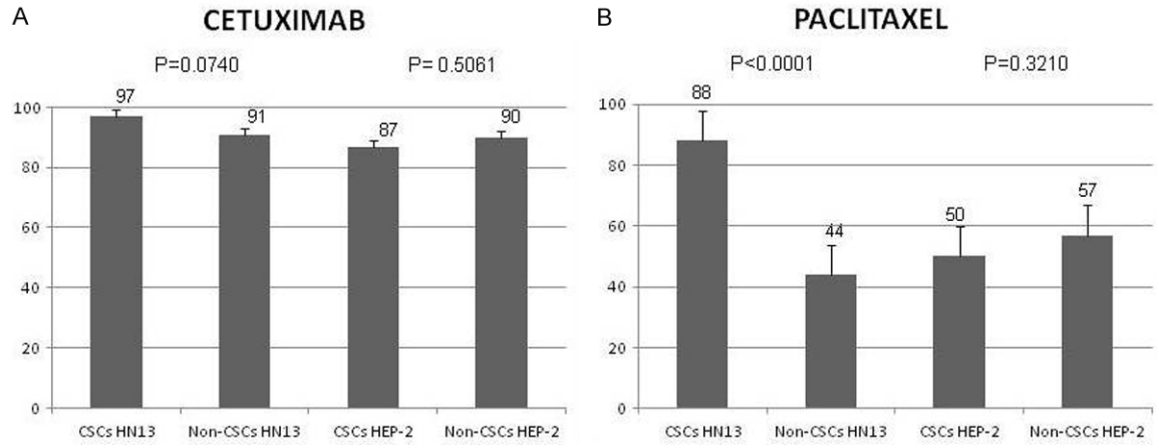

Figure 3. Cell proliferation of CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with chemotherapies after 24 hours. A. CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with Cetuximab. B. CSCs and Non-CSCs subpopulations of HN13 and HEP-2 cell lines treated with Paclitaxel chemotherapeutic.

The subpopulation of sorted HEP2 cell line with $CD44^{high}/CD133^{high}/CD117^{high}$ (CSCs) was detected in 0.8% and isolated of Non-CSCs. The expression of CD44, CD117 and CD133 were 0.3%, 0.4% and 0.1%, respectively.

Confirmation of presence of CSCs

After sorting, CSCs and Non-CSCs subpopulations were then collected and cultured separately under the same conditions, as described above. As shown in **Figures 1** and **2**, CSCs demonstrated increased invasive capacity as compared with Non-CSCs subpopulations after 24 hours in both cell lines. In CSC HN13 the migration was 92% and in Non-CSC HN13 was 53%. In CSC HEP-2 the migration was 94% and in Non-CSC HEP-2 was 13%.

Drug sensitivity of CSCs after treatment

Both CSCs and Non-CSCs subpopulations of cell lines were treated with Cetuximab and Paclitaxel agents, and then cell proliferation was assessed using MTS assay. As shown in **Figure 3**, CSCs subpopulation cells demonstrated more cell proliferation when compared with Non-CSCs subpopulation in HN13 and HEP-2 cell lines.

Expression of genes related to stem cell and cancer drug resistance in Non-CSCs and CSCs subpopulations

To exanimate the difference in the expression of genes related to stem cell and cancer drug resistance between Non-CSCs and CSCs subpopulations cells, we used delta threshold cycle (Ct) method according to mathematical following formula: Expression level of target gene = 2-(Delta Ct) × 1,000 Delta Ct = Ct of target gene - (Mean Ct of β-actin and GAPDH genes). Regarding to HN13 cell line, we found that CD44 gene presented up-regulation (rate < 2.0) in CSCs when compared with Non-CSCs and, EGFR gene presented down-regulation (rate > 2.0) in CSCs when compared with Non-CSCs. For HEP-2 cell line the results showed that CD44 gene presented down-regulation (rate < 2.0) in CSCs when compared with Non-CSCs and, EGFR gene presented up-regulation (rate > 2.0) in CSCs when compared with Non-CSCs (**Table 1**).

Discussion

We firstly characterized CSCs in two head and neck cell lines with CD44, CD133 and CD117 biomarkers. So we treated CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel chemoterapies and found that CSCs subpopulations demonstrated more resistance to Paclitaxel, as compared with Non-CSCs subpopulations in HN13 cell line. These HN13 CSCs subpopulations presented up-regulation of *CD44* gene and down-regulation of *EGFR* gene when compared with Non-CSCs subpopulations while HEP-2 CSCs presented down-regulation of *CD44* gene and up-regulation of *EGFR* gene when compared with Non-CSCs subpopulations.

Table 1. *CD44* and *EGFR* gene expression in CSCs HN13 and CSCs HEP-2 cell lines

Gene symbol	Gene description	GenBank	Fold change			
			CSCs HN13	Non-CSCs HN13	CSCs HEP-2	Non-CSCs HEP-2
CD44	The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration.	NM_000610.3	102.775859	1 (REF)	0.65892	1 (REF)
EGFR	EGFR and its ligands are cell signaling molecules involved in diverse cellular functions, including cell proliferation, differentiation, motility, and survival, and in tissue development	NM_001346897.1	0.741344907	1 (REF)	7.55986	1 (REF)

Regarding to characterization, the culture condition was capable of expanding $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells from HN13 and HEP-2 cell lines. CD44 biomarker is a cell surface hyaluronan receptor protein involved in cell adhesion, cell-cell interactions and cell proliferation besides being receptor for hyaluronic acid [21, 22]. CD44 was firstly identified in head and neck cancer in 2007 by Prince and collaborators and found that positive CD44 cells initiated tumor growth with high tumorigenic potential and differentiation capacity when compared with negative CD44 cells confirming that positive CD44 population of human head and neck cancer has properties of cancer stem cells and head and neck cancer contain a subpopulation of CSC, which was confirmed in our study in HNC cell lines [16].

CD133 biomarker is a cell-surface glycoprotein comprising five trans-membrane domains associated with cell membrane topology organization. It is often expressed on adult stem cells with function of maintaining stem cell properties by suppressing differentiation [23, 24]. CD133 also has been identified human tongue, laryngeal and bucal cancer cell lines with ability of tumorigenic, power of cell proliferation and differentiation when compared to CD133-subpopulations, now we confirmed the identification of CD133+ cells in oral cancer cell line [25-28].

CD117 biomarker is a transmembrane receptor for MGF (mast cell growth factor, also known as stem cell factor) with cellular function not entirely known, however studies show that CD117 promotes the proliferation, survival, and metastasis of tumor cells and has been regarded as a cancer stem cell biomarker, but is not yet evaluated in oral cancer. We found CD117 high in oral cancer cell line, however more studies is needed to evaluate the importance of this biomarker is cancer stem cells development [30-32].

Regarding to treatment of CSCs and Non-CSCs subpopulations with Cetuximab and Paclitaxel we found more resistance to Paclitaxel chemoterapy. As compared with Non-CSC subpopulations in both cell lines suggesting that $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells should be considered as targets in future therapies with Paclitaxel.

This is the first study that isolates cancer stem cells of head and neck cancer cell lines through of CD44/CD133/CD117 biomarkers in combination and evaluated the cancer treatment with Cetuximabe and Paclitaxel chemotherapies to single-modality treatment. Literature studies already evaluated these biomarkers alone and found that $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cells besides presenting stem cell properties also has ability to resist chemotherapeutic agents in cancer treatment, including head and neck cancer. Furthermore CSCs often have enhanced telomerase and DNA repair activities, as well as, membrane bound ATP-binding cassette transporters (ABC “drug” transporters) whose normal functions are to exclude xenobiotics, as chemotherapies [33-36].

Cetuximab is a monoclonal antibody binding the epidermal growth factor receptor (EGFR) on both normal and tumor cells. It is a functional antagonist of the EGF and TGF ligands and is thus inhibitors of the EGFR-dependent signaling pathways leading to inhibition of cancer cell division in the G1 phase and metastatic spread because of the lack of transcription factors [37]. In our study we found the Cetuximab is not effective in CSCs subpopulation of head and

neck cancer cell lines. There is a suggestion of pathways activated in head and neck cells by EGFR increase the migratory potential of cells and interfere with their sensitivity to single-modality treatment with cetuximab, as our study [38, 39].

Paclitaxel chemotherapy is a mitotic inhibitor used in cancer chemotherapy that interferes with the normal function of microtubule growth. It binds to the β subunit of tubulin, that is the "building block" of mictotubules, and the binding of paclitaxel locks these building blocks. The resulting microtubule/paclitaxel complex affects cell function leading to mitotic arrest, prevention of cell division, and eventually apoptosis [40]. In our study the Paclitaxel is not effective in CSCs subpopulation of oral cancer cell line. Studies show that mesenchymal stem cells have been shown to be highly resistant to the cytotoxic effects of Paclitaxel and other chemotherapeutic agents due to regulation of the cell cycle [41, 42].

Besides that we found high expression of CD44 gene in HN13 CSCs and down expression of CD44 gene in HEP-2 CSCs suggesting that the exact influence of CD44 gene expression in resistant to chemotherapy is not entirely clear. The mechanistic origins can be associated with DNA repair, resistance to apoptosis, low mitotic rate, and increased tolerance of DNA damage [48, 49] According literature data the high expression of CD44 has been identified in treatment resistant in cancer with CSCs properties, including head and neck cancer, as our study [34, 43-47]. The high expression of CD44 gene in CSCs and resistance treatment can be explained due to association of this gene with cell-cell interactions, cell adhesion and migration that is increased in CSCs.

We also found down expression of CD44 in laryngeal cancer cell line, reports confirmed that levels of CD44 expression are linked to stem cell properties [50, 51]. The HEP-2 cell line presented decreased rate of population expansion with cancer stem cell characteristics which may justify this finding. However several signalling pathways can be associated with CSCs survival and therapies that target such pathways might be therapeutically effective [52].

Regarding to EGFR gene expression, our study found that the HN13 CSCs showed down expression of EGFR and HEP-2 CSCs showed high expression of EGFR. The EGFR is found in surface of cells to which epidermal growth factor (EGF) binds. When EGF attaches to EGFR, it activates tyrosine kinase activity, triggering reactions that cause the cells to grow and multiply this way activates a wide variety of intracellular cascades and induces the regulation of target genes, leading to a specific cellular response [53, 54].

The blocking EGFR signaling has provided less therapeutic benefit and this may be related to the presence of sub-populations of CSCs and heterogeneity of tumours [55, 56]. Literature data confirm that head and neck patient tumors express EGFR (~98%), however only approximately 15-20% of patients respond positively and benefit from treatment [57, 58]. Our results suggest that 80-85% of patients may present tumor with CSCs and, consequently, alterations in EGFR expression, what can contribute to treatment resistance but the mechanisms are still unclear and need to be further studied in another cell lines and primary tumor.

In conclusion, our results show that the combination of CD44, CD133 and CD117 biomarkers have stem cell properties and ability to resist Paclitaxel chemoterapy. CSCs present high expression of CD44 gene and down expression of EGFR gene in oral cancer cell line. CSCs in laryngeal cell line presents down expression of CD44 gene and high expression of EGFR gene when compared with cells without characteristics of cancer stem cells.

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Disclosure of conflict of interest

None.

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