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The role of myeloid derived suppressor cells and CXCR4 genes expression for nasopharyngeal carcinoma progression

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Nasopharyngeal carcinoma (NPC) is radiosensitive, but prognosis remains poor with a 5-year survival around 50% due to secondary spread of tumor cells. Tumors growth is influenced by many factors, generally an interaction between genetic and environmental factors, particularly the microenvironment tumor. There is the role of myeloid derived suppressor cells (MDSC) in the process of tumors growth. MDSC are immature myeloid cells produced by bone marrow precursor cells that are increased in a variety of disease. Most significantly, MDSC are increased in cancer patients and significantly contribute to the immunosuppression. MDSC are then recruited to the tumor by such chemotactic factors as tumor derived CXCL12 and stem cell factor that bind to and active their respective receptors CXCR4 on MDSC. The main role of MDSC appeared to be due to immunosuppression of anti-tumor effectors and had significant effects of tumor progression. This study identified specific markers that can be used to identify MDSC and the relation with CXCR4 for NPC progressivity prediction. Peripheral blood specimen and biopsy from primary tumor were collected from 16 nasopharyngeal carcinoma patients. The samples collected underwent qRT-PCR. Data were analyzed by $2^{-\Delta\Delta Ct}$ methods and statistical analysis. CD14, CD15, and CXCR4 genes are expressed in peripheral blood and primary nasopharyngeal carcinoma. We found that patients with advanced stage had elevated number of circulating CD14, CD15, and CXCR4 although the increase of CXCR4 did not reach the significant level. MDSC and CXCR4 correlated significantly with T and N classification and clinical stage also. We concluded that expression of MDSC and CXCR4 play an important role in tumor progression and invasion in NPC.

Key words: Nasopharyngeal carcinoma, qRT-PCR, MDSC, CXCR4, clinical stage.

INTRODUCTION

In Indonesia, nasopharyngeal carcinoma (NPC) is a frequent cancer, rating as the fourth for all malignancy and the most common malignancy in the head and neck. NPC is prevalent among different native people and presents a major socioeconomic problem, with an overall incidence estimated at 6.2/100.000 or about 12.000 new cases per year (Adham et al., 2012).

NPC is a tumor derived from epithelial cells located in the posterior nasopharynx (Adham et al., 2012; Brennan, 2006; Thompson, 2007). Although the primary tumor is sensitive to radiotherapy, mortality and morbidity occur because of secondary spread of tumor cells (Wang et al., 2005). These tumors are highly malignant with extensive and early lymphatic spread and a high incidence of hematogenous spread (Thompson, 2007). The prognosis remains poor with a 5-year survival around 50% (Wang et

al., 2005).

NPC patients generally come with an advanced stage at the time of diagnosis; there are metastatic lymph nodes of the neck. Early diagnosis of NPC is still difficult to do because the symptoms are not typical at the initial examination.

Tumors growth is influenced by many factors, generally an interaction between genetic and environmental factors, particularly the microenvironment tumor. There is the role of myeloid derived suppressor cells (MDSC) in the process of tumors growth.

MDSC are immature myeloid cells produced by bone marrow precursor cells that are increased in a variety of disease. Most significantly, MDSC are increased in cancer patients and significantly contribute to the immunosuppression (Sherger et al., 2012). MDSC are heterogeneous populations of cells comprising immature myeloid progenitors for neutrophils, monocytes, and DC (Murdoch et al., 2008). Predominantly, granulocytic (expressing markers such as CD15) or monocytic (expressing CD14) are phenotype described for MDSC (Brandau et al., 2011; Diaz-Montero et al., 2009; Gabrilovich and Nagaraj, 2009; Greten et al., 2011; Murdoch et al., 2008; Sawanobori et al., 2008; Zhang et al., 2013; Zhi et al., 2012). MDSC have also been described, such as the promotion of tumor angiogenesis, tumor-cell invasion and metastasis (Gabrilovich and Nagaraj, 2009), and the presence of MDSC in the peripheral blood and tumors of patients with various forms of cancer (Murdoch et al., 2008)

MDSC are then recruited to the tumor by such chemotactic factors as tumor derived CXCL12 and stem cell factor that bind to and active their respective receptors CXCR4 on MDSC. The main role of MDSC appeared to be due to immunosuppression of anti-tumor effectors and had significant effects of tumor progression (Murdoch et al., 2008).

Chemokine involved in cell activation, differentiation, and trafficking, bind to multiple receptors, and the same receptor may bind to more than one chemokine, for example, CXCL12 binds to CXCR4 and CXCR7 (Teicher and Fricker, 2010). Chemokine have been reported to participate in tumor growth and metastatic by promoting angiogenesis or by inducing directional migration of tumor cells (Hu et al., 2005; Teicher and Fricker, 2010; Wang et al., 2005). CXCL12 is overproduced by stromal and tumor cell in different tumor microenvironment (Obermajer et al., 2011). The chemokine CXCR4 is highly expressed by human primary NPC tissue and established cell lines and contributes to the progressivity of tumor (Hu et al., 2005). Immunosuppressive cell recruited and retained in tumor microenvironment limits the effectiveness of immune response (Obermajer et al., 2011).

This study identified specific markers that can be used to identify specific myeloid cell population and the relation with CXCR4 for nasopharyngeal progressivity prediction.

MATERIALS AND METHODS

Clinical samples

Peripheral blood specimen and biopsy from primary tumor, collected from 16 nasopharyngeal carcinoma patients in oncology head and neck clinic, Departement of Otorhinolaryngology, Hasan Sadikin General Hospital Bandung, Indonesia, with total numbers of samples, were 32 (mean age 49.12; median 53; range 16-69; standard deviation ± 14.98) with newly diagnosed. Early stage are stages I/II (n=8) and advanced stage are III/IV (n=8) in accordance with American Joint Committee on Cancer (AJCC), Cancer Staging Manual, 7th edition 2010. All samples were collected after obtaining informed consent from the patient. 3 ml of venous blood was collected in EDTA tube and tissue from biopsy were collected and transferred into sterile tubes containing RNA later solution (Ambion, USA). This study was approved by the Research Ethics Board of the Faculty of Medicine, Padjadjaran University, Bandung Indonesia. Samples are divided into four groups; group I were blood samples from early stage NPC patients; group II were tissue samples from early stage; group III were blood samples from advanced stage; and group IV were tissue from advanced stages NPC patients.

According to TNM classification with AJCC, Cancer Staging Manual in nasopharyngeal carcinoma are for primary tumor (T); T1 (Tumor confined to the nasopharynx; T2 (tumor extends to soft tissue), T2a (tumor extends to the oropharynx and/or nasal cavity without parapharyngeal extension, T2b (any tumor with parapharyngeal extension); T3 (tumor involves bony structures and or/paranasal sinuses); T4 (tumor with intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, orbit, or masticator space). For regional lymph node (N); N0 (no regional lymph node metastasis); N1 (unilateral metastasis in lymph node, 6 cm or less in greatest dimension, above the supraclavicular fossa; N2 (bilateral metastasis in lymph node, 6 cm or less in greatest dimension, above the supraclavicular fossa; N3 (metastasis in a lymph node >6 cm and/or to supraclavicular fossa, N3a (greater than 6 cm in dimension), N3b (extension to the supraclavicular fossa). For distant metastasis (M); M0 (no distant metastasis); M1 (distant metastasis) (Cancer American Joint Committee, 2010).

Quantitative real time polymerase chain reaction (qRT-PCR)

The expression levels of mRNA were determined using universal one-step qRT-PCR kit according to the manufacturer's instructions (Kapa Biosystems, USA). The primers consisted of forward and reverse for human

Table 1. Primers used for qRT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GADPH	TGTTCCAATATGATTCCACCCAT	AGCCACACCATCCTAGTTGC
CD14	AGAATCCTTCCTGTTACGGT	CTCTGACAGTTTATGTAATCCT
CD15	CTTTGTGCCTTATGGCTACC	TTGGCTCAGTTGGTGGTAGT
CXCR4	CAATGGATTGGTCATCCTGG	GACTGATGAAGGCCAGGATG

glyceraldehyde-3-phosphate dehydrogenase (GADPH), CD14, CD15, and CXCR4 (Table 1). The qRT-PCR reaction was subjected to reverse transcription (RT) for five minutes at 42°C, followed by enzyme inactivation at 95°C for 3 min, and the cycle of PCR for 40 cycles at 95°C for 30 s (denature), 60°C for 20 s (annealing) and 72°C for 20 s (extension data acquisition). All reactions were run in duplicate. The result can be seen in the form of threshold cycle (Ct curves), by applying the relative changes in targeting genes expression (Budiman et al., 2015; Livak and Schmittgen, 2001; Shahib et al., 2015). Ct (Cycle of threshold) is reported as the PCR cycle number that crosses an arbitrary placed signal threshold (Budiman et al., 2015; Schmittgen and Zakrajsek, 2000). The average Ct was calculated for both targets gene and internal control (GADPH) and the ΔCt were equal to the difference in the threshold cycles for target and GADPH (Ct target-Ct GADPH).

Data analysis

qRT-PCR was analyzed by calculating the fold difference individually for each gene. Ct is defined as the number of PCR cycles at which the fluorescence signal rises above the threshold value and is inversely propositional to the amount of template present in the reaction.

The $2^{-\Delta\Delta Ct}$ methods used to calculate relative changes in gene expression were determined from qRT-PCR. The data were analyzed using:

$$\Delta\Delta Ct = (Ct._{target}\text{-}Ct_{GADPH})_{\text{time x}} - (Ct._{target}\text{-}Ct_{GADPH})_{\text{time 0}} \quad (1)$$

Time $_x$ is any time point and time $_0$ represents the 1x expression of the target gene normalized to GADPH. The mean Ct values for both the target and control gene were determined at time zero and was used in Equation 1. The mean and SD are then determined from the samples at each time point (Livak and Schmittgen, 2001; Shahib et al., 2015).

We categorized the Ct values varying from 15 to 40; 15-20 was very high expression; >20-25 was high expression; >25-30 was moderate expressions; >30-35 was low expression; and >35-40 was very low or no expression. We also adopted the possibilities of genes expression under 15 of Ct value were over expression (Budiman et al., 2015; Shahib et al., 2015). All these categories were confirmed with gel electrophoretic data.

Statistical analysis

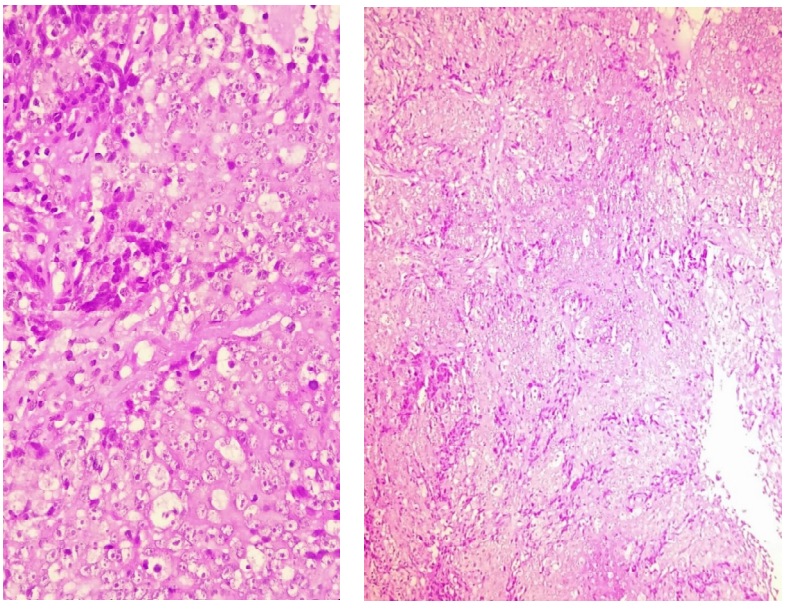
Statistical data were converted to the linear form by the ΔCt calculations. Pearson analysis was used to evaluate the normal data distribution, and alternative Spearman analysis was used if the data were not normally distributed. Spearman was used to analyse the correlation between ordinal and numeric data and correlation between nominal and numeric data by Eta analysis. A p-value <0.05 was considered significant and statistical test were performed using the software SPSS version 21 (SPSS Inc., Chicago, Illinois).

RESULTS

Freshly drawn whole blood and tissue from nasopharyngeal carcinoma patients was therefore labeled with CD14 and CD15 for myeloid marker and CXCR4 gene expression, and GADPH by analysis the mRNA transcripts. Among those, 16(100%) undifferentiated nasopharyngeal carcinoma (Figure 1); 22(68.8%) are male and 10(31.3%) are female. The mRNA transcripts were determined by Real-Time PCR, which identified 32 genes expressions. The mean Ct values for four group I, II, III, and IV were determined and the amount of the transcripts = $2^{-\Delta\Delta Ct}$.

For AJCC clinical stage I, there was one patient. While for stage II, there were seven patients, stages III were two patients, and stages IV were six patients. For N classification, there were two patients for NO, eight patients for N1, two patients for N2, and four patients for N3. For T classifications T1 there were two patients, while for T2 classifications were eight patients, one patient for T3 and five patients for T4 classification.

The order of two targeting genes was placed from lower to highest based on their Ct values compared to GADPH. The ΔCt value that was lower than GADPH was placed in a group of negative ΔCt (-ΔCt). –ΔCt suggested a higher expression than GADPH. Calculation of data was adapted from Livak and Schimttgen (2001) and Shahib et al. (2015). ΔΔCt = ΔCt (target gene) – ΔCt (GADPH). The mean Ct values for both peripheral blood and tissue from early and advanced stage of



(a) (b)

Figure 1. Tumor masses that consist of cells that are round oval, arranged in groups, polymorphic cell nucleus, and vesicular, prominent core, around it look lymphocytes. **(a)** Magnification 400 times; **(b)** Magnification 40 times.

nasopharyngeal carcinoma were determined and the amount of the transcripts=$2^{-\Delta\Delta Ct}$.

By using GADPH as a reference gene, we determined ΔCt that is equal to the difference in Ct for target and reference gene. The expression were studied and completed by analyzing the Ct values of 32 gene expressions compared to GAPDH expressions of peripheral blood and tissue of nasopharyngeal carcinoma in early and advanced stage.

In Table 2, the variations of expressions of the peripheral blood and tissue from early and advanced stage of nasopharyngeal carcinoma, were high expression of CD14 and CD15, and it was found the overexpression in CXCR4 gene in all specimens.

In Table 2, of each eight-gene expression in blood from early stage of NPC, all gene expression was –ΔCt varying from -0.93 to -19.83. The result for advanced stage, whole sample was -ΔCt varying from -1.76 to -20.43. Of each eight from early stage of nasopharyngeal carcinoma tissue was –ΔCt varying from -6.22 to -24.93. Otherwise for advanced stage of nasopharyngeal carcinoma tissue was –ΔCt varying from -4.06 to -21.45.

The profile of gene expressions is also shown in Table 3 that CD14, CD15, and CXCR4 were increased 52.42, 38.03, and 33.30 in blood of advanced stage of nasopharyngeal carcinoma. In the tissue of advanced stage of nasopharyngeal carcinoma was also fold increased of CD14, CD15, and CXCR4 were 6.94, 0.28, and 1.03. All gene were up regulated, the remaining CD14 and CD15 in blood were significantly different.

Analysis of correlation between CD14 and CD15 using Rank Spearman statistical analysis had significance or p value = 0.0001 and r = 0.856. This indicated a statistically significant and there was a strong correlation. It can be concluded that CD14 and CD15 were MDSC sub variable.

CXCR4 expressions were significantly related to clinical stage (r=-0.452; p=0.009), T classification (r=0.367; p=0.039), and N classification (r=-0.454; p=0.009) but not significant for age (r=0.108; p=0.558) and gender (r=0.015; p=1.000). MDSC were significantly related to age (r=-0.517; p=0.002), T classification (r=-0.515; p=0.003), N classification (r=-0.472; p=0.006), and clinical stage (r=-0.601; p=0.001). No significant correlation was detected between MDSC and gender (r=0.032; p=1.000) (Table 4).

CXCR4 expression was significantly correlated with MDSC in clinical samples of NPC (r=0.640; p=0.000) (Table 4).

DISCUSSION

Analysis of early and advanced stage from 16 NPC patients was determined based on the $2^{-\Delta\Delta Ct}$ methods varied from the lowest to highest expression (Table 2). CD14 and CD15 gene expression were found higher and overexpression at CXCR4 gene in peripheral blood and tissue of NPC patients (Table 2). These results suggest that CD14, CD15, and CXCR4 genes are expressed in peripheral blood and primary nasopharyngeal carcinoma. Additional experiments showed that nasopharyngeal carcinoma tissue expressing CD14, CD15, and CXCR4

Table 2. Ct value for 32 transcripts in total RNA samples from blood and tissue of nasopharyngeal carcinoma.

Targeting genes in (n=8)	GADPH Ct	Genes Ct	ΔCt (avg. target gene Ct - avg. GADPH Ct)		ΔΔCt	Normalized target gene relative to GADPH 2^-ΔΔCt	Categories of expression based on the Ct Values
			+ΔCt	-Δ Ct			
Group I							
GADPH	24.26±4.39		0.00				
CD15		23.33±2.46		-0.93±4.58	-0.93	1.91	High
CD14		22.25±4.76		-2.01±3.39	-2.01	4.03	High
CXCR4		4.43±1.02		-19.83±5.18	-19.83	932019.10	Over expression
Group II							
GADPH	24.44±2.64		0.00				
CD14		22.68±4.58		-1.76±2.90	-1.76	3.39	High
CD15		20.73±1.54		-3.71±2.35	-3.71	13.09	High
CXCR4		3.95±0.23		-20.43±2.67	-20.43	1412676.80	Over expression
Group III							
GADPH	29.09±2.07		0.00				
CD15		22.87±1.34		-6.22±1.21	-6.22	74.54	High
CD14		21.33±1.47		-7.75±1.06	-7.75	215.27	High
CXCR4		4.16±0.47		-24.93±2.31	-24.93	31965226.93	Over expression
Group IV							
GADPH	25.13±1.69		0.00				
CD15		21.07±1.67		-4.06±1.35	-4.06	16.68	High
CD14		20.38±2.50		-4.75±2.04	-4.75	26.91	High
CXCR4		3.68±0.09		-21.45±1.64	-21.45	2864794.06	Over expression

Table 3. The relative fold change of gene expressions in peripheral blood and tissue of nasopharyngeal carcinoma of early stage compared to the advanced stage.

Target Genes	Early Stage	Advanced Stage	Fold increased (↑) fold decreased (↓)	p-value
Blood				
CD14	4.03	215.27	52.42(↑)	0.011**
CD15	1.91	74.54	38.03(↑)	0.012**
CXCR4	932019.10	31965226.93	33.30(↑)	0.292
Tissue				
CD14	3.39	26.91	6.94(↑)	0.333
CD15	13.09	16.80	0.28(↑)	0.154
CXCR4	1412676.80	2864794.06	1.03(↑)	0.163

Note: p value was analyzed by unpaired t test for numeric data when normally distributed, alternative Mann Whitney if the data was not normally distributed. Significant if $p<0.05$. Double asterisk (**) is significant.

genes especially peripheral blood containing high level of mRNA as determined by RT-PCR suggesting that this genes is more widely expressed in peripheral blood NPC than tissue.

We found that patients with advanced stage had elevated number of circulating CD14, CD15, and CXCR4

Table 4. The Correlation of CXCR4 and MDSC with Age, Gender, Clinical Stage, T, and N classification.

Variable	r	p
CXCR4 and MDSC	0.640	0.000**
CXCR4 and Age	0.108	0.558
CXCR4 and Gender	0.015	1.000
CXCR4 and Clinical Stage	-0.452	0.009*
CXCR4 and T	0.367	0.039*
CXCR4 and N	-0.454	0.009*
MDSC and Age	0.517	0.002**
MDSC and T	-0.515	0.003**
MDSC and N	-0,472	0.006**
MDSC and Gender	0.032	1.000
MDSC and Clinical Stage	-0.601	0.001**

Note: Analyzed by Rank-Spearman Correlation. Significant if $p<0.05$. Double asterisk (**) is significant. r: Correlation Coefficient .

although the increased of CXCR4 did not reach the significant level (Table 3). Closed correlation between advanced stage and circulating MDSC may play role in tumor invasion and metastasis. It means that MDSC in blood of nasopharyngeal carcinoma is one of critical players in mediating cancer immune evasion that suppressed T cell proliferation and cytokine production (Zhang et al., 2013).

Similar to peripheral blood, we also found the markedly elevated level of CD14, CD15, and CXCR4 in tumor tissues of advanced stage compared to early stage of NPC but did not reach the significant level (Table 3).

NPC cell induced the expansion of tumor-induced MDSC; in turn MDSC enchanted NPC cell migration and metastasis (Li, 2015). Li (2015) mentions that up regulation of tumor cyclooxygenase-2 (COX-2) expression positively correlated with the expansion of MDSC in NPC patients and both of COX-2 and MDSC were poor predictors for disease free survival. Wang et al. (2005) found that CXCR4 was highly expressed in NPC cell lines. Among the known factors associated with NPC induction, Epstein Barr Virus (EBV) infection plays an important role. Latent membrane protein (LMP)-1 is the EBV-encoded protein with the most significant oncogenic properties. In addition, LMP-1 induces NF-κB activation, which has important effects on EBV-infected. NF-κB regulates the motility of cells by directly up regulating the expression of CXCR4 (Wang et al., 2005).

Pro tumor action of MDSC is not limited to their immunosuppressive properties; these cells have also been shown to favor cancer progression by promoting angiogenesis, cancer cell proliferation, invasion, and metastasis (Wesolowski et al., 2013; Zhi et al., 2012). The expansion, activation, and accumulation of MDSC in peripheral tissue can be driven by multiple factors produced by tumor cells, tumor stromal cell, or by activated T cells. These mediators include chemokine CXCL12. These agents either promote MDSC expansion through the JAK2/STAT3 signaling pathway or induce the activation of MDSC via STAT1, STAT6, or through NF-κB-dependent mechanism (Wesolowski et al., 2013; Zhi et al., 2012).

MDSC have been also demonstrated to support neoangiogenesis, tumor growth, and metastasis. These cells are known to produce VEGF, basic fibroblast growth factors (bFGF), hypoxia-induced factor (HIF)-1, TGF-β, and MMP9 that promote angiogenesis and create a pre-metastatic environment (Sevko and Umansky, 2013).

Hu et al. (2005) suggested that CXCR4 expression correlate with the state of tumor cell differentiation grade and proliferation and metastatic potential of human NPC cells. Wang et al. (2005) identified that CXCR4 could be involved in NPC progression and could be used as a predictor for NPC prognosis. Diaz-Montero et al. (2009) found that circulating levels of MDSC were prevalent in cancer patient and correlated with clinical stage.

Our study results showed that both MDSC and CXCR4 expressions significantly correlated with T and N classification and clinical stage also, thus MDSC and CXCR4 play role in tumor progression of nasopharyngeal carcinoma.

The pathogenesis of NPC is closely related to EBV infection with up to 70% of patients with NPC were EBV latent membrane protein-1 (LMP-1) positive (Morris et al., 2008; Munz and Moormann, 2008; Savitri and Mubarika, 2012). It has been reported that LMP-1 has the capacity to cause activation of nuclear factor-κB (NF-κB), a potent transcription factor. It is relevant that activation of NF-κB to enhance the expression of CXCR4 may promote tumor cell migration and metastasis (Hu et al., 2005).

Obermajer et al. (2011) showed that tumor-associated inflammatory mediator, PGE2 induces both CXCL12/SDF-1 chemokine production in tumor microenvironment, and CXCR4 expression to CXCL12.

MDSC is critically important for their continued expression of CXCR4 and responsiveness to CXCL12, promoting the attraction and retention of MDSC in tumor microenvironment (Obermajer et al., 2011). Chemokines play a crucial role in immune and inflammatory reactions; they have an equally important role in the development of a variety of cancers, being involved in cell transformation, survival, growth metastasis, and tumor-associated angiogenesis (Obermajer et al., 2011).

The study performed also supports the significant correlation between MDSC and CXCR4. MDSC expressed CXCR4 to maintain inflammatory and immune suppression in tumor and blood.

Conclusion

From our study, we concluded that expression of MDSC and CXCR4 play important role in tumor progression and invasion in NPC. We suggest that precise identification of specific changes in MDSC subpopulations and their distribution during tumor development is required for an improved understanding of the roles of MDSC in carcinogenesis of NPC.

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