Profiling of inhibitory immune checkpoints in glioblastoma: Potential pathogenetic players

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Abstract. Glioblastoma (GBM) represents the most frequent glial tumor, with almost 3 new cases per 100,000 people per year. Despite treatment, the prognosis for GBM patients remains extremely poor, with a median survival of 14.6 months, and a 5-year survival less than 5%. It is generally believed that GBM creates a highly immunosuppressive microenvironment, sustained by the expression of immune-regulatory factors, including inhibitory immune checkpoints, on both infiltrating cells and tumor cells. However, the trials assessing the efficacy of current immune checkpoint inhibitors in GBM are still disappointing. In the present study, the expression levels of several inhibitory immune checkpoints in GBM (CD276, VTCN1, CD47, PVR, TNFRSF14, CD200, LGALS9, NECTIN2 and CD48) were characterized in order to evaluate their potential as prognostic and eventually, therapeutic targets. Among the investigated immune checkpoints, TNFRSF14 and NECTIN2 were identified as the most promising targets in GBM. In particular, a higher TNFRSF14 expression was associated with worse overall survival and disease-free survival, and with a lower Th1 response.

Introduction

According to the World Health Organization (WHO) classification of the central nervous system (CNS) tumors, glioblastoma (GBM) is defined as a grade IV astrocytoma (1). GBM represents the most malignant glioma and it is characterized by necrosis, neovascularization and histological heterogeneity (2). GBM represents the most frequent glial tumor, with almost 3 new cases per 100,000 people per year (3). The current standard of care for GBM consists of surgical resection, followed by radiotherapy and chemotherapy with temozolomide (4). Despite treatment, the prognosis for GBM patients remains extremely poor, with a median survival period of 14.6 months, and the 5-year survival is less than 5% (4).

In recent years, great progress has been made in the area of immunotherapy and accumulating preclinical and clinical data seem to suggest potential novel therapeutic avenues for GBM patients (5,6). It is generally believed that GBM creates a highly immunosuppressive immunoregulatory microenvironment. Several checkpoint molecules capable of inhibiting the immune responses against neo-antigens, including CTLA4 and PD1/PDL-1, are expressed on both T cells and cancer cells. Immune checkpoint inhibitors, such as nivolumab, ipilimumab and pembrolizumab, have strikingly improved patient survival in solid tumors, such as non-small lung cancer and melanoma. However, the trials assessing the efficacy of immune checkpoint inhibitors in GBM are still disappointing (7). A retrospective study of the use of pembrolizumab in the treatment of recurrent CNS tumors, including GBM, demonstrated that patients treated with Pembrolizumab did not have improved survival (7). Another Phase III randomized trial comparing radiation and concomitant temozolomide with or without nivolumab showed that no progression-free survival benefits were obtained by the addition of nivolumab. However, in a Phase II trial, preoperative administration of nivolumab increased chemokine expression and T-cell receptor clonal diversity, which likely promotes immune-cell infiltration and antitumor immune response (7).

It is reasonable that targeting multiple immune checkpoints in combination with cytotoxic drugs could represent a promising strategy for GBM. The present study characterized the expression levels of several inhibitory immune checkpoints in GBM (i.e., CD276, VTCN1, CD47, PVR, TNFRSF14, CD200, LGALS9, NECTIN2 and CD48) in order to evaluate their prognostic value. Moreover, their potential effects in regulating immune-cell infiltration was investigated.

Materials and methods

Profiling of inhibitory immune checkpoints in GBM. In order to evaluate the expression levels of inhibitory immune check-
Expression of inhibitory immune checkpoints in GBM. A significant upregulation in the expression levels of CD276, 

\[ \text{Figure 1. Expression of immune checkpoints in glioblastoma. Relative expression levels of the selected inhibitory immune checkpoints in glioblastoma, lower grade astrocytomas and normal brain samples are presented as heatmap (A). Correlation of the selected inhibitory immune checkpoints (B). Pearson correlation coefficient is presented in blue-red gradient and significance in yellow gradient.} \]
Table I. Expression of selected immune checkpoints in gliomas.

|                      | CD276 (Log mean ± SD) | VTCN1 (Log mean ± SD) | CD47 (Log mean ± SD) | PVR (Log mean ± SD) | TNFRSF14 (Log mean ± SD) | CD200 (Log mean ± SD) | LGALS9 (Log mean ± SD) | CD48 (Log mean ± SD) | NECTIN2 (Log mean ± SD) |
|----------------------|------------------------|-----------------------|---------------------|-------------------|--------------------------|------------------------|------------------------|----------------------|-------------------------|
| Glioblastoma         | 11.47±0.61             | 2.09±1.61             | 11.3±0.45           | 9.17±0.55         | 9.09±0.84                 | 10.2±0.89             | 6.77±1.43             | 10.37±0.61           |
| Anaplastic astrocytoma| 10.46±0.70             | 2.78±1.54             | 11.10±0.44          | 8.77±0.55         | 8.19±1.14                 | 9.03±0.71             | 4.77±2.10             | 9.7±0.69             |
| Astrocytoma grade II | 9.99±0.63              | 2.98±1.36             | 11.07±0.52          | 8.62±0.52         | 7.81±0.78                 | 9.17±0.75             | 9.69±0.94             | 3.81±1.97            |
| Normal               | 8.79±0.36              | 0.15±0.83             | 12.17±0.13          | 9.69±0.51         | 7.75±0.46                 | 10.91±0.46            | 8.59±0.49             | 3.21±0.93            |

Glioblastoma vs. anaplastic astrocytoma
Adjusted P-value 1.38896E-30

Glioblastoma vs. astrocytoma grade II
Adjusted P-value 9.64928E-39

Glioblastoma vs. normal
Adjusted P-value 7.41774E-16

Anaplastic astrocytoma vs. astrocytoma grade II
Adjusted P-value 0.000115669

Anaplastic astrocytoma vs. normal
Adjusted P-value 6.24305E-07

Astrocytoma grade II vs. normal
Adjusted P-value 0.000601994
Table II. Overall survival for the selected immune checkpoints in glioblastoma.

|         | Mean Estimate | SE  | Lower bound | Upper bound | Median Estimate | SE  | Lower bound | Upper bound | Log-rank (Mantel-Cox) |
|---------|---------------|-----|-------------|-------------|----------------|-----|-------------|-------------|-----------------------|
|         | 95% CI        |     |             |             | 95% CI         |     |             |             | Chi-square            | Significance          |
| CD276   | Low           | 2,106.954 | 437.733     | 1,248.998   | 2,964.910      |     |             |             |                       |                       |
|         | High          | 1,170.322 | 156.700     | 863.189     | 1,477.455      |     |             |             |                       |                       |
|         | Overall       | 1,750.302 | 291.264     | 1,179.424   | 2,321.180      |     |             |             |                       |                       |
| VTCN1   | Low           | 1,510.133 | 334.027     | 846.440     | 2,155.825      |     |             |             |                       |                       |
|         | High          | 2,332.769 | 419.625     | 1,510.304   | 3,155.233      |     |             |             |                       |                       |
|         | Overall       | 1,915.041 | 270.368     | 1,385.119   | 2,444.963      |     |             |             |                       |                       |
| CD47    | Low           | 1,550.759 | 265.609     | 1,030.166   | 2,071.352      |     |             |             |                       |                       |
|         | High          | 1,538.345 | 268.458     | 1,012.168   | 2,064.523      |     |             |             |                       |                       |
|         | Overall       | 1,565.487 | 229.512     | 1,115.643   | 2,015.332      |     |             |             |                       |                       |
| PVR     | Low           | 1,812.897 | 344.718     | 1,137.251   | 2,488.544      |     |             |             |                       |                       |
|         | High          | 1,249.227 | 179.475     | 897.456     | 1,600.999      |     |             |             |                       |                       |
|         | Overall       | 1,579.214 | 214.791     | 1,158.224   | 2,000.205      |     |             |             |                       |                       |
| TNFRSF14| Low           | 2,376.561 | 534.969     | 1,328.021   | 3,425.100      |     |             |             |                       |                       |
|         | High          | 1,249.227 | 179.475     | 897.456     | 1,600.999      |     |             |             |                       |                       |
|         | Overall       | 1,696.854 | 245.369     | 1,215.931   | 2,177.777      |     |             |             |                       |                       |
| CD200   | Low           | 1,538.345 | 268.458     | 1,012.168   | 2,064.523      |     |             |             |                       |                       |
|         | High          | 1,960.634 | 491.408     | 997.474     | 2,923.794      |     |             |             |                       |                       |
|         | Overall       | 1,647.845 | 242.431     | 1,172.680   | 2,123.010      |     |             |             |                       |                       |
| LGALS9  | Low           | 1,678.101 | 252.097     | 1,183.991   | 2,172.212      |     |             |             |                       |                       |
|         | High          | 1,775.014 | 291.699     | 1,203.283   | 2,346.744      |     |             |             |                       |                       |
|         | Overall       | 1,754.181 | 202.451     | 1,357.377   | 2,150.985      |     |             |             |                       |                       |
| NECTIN2 | Low           | 1,914.122 | 383.529     | 1,162.406   | 2,665.839      |     |             |             |                       |                       |
|         | High          | 1,467.364 | 265.874     | 946.251     | 1,988.477      |     |             |             |                       |                       |
|         | Overall       | 1,693.007 | 232.899     | 1,236.525   | 2,149.490      |     |             |             |                       |                       |
VTCN1, TNFRSF14, LGALS9, NECTIN2 and CD48 was observed in GBM as compared to normal brain samples (Fig. 1A, Table I). On the contrary, a significant downregulation of CD47 and CD200 was observed in GBM as compared to normal brain samples, while a trend of downregulation was observed for PVR (Fig. 1A, Table I). Along the same lines, with the exception of LGALS9 and CD200, a significant modulation in the expression levels of the investigated immune checkpoints was observed between the GBM and anaplastic astrocytoma groups of samples (Fig. 1A, Table I). Moreover, the expression levels of these immune checkpoints were associated with patient survival, as shown in Figures 2 and 3. Table II. Continued.

| Median | 95% CI | Log-rank (Mantel-Cox) | Chi-square | Significance |
|--------|--------|------------------------|------------|--------------|
| Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound |
| CD48 Low | 1,535.158 | 253.307 | 1,038.675 | 2,031.640 | 1,376.000 | 182.438 | 1,018.422 | 1,733.578 | 1,376.000 | 182.438 | 1,018.422 | 1,733.578 | 0.001 | 0.970 |
| High | 1,685.861 | 357.415 | 985.327 | 2,386.395 | 1,275.000 | 260.223 | 764.962 | 990.956 | 1,275.000 | 260.223 | 764.962 | 990.956 | 0.001 | 0.970 | 0.001 | 0.970 |
| Overall | 1,630.422 | 240.486 | 1,159.068 | 2,101.775 | 1,298.000 | 156.655 | 990.956 | 1,605.044 | 1,298.000 | 156.655 | 990.956 | 1,605.044 | 0.001 | 0.970 | 0.001 | 0.970 | 0.001 | 0.970 |

Figure 2. Effect of immune checkpoint expression on overall survival in glioblastoma. Kaplan-Meier curve for the overall survival of glioblastoma patients stratified on the expression levels of TNFRSF14.

Figure 3. Effect of immune checkpoint expression on disease-free survival in glioblastoma. (A) Kaplan-Meier curve for the disease-free survival of glioblastoma patients stratified on the expression levels of CD276; (B) Kaplan-Meier curve for the disease-free survival of glioblastoma patients stratified on the expression levels of VTCN1; (C) Kaplan-Meier curve for the disease-free survival of glioblastoma patients stratified on the expression levels of TNFRSF14; (D) Kaplan-Meier curve for the disease-free survival of glioblastoma patients stratified on the expression levels of NECTIN2.
Table III. Disease-free survival for the selected immune checkpoints in glioblastoma.

|                | Mean 95% CI | Median 95% CI | Log-rank (Mantel-Cox) |
|----------------|-------------|---------------|-----------------------|
|                | Estimate    | SE            | Lower bound | Upper bound | Estimate | SE | Lower bound | Upper bound | Chi-square | Significance |
| **CD276**      |             |               |            |            |          |    |            |            |            |             |
| Low            | 19.417      | 4.607         | 10.386     | 28.447     | 11.270   | 1.527 | 8.277       | 14.263      | 10.000     | 0.002        |
| High           | 6.726       | 1.132         | 4.507      | 8.945      | 4.300    | 0.760 | 2.810       | 5.790       |            |              |
| Overall        | 13.590      | 2.671         | 8.354      | 18.825     | 7.590    | 1.724 | 4.211       | 10.969      |            |              |
| **VTCN1**      |             |               |            |            |          |    |            |            |            |             |
| Low            | 8.510       | 1.615         | 5.346      | 11.675     | 4.730    | 0.966 | 2.836       | 6.624       |            |              |
| High           | 19.102      | 4.450         | 10.380     | 27.824     | 9.460    | 2.380 | 4.795       | 14.125      |            |              |
| Overall        | 13.618      | 2.366         | 8.981      | 18.255     | 5.980    | 1.070 | 3.883       | 8.077       | 5.944      | 0.015        |
| **CD47**       |             |               |            |            |          |    |            |            |            |             |
| Low            | 11.583      | 2.051         | 7.562      | 15.603     | 8.510    | 1.797 | 4.987       | 12.033      |            |              |
| High           | 11.562      | 2.939         | 5.801      | 17.323     | 5.390    | 0.833 | 3.757       | 7.023       |            |              |
| Overall        | 11.544      | 1.811         | 7.994      | 15.094     | 7.030    | 1.056 | 4.960       | 9.100       | 0.182      | 0.670        |
| **PVR**        |             |               |            |            |          |    |            |            |            |             |
| Low            | 13.275      | 3.278         | 6.851      | 19.699     | 5.910    | 2.202 | 1.593       | 10.227      |            |              |
| High           | 6.936       | 1.188         | 4.608      | 9.264      | 4.860    | 0.715 | 3.458       | 6.262       |            |              |
| Overall        | 10.343      | 1.906         | 6.608      | 14.078     | 5.190    | 0.506 | 4.199       | 6.181       | 2.563      | 0.109        |
| **TNFRSF14**   |             |               |            |            |          |    |            |            |            |             |
| Low            | 19.741      | 5.110         | 9.725      | 29.756     | 7.620    | 1.766 | 4.158       | 11.082      |            |              |
| High           | 7.659       | 1.249         | 5.455      | 9.862      | 5.390    | 0.715 | 3.988       | 6.792       |            |              |
| Overall        | 13.405      | 2.628         | 8.253      | 18.557     | 5.910    | 0.974 | 4.000       | 7.820       | 4.168      | 0.041        |
| **CD200**      |             |               |            |            |          |    |            |            |            |             |
| Low            | 8.966       | 1.925         | 5.194      | 12.738     | 5.160    | 0.873 | 3.448       | 6.872       |            |              |
| High           | 17.597      | 4.920         | 7.954      | 27.239     | 8.410    | 1.419 | 5.628       | 11.192      |            |              |
| Overall        | 12.064      | 2.308         | 7.539      | 16.588     | 6.670    | 0.761 | 5.179       | 8.161       | 2.805      | 0.094        |
| **LGALS9**     |             |               |            |            |          |    |            |            |            |             |
| Low            | 14.228      | 2.693         | 8.950      | 19.507     | 10.580   | 2.960 | 4.779       | 16.381      |            |              |
| High           | 10.808      | 2.001         | 6.887      | 14.729     | 6.340    | 1.336 | 3.722       | 8.958       |            |              |
| Overall        | 12.388      | 1.641         | 9.171      | 15.604     | 7.620    | 1.210 | 5.248       | 9.992       | 1.283      | 0.257        |
| **NECTIN2**    |             |               |            |            |          |    |            |            |            |             |
| Low            | 14.843      | 3.950         | 7.101      | 22.584     | 7.030    | 1.789 | 3.524       | 10.536      |            |              |
| High           | 7.493       | 1.462         | 4.627      | 10.359     | 4.860    | 0.698 | 3.491       | 6.229       |            |              |
| Overall        | 10.843      | 1.991         | 6.942      | 14.745     | 5.190    | 0.480 | 4.249       | 6.131       | 4.010      | 0.045        |
CD276, TNFRSF14, LGALS9 and CD48 resulted significantly upregulated in anaplastic astrocytoma samples as compared to grade II astrocytomas (Fig. 1A, Table I). A significant direct correlation was observed for CD276, PVR, TNFRSF14, NECTIN2 and CD48 (Fig. 1B). Among the GBM samples, a significant negative correlation was instead observed between VTCN1 and PVR, NECTIN2 and CD48 (Fig. 1B).

Survival analysis. Samples were stratified in quartiles based on the expression of the genes of interest, and samples in the upper and lower quartiles were selected for comparison. As shown in Table II and Fig. 2, higher expression levels of TNFRSF14 in GBM were associated to a significantly lower overall survival. No significance was observed for any of the other immune checkpoints. Accordingly, higher TNFRSF14 levels were associated to a shorter disease-free time (Fig. 3 and Table III). Lower levels of CD276 and NECTIN2 were also significantly associated to better disease-free time (Fig. 3 and Table III). Unexpectedly, higher levels of VTCN1 were associated to a longer disease-free time (Fig. 3 and Table III).

Deconvolution analysis. Deconvolution analysis of cell infiltration in GBM was performed on samples dichotomized on the expression levels of the immune checkpoints associated to a significant modulation of survival, i.e., CD276, VTCN1, TNFRSF14 and NECTIN2. As shown in Fig. 4, higher levels of CD276, TNFRSF14 and NECTIN2 were associated with a significant lower proportion of infiltrating plasma cells. Higher VTCN1 levels were associated to higher proportions of infiltrating plasma cells, along with higher infiltration of Th1, aDCs and cDCs (Fig. 4B). Samples with high expression levels of TNFRSF14 were characterized by a significant lower infiltration of Th1 cells and cDC, and higher proportions of iDCs, aDCs, pDCs and of macrophages (both M1 and M2) (Fig. 4C). A significantly higher infiltration of iDCs, aDCs and M1 macrophages, along with reduced proportions of Th1, Th2 and CD8 T cells, were observed in GBM samples with high NECTIN2 expression levels (Fig. 4D).

Discussion

Conventional immune checkpoint inhibitors, Nivolumab/Pembrolizumab for PD-1/PDL1 blockade or Ipilimumab for CTLA4, have proven beneficial effects on the clinical course of different cancer types, including metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, and Hodgkin lymphoma (9-11). However, these treatments have often failed in gliomas (12-14). A possible explanation for this outcome seems to be due to two main glioma features: the low tumor mutational burden (TMB) and a highly immunosuppressive microenvironment. Identifying genomic markers of response to immune checkpoint may benefit cancer patients by providing predictive biomarkers for patient stratification and identifying resistance mechanisms for therapeutic targeting.

The present investigation evaluated the potential role of a series of inhibitory immune checkpoints not previously studied or only marginally characterized in GBM, i.e., CD276, VTCN1, CD47, PVR, TNFRSF14, CD200, LGALS9, NECTIN2 and CD48. To this aim, a computational analysis of RNA-seq data obtained from the TCGA (The Cancer Genome
Atlas) database was performed. Whole-genome expression data was largely used (15) to identify pathogenic pathways and therapeutic targets for several disorders, including autoimmune diseases (16-23) and cancer (24-29).

We found that VTCN1 and CD200 are highly over-expressed in GBM, anaplastic astrocytoma and astrocytoma grade II compared to normal brain. Previously, Yao et al (30) showed that VTCN1 has a crucial role in the creation and maintenance of the immunosuppressive microenvironment in gliomas, correlating with prognosis and malignant grades. Furthermore, lower levels of VTCN1 are associated with a higher survival in a clinical trial of DC based vaccination (31). This is in contrast with our observations, which appears to show a protective role for VTCN1 in GBM. The reasons for this counterintuitive data is currently object of further exploration.

On the contrary, CD200 expression levels resulted in significantly reduced astrocytomas in comparison to normal brain. CD200 is a type I transmembrane glycoprotein that plays an inhibitory role in the activation of microglia. For this reason, many studies have shown that its expression is enhanced in brain tumors (32), and especially in higher grade tumors (33). However, its role is still controversial, indeed in the same study Wang et al (33) found that CD200 down-expression can lead to a particular microglia tumor microenvironment that promotes tumor progression, in agreement with our results. Recent studies in dogs also showed that targeting CD200, enhanced the capacity of antigen-presenting cells to prime T-cells to mediate an anti-glioma response (34).

PVR and CD47 were also found down-expressed in astrocytomas when compared to normal brain, while higher levels of expression were found for LGALS9, TNFRSF14, CD48, CD276 and NECTIN2. PVR has been described as regulator of cell adhesion in a rat model of GBM (35) and a recent study in mice proved that the combination of anti-PD-1 and anti-PVR leads to a better survival (36).

CD47 is a member of the immunoglobulin superfamily that activates the signal regulatory protein-α (SIRP-α) expressed on macrophages, preventing phagocytosis. In contrast with previous studies (37,38), we found decreased levels in gliomas compared to normal brain. We consider that this down-expression can represent an attempt to maintain homeostasis. Recent studies have associated CD47 with the tumor-associated macrophages (TAMs) in the GBM microenvironment. Zhang et al (39) have also proven that anti-CD47 treatment leads to enhanced tumor cell phagocytosis by both M1 and M2 macrophage subtypes with a higher phagocytosis rate by M1 macrophages. A combination of anti-CD47 treatment and temozolomide has also been reported (40).

TNFRSF14 was found to be elevated in aggressive gliomas and its expression seemed to be associated with amplification of EGFR and loss of PTEN (41). TNFRSF14 plays an important role in the recruitment and activation of immune system in the tumor microenvironment. We showed that TNFRSF14 seems to have a significant impact on both the overall survival and the disease-free time. Interestingly, in metastatic melanoma, TNFRSF14 shows a similar behavior (42), further reinforcing our observations and suggesting that similar mechanisms can be shared also in glioma and that a combinatory blocking strategy can improve patients outcome.

Finally, we performed a deconvolution analysis showing that higher levels of CD276, TNFRSF14 and NECTIN2 are associated with a significant lower proportion of infiltrating plasma cells, while higher levels of VTCN1 were associated to higher proportions of infiltrating plasma cells, Th1, aDCs and cDCs. Higher levels of TNFRSF14 were associated with a major infiltration of iDCs, aDCs, pDCs and macrophages, but lower levels of Th1 cells and cDCs. Higher expression of NECTIN2, associated with shorter survival, is associated with reduced proportions of Th1, Th2 and CD8 T cells. Together these findings suggest that the main immune cell types that
help to reduce the tumor mass and improve the survival are Th1 and cDCs, and that their expression is strictly dependent on these immune checkpoints. In agreement with our hypothesis, previous studies have shown that in gliomas, there is a prevalent Th2 response and that switching from Th2 to Th1 can help to block glioma growth (43). Additionally, recent studies have proven that combinational therapy that blocks more immune checkpoints is a possibility to create a more vigorous Th1 antitumor response (44,45) and its association with better outcome (46). Future preclinical and clinical studies are necessary to ascertain whether, in addition to the prognostic value we have highlighted, the dysregulated expression of the inhibitory immune checkpoint presently studied may translate into clinical applications, as novel immunotherapeutic approaches for the treatment of gliomas and possibly other types of cancers.

Collectively, in this study, we evaluated the expression of several inhibitory immune checkpoints that can play a role in glioma progression. Among the investigated immune checkpoints, TNFRSF14 and NECTIN2 were identified as the most promising targets in GBM. In particular, TNFRSF14 expression is associated with worse overall survival and disease-free survival, correlating with a lower Th1 response and suggesting that it could become an interesting biomarker or therapeutic target.

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Availability of data and materials

All the data in this study are available for download from TCGA (The Cancer Genome Atlas) databank.

Authors’ contributions

Conceptualization: FN and PF; data curation: SDL, RB, KM and PF; formal analysis: MP and KM; funding acquisition: AB, PB and FN; investigation: RC; project administration: PB; supervision: FN; visualization: MSB; writing-original draft: SDL, RC, MSB, MP and RB; writing-review and editing: AB, KM, PB, FN and PF.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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