Research Article

Down-regulation of siglec-2 (CD22) predicts worse overall survival from HBV-related early-stage hepatocellular carcinoma: a preliminary analysis from Gene Expression Omnibus

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second most common cause of cancer-related deaths [1–3]. In the past two decades, a marked increase in HCC-related annual death rates was observed [2,4]. And, the incidence of HCC will continue to rise until 2030 based on a SEER registry projects study [5]. Previous research revealed that the prediction of prognosis plays a critical role in therapeutic options of HCC. But, little tumor markers have been externally validated in HCC survival prediction [6]. To find novel biomarkers for predicting HCC prognosis, and to reveal HCC target for treatment is urgently required.

As a characteristic of cancer, immune evasion is more prevalent in organs with high immune tolerance including the liver [7]. The sialic-acid-binding immunoglobulin-like lectins (siglec), a novel family of immunoregulatory, have received more and more attention for their capacity to mediate cell death, anti-proliferative effects and to regulate a variety of cellular activities [8]. Currently, pharmacological strategies using siglec agonistic cross-linking therapeutics are discussed. Modulation of immune responses by targeting siglecs using agonistic or antagonistic therapeutics may have important clinical implications and may be a novel pharmacological strategy in tumor immunotherapy [8]. A recent research has revealed...
that high expression of siglec-10 on NK cells mediates impaired NK cell function, and siglec-10 expression in tumors is associated with poorer survival of HCC patients [9]. However, roles of siglec family in HCC development were little discussed.

According to the potential value of sigles in HCC development, this study aimed to evaluate the associations between siglec family and outcomes from hepatitis B virus (HBV)-related HCC patients, hoping that the data may provide potential biomarker candidates and useful insights into the pathogenesis and progression of HCC.

**Materials and methods**

**Patients**

Using GSE14520 profile from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database, 247 patients with HCC were identified. Twenty-seven patients were excluded for the unavailable siglec gene expression or insufficient clinical outcome data. Finally, 220 HCC cases were included in the analysis. All the HCC patients had a history of HBV infection or HBV-related liver cirrhosis; the diagnosis of HCC was made in all cases by two independent pathologists who had detailed information on clinical presentation and pathological characteristics as declared by Roessler et al. [10].

All liver tissue was obtained with informed consent from patients who underwent radical resection between 2002 and 2003 at the Liver Cancer Institute and Zhongshan Hospital, Fudan University. The study was approved by the Institutional Review Board of the participating institutes [10]. All participants provided written informed consent, as reported by Roessler et al. [10,11].

**Data extraction and end points**

We extracted the GSE14520 microarray expression profile. Tumor sample and microarray processing were reported by Roessler et al. [10,11] and are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520. The experiment protocols and data processing methods are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM362949. Siglec gene expression levels were calculated using the matchprobes package in the R program and the log2 RMA-calculated signal intensity was reported. Nine sigles including siglec-1, siglec-2, siglec-3, siglec-4, siglec-5, siglec-6, siglec-7, siglec-8 and siglec-9 were searched and included in our analysis. Overall survival (OS) was defined as the time from surgery to death from any disease.

**Statistical analysis**

PASW Statistics software version 22.0 from SPSS Inc. (Chicago, IL, USA) was used for statistical analysis. Student’s t-test, Mann–Whitney U-test and Chi-squared test were used for normally distributed continuous data, non-normally distributed continuous data and categorical variables, respectively. Univariate analysis and multivariate Cox and logistic regression were assessed for identifying factors associated with OS and clinico-pathological features. The Kaplan–Meier curve by log rank method was used to compare OS between different groups. A two-tailed $P < 0.05$ were considered statistically significant.

**Results**

**Siglec levels comparison between tumor and non-tumor tissues**

Nine members of siglec family were identified, including siglec-1 to siglec-9. As shown in Figure 1, all siglec were overexpressed in non-tumor tissues compared with those in tumor tissues (all $P < 0.05$, Figure 1).

**Relationship between sigles and HCC overall survival**

As shown in Table 1, univariate analysis showed that siglec-2 and siglec-4 were potential factors associated with HCC OS ($P = 0.065$ and $P = 0.061$, respectively). When all siglec were evaluated by a multivariate model using enter selection, up-regulation of siglec-2 in tumor tissues showed protective potentials for HCC OS (HR = 0.883, 95%CI = 0.806–0.966, $P = 0.007$). In contrast, siglec-4 overexpression was negatively associated with HCC OS (HR = 1.059, 95%CI = 1.025–1.094, $P = 0.001$).

Furthermore, we performed R software analysis to determine the cut-off values of siglec-2 and siglec-4 for the prediction of OS in the training set. Then, we transformed the continuous data above into dichotomous variables according to the determined cut-off values. Unfortunately, no statistical significance was found between siglec-4 and HCC OS in training set based on randomized sampling. According to R language analysis, we grouped siglec-2 using cut-off values of 11.6 into siglec-2 low group and siglec-2 high group. This demonstrated that patients in siglec-2
**Figure 1.** Differential expression of siglecs between non-tumor and tumor tissues in HCC patients

**Table 1** Univariate and multivariate Cox regression analysis of siglecs and HCC overall survival

| Siglec  | Univariate analysis | Multivariate analysis |
|---------|---------------------|-----------------------|
|         | HR (95%CI)          | *P* value             | HR (95%CI)          | *P* value             |
| Siglec-1| 0.988 (0.971–1.006) | 0.18                  |                      |                      |
| Siglec-2| 0.932 (0.65–1.004)  | 0.065                 | 0.883 (0.806–0.966)  | 0.007                 |
| Siglec-3| 1.005 (0.979–1.032) | 0.708                 |                      |                      |
| Siglec-4| 1.028 (0.999–1.058) | 0.061                 | 1.059 (1.025–1.094)  | 0.001                 |
| Siglec-5| 1.025 (0.968–1.084) | 0.397                 |                      |                      |
| Siglec-6| 0.995 (0.911–1.087) | 0.917                 |                      |                      |
| Siglec-7| 1.003 (0.94–1.07)   | 0.939                 |                      |                      |
| Siglec-8| 1.018 (0.898–1.153) | 0.783                 |                      |                      |
| Siglec-9| 1.004 (0.884–1.167) | 0.957                 |                      |                      |
Relationship between siglecs and HCC clinico-pathological features

We grouped HCC patients with siglec-2 cut-off of 11.6 and compared differences of clinico-pathological features between these two groups. As shown in Table 2, more patients had higher alpha-fetoprotein (AFP) levels in siglec-2 low group than those in siglec-2 high group (60% vs. 41.7%, \( P = 0.043 \)). Additionally, no differences were found in patients’ clinico-pathological features including HBV virus status, ALT levels, tumor size, multinodular, cirrhosis and tumor staging (all \( P > 0.05 \)).

We performed logistic regression analysis to identify the relationship between siglecs and HCC clinico-pathological features. This was summarized in Table 3. Univariate analysis showed that siglec-2 was a potential factor associated with AFP levels in HCC patients (\( P = 0.012 \)). When all siglecs were evaluated by a multivariate model using enter selection, siglec-2 overexpression is negatively associated with HCC patients’ AFP level (OR = 0.822, 95%CI = 0.724–0.934, \( P = 0.003 \)). To evaluate the predictive accuracy of siglec-2 and siglec-4 for AFP levels in HCC patients, we analyzed ROCs and found that elevated siglec-2 significantly and accurately predicted lower AFP level (AUC = 0.607, \( P = 0.007 \), Figure 3).

Figure 2. Association between siglec-2 expression and OS in HCC patients

Higher siglec-2 levels are associated with better OS in HCC patients, in training set (A), validation set (B) and total database (C).
Table 2 Clinico-pathological features based on siglec-2 expression in HCC patients

| Clinico-pathological features | High siglec-2 group (n = 180) | Low siglec-2 group (n = 40) | P value |
|------------------------------|-------------------------------|----------------------------|---------|
| Gender (male/female), n      | 156/24                       | 34/6                       | 0.781   |
| Age (≥50 years/≤50 years), n | 99/81                        | 25/15                      | 0.387   |
| HBV viral status (AVR-CC/no/NA), n | 47/128/5                    | 9/27/4                     | 0.111   |
| ALT (≥50/<50/NA), IU         | 76/104                       | 14/26                      | 0.401   |
| Main tumor size (≥5/<5/NA), cm | 66/114/0                    | 14/25/1                    | 0.104   |
| Multinodular (yes/no), n     | 37/143                       | 7/33                       | 0.662   |
| Cirrhosis (yes/no), n        | 163/17                       | 39/1                       | 0.147   |
| TNM staging (I/II/III/NA), n | 138/40/2                     | 31/8/1                     | 0.763   |
| BCLC staging (0-A/B-C/NA), n | 138/41/1                     | 30/9/1                     | 0.503   |
| CLIP staging (0/1/2/3/4/5/NA), n | 81/61/25/8/2/1/2             | 15/13/9/1/1/0/1            | –       |
| AFP (>300/<300/NA), ng/ml    | 75/102/3                     | 24/16/0                    | 0.043   |

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AVR-CC, active viral replication chronic carrier; NA, not available.

Table 3 Relationship between siglecs and HCC clinico-pathological characteristics by logistic regression analysis

| Siglecs, per increase of 1 unit | OR (95%CI) | P value | OR (95%CI) | P value |
|---------------------------------|------------|---------|------------|---------|
| Siglec-1                        | 1.001 (0.984–1.018) | 0.936   |            |         |
| Siglec-2                        | 0.891 (0.815–0.975) | 0.012   | 0.822 (0.724–0.934) | 0.003   |
| Siglec-3                        | 1.0 (0.967–1.035)   | 0.992   |            |         |
| Siglec-4                        | 1.034 (0.969–1.102) | 0.313   |            |         |
| Siglec-5                        | 1.028 (0.944–1.112) | 0.523   |            |         |
| Siglec-6                        | 1.045 (0.932–1.173) | 0.449   |            |         |
| Siglec-7                        | 1.044 (0.959–1.137) | 0.316   |            |         |
| Siglec-8                        | 1.063 (0.908–1.245) | 0.448   |            |         |
| Siglec-9                        | 0.861 (0.714–1.038) | 0.117   |            |         |

Figure 3. ROC curve of siglec-2 for AFP > 300 ng/ml
Siglec-2 coexpression genes and pathways enrichment

Using the GSE14520 microarray database, coexpressed genes of siglec-2 in HCC were searched in HCC. As shown in Table 4, 137 genes were found to be positively coexpressed with siglec-2. On the other hand, 352 genes were negatively coexpressed with siglec-2 as shown in Table 5.

Additionally, gene set enrichment analysis (GSEA) was used for identification of putative KEGG pathways associated with siglec-2 coexpressed genes. Consequently, pathways including MAPK signaling pathway and calcium signaling pathway, which have been proved in liver cancer, were significantly enriched with siglec-2 positively coexpressed genes ($FDR < 0.05$, Figure 4), While siglec-2’s negatively coexpressed genes contributed to tumor cell...
phenotype including cell cycle, spliceosome, DNA replication, ubiquitin-mediated proteolysis, proteasome, oocyte meiosis, mismatch repair, ribosome, pathways in cancer and pathogenic Escherichia coli infection (FDR < 0.05, Figure 5).

**Discussion**

Immunotherapy for HCC has shown some success [7]. However, in most HCC patients or animal models, tumors progressed in spite of tumor-specific immune responses [12]. Thus, to find new immune markers of HCC development is still of significant importance. Functionally, siglecS participate in regulating the innate and adaptive immune responses through the recognition of their glycan ligands [13]. They have been demonstrated to be involved in a series of inhibitory processes, cell–cell interaction processes and endocytosis [8,14–16]. In our analysis, we found that all siglecS including siglec-1 to siglec-9 were significantly suppressed in HCC tumors, which may serve as anti-oncogenes. Recently, several studies revealed that siglec deficiencies contributed to the potential for generation of malignancy like lymphomas and leukemias [17,18]. As reviewed by Macauley et al., siglecS played a role in regulating of immune surveillance of cancer by keeping with their roles aiding immune cells in distinguishing between self and non-self [13]. They concluded that siglecS effectively reduce innate immune responses against cancer cells by down-regulating immune cells that express them through recognition of sialofucosyl ligands on the cancer cell itself or soluble mucins produced by the cancer cell [13].

Serum AFP levels increase by 20–80% in HCC patients and are strongly associated with tumor aggressiveness [19–21]. High level of AFP is correlated with tumor size, vascular invasion and poorly differentiated HCC [19,22,23]. In our analysis, we found that siglec-2 expression in tumor tissues was significantly negatively associated with AFP elevation. Although the immunogenicity of AFP is weak, it could induce the immune escapes through inhibiting the function of dendritic cells, natural killer cells and T lymphocytes [24,25]. Several studies demonstrated that AFP is involved in immunosuppression [25,26]. It can impair the function of macrophages leading to decreased phagocytosis and impaired antigen-presenting abilities [27]. AFP-modified immune cell vaccine or peptide vaccine has displayed the specific antitumor immunity against AFP-positive tumor cells [28,29]. Hence, siglec-2 could play antitumor effects via enhancing immune responses by inhibition AFP levels. Although the proportion of patients with elevated AFP in
| Gene set name                          | Genes in Overlap | P value  | FDR q-value |
|---------------------------------------|------------------|----------|-------------|
| Cell cycle                            | 21               | 3.46E-22 | 6.44E-20    |
| Spliceosome                           | 17               | 1.35E-16 | 1.26E-14    |
| DNA replication                       | 11               | 1.73E-15 | 1.08E-13    |
| Ubiquitin mediated proteolysis        | 13               | 4.54E-11 | 2.11E-9     |
| Proteasome                            | 9                | 8.4E-11  | 3.13E-9     |
| Oocyte meiosis                        | 11               | 1.11E-9  | 3.43E-8     |
| Mismatch repair                       | 6                | 1.5E-8   | 3.99E-7     |
| Ribosome                              | 9                | 2.2E-8   | 5.12E-7     |
| Pathways in cancer                    | 15               | 3.38E-8  | 6.99E-7     |
| Pathogenic Escherichia coli infection | 7                | 2.97E-7  | 5.52E-6     |

Figure 5. KEGG functional enrichment of siglec-2 with its negative coexpressed genes

siglec-2 low expression group was significantly higher than that in siglec-2 high expression group (60.0% vs. 41.7%), the biologic value is not strong. Further research with larger samples is needed.

Our results also showed that siglec-2 elevation predicts better survival in HCC. Siglecs including siglec-2 have been reported to regulate cell growth and survival, by both inhibition of proliferation and/or induction of apoptosis [13]. Throughout the last decade, several novel therapeutic agents that target siglec-2 are being developed as an alternative approach for cancer treatment [17,18,30]. Previous reports showed that siglec-2 as a B-cell-associated adhesion protein appeared to play a critical role in establishing signaling thresholds for B-cell activation, mediating normal antibody response to thymus-independent antigens and regulating the lifespan of mature B cells [31,32]. Therefore, down-regulating of siglec-2 in tumor tissues might risk the tumor progress by reducing innate immune response and mature B cells proliferation in HCC patients. Recently, it is gradually recognized that some B-cell subpopulations including regulatory B cells can impair CD4+ T cell activation or produce cytokines promote tumor progression [33-35]. Leading to dramatically suppress antibody and inhibit antitumor effector T cells [34,36]. Lymphotoxin secreted from tumor-infiltrating B cells also promotes tumor growth [37]. Therefore, serves as B cell receptor inhibitor, siglec-2 might suppress tumor progress and development, contributing to a prolonging survival in HCC patients. Additionally, we enriched coexpressed genes of siglec-2 and its functional pathways. Siglec-2 and its coexpressed genes participate in the tumor cell phenotype including cell cycle, spliceosome, DNA replication, ubiquitin mediated proteolysis, proteasome, mismatch repair and pathways in cancer like MAPK signaling pathway and calcium signaling pathway, which should be the main research directions of siglec-2 mechanism in HCC in future.

Although siglec-4 levels in tumor tissues might associate with HCC OS in our Cox regression analysis, no significance was found in log-rank methods. Known as myelin-associated glycoprotein (MAG), siglec-4 is selectively localized in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths [38] and plays a role in axon-myelin stabilization and inhibition of axon regeneration after injury [39,40]. Since siglec-4 is only found in
the nervous system, even though siglec-4 showed some significance for HCC OS in our analysis, deep research of this gene in HCC development should be cautious and well-designed.

The present study has some limitations: First, our research was a preliminary analysis from GEO database, no further mechanism data were shown. Second, we included siglecs as a continuous variable in the logistic and Cox regression process, leading to a small HRs of the siglecs biomarker candidates. Third, only siglec-1 to siglec-9 were included in this analysis, other siglec family members like siglec-10 to siglec-15 were not available in this gene database. Fourth, we did not conduct mechanism research in siglec-2 protein level. Even with these limitations, the results might provide useful insights for HCC research in therapeutic strategy.

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Author contribution
X.Q. and X.R. conceived and designed the study. X.R. wrote the manuscript. X.R and Y.J. analyzed and interpreted the data. X.J. helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations
AFP, alpha-fetoprotein; GEO, Gene Expression Omnibus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OS, overall survival; siglec, sialic-acid-binding immunoglobulin-like lectin.

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