HAVCR1 expression might be a novel prognostic factor for gastric cancer

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Abstract

Hepatitis A virus cellular receptor 1 (HAVCR1), which is also known as T-cell immunoglobulin and mucin domain 1 (TIM-1) is a TIM gene family member. In this study, we aimed to characterize the expression profile of HAVCR1 in GC, its prognostic value and the potential epigenetic mechanism leading to its dysregulation. Bioinformatic analysis was performed by using genomic, clinicopathological and survival data in the human protein atlas (HPA) and the Cancer Genome Atlas (TCGA). Results showed that HAVCR1 was significantly upregulated at the mRNA and protein level in GC tissues compared to the adjacent normal tissues. In addition, HAVCR1 upregulation was an independent indicator of shorter OS (HR: 1.698, 95%CI: 1.221–2.361, p = 0.002), after adjustment of older age, differentiation status, pathological stages and the presence of residual tumor and was also an independent indicator of shorter RFS (HR: 2.577, 95%CI: 1.583–4.197, p < 0.001), after adjustment of gender and histological grade. The methylation level of two CpG sites (cg11188031 and cg07320595) was negatively correlated with HAVCR1 expression. However, only high methylation level of cg07320595 was associated with significantly longer OS (p = 0.018) and RFS (p = 0.021). Based on these findings, we infer that HAVCR1 upregulation might serve as a valuable prognostic marker in terms of OS and RFS in GC patients. Cg07320595 might be a critical CpG site influencing HAVCR1 expression.

Introduction

Hepatitis A virus cellular receptor 1 (HAVCR1), also known as T-cell immunoglobulin and mucin domain 1 (TIM-1) is a TIM gene family member [1]. It is a class I integral membrane glycoprotein, which contains an N-terminal extracellular immunoglobulin (Ig)-like domain, an extended mucin-like domain, a single transmembrane domain, and a C-terminal short cytoplasmic tail, allowing accessibility to interactions with extracellular proteins [2].
Although HAVCR1 is expressed in many human tissues, its functional role has not been fully investigated [3]. Previous studies found that the members of this family widely involve in regulating immune cell activity [4, 5]. HAVCR1 is preferentially expressed on Th2 cells, inducing T-cell activation and inhibiting the development of peripheral tolerance [6, 7]. In addition, this molecule is also involved in the moderation of allergic response and asthma [5]. Previous studies found that HAVCR1 is overexpressed in numerous cancers and its upregulation might be associated with cancer development and progression. In clear cell renal carcinoma (RCC) cells, HAVCR1 upregulation and its shedding activate the IL-6/STAT-3/HIF-1A axis, which is a signaling pathway enhancing angiogenesis and tumor growth [8]. HAVCR1 overexpression results in reduced formation and integrity of tight junctions, which have an imperative role in cell to cell adhesion [9]. The disruption of tight junctions is thought to be a cause of enhanced cancer cell dissemination and cancer metastasis [3, 9]. In addition, the cleaved ectodomain of HAVCR1 can be detected in the urine samples from RCC patients, making it a possible biomarker for early detection of RCC [10].

One previous study found that blocking the interaction of TIM-1 and TIM-4 can enhance DC vaccine against gastric cancer (GC) [11]. In this study, using genomic, clinicopathological and survival data in multiple databases, we characterized the expression profile of HAVCR1 in GC, its prognostic value and the potential epigenetic mechanism leading to its dysregulation.

**Patients and methods**

**Secondary analysis using data in TCGA-STAD**

The level-3 data in TCGA-STAD was downloaded using the UCSC Xena Browser (https://xenabrowser.net/). This database included 415 cases of primary GC tumors and 35 cases of matched normal stomach tissues. These tissues had gene expression quantified by IlluminaHiSeq analysis. No patient had the history of neoadjuvant treatment. Among the patients with RNA-seq data available, 388 cases had intact OS data recorded, while 324 cases had intact RFS data recorded. The genomic, clinicopathological and survival data of the patients, including HAVCR1 expression (IlluminaHiSeq), age at initial diagnosis, gender, pathological stage, reflux history, histological grade, radiation therapy, targeted molecular therapy, H. pylori infection, primary therapy outcomes, the presence of residual tumor, living status and recurrence status were downloaded. Primary therapy outcomes were defined as complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD).

The DNA methylation data of HAVCR1 (measured by Illumina Infinium Human Methylation 450K BeadChip) were also downloaded using the UCSC Xena Browser. Among the GC cases with HAVCR1 DNA methylation available, 360 GC cases had intact OS data recorded, while 305 cases had intact RFS data recorded.

**Data mining in the Human Protein Atlas**

RNA-seq data of HAVCR1 RNA and protein expression in normal human tissues and human cancer tissues were reviewed via using data generated by the Human Protein Atlas (HPA) (http://www.proteinatlas.org/) [12, 13]. The protein expression was examined by immunohistochemistry and the expression level was scored as not detected, low, medium or high, which is a combination of staining intensity and fraction of stained cells.

**Statistical analysis**

Statistical analysis was performed using SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA) or using GraphPad Prism 7.0 (GraphPad Inc., La Jolla, CA, USA). Gene expression
between different groups was assessed using one-way ANOVA followed by Turkey’s post-hoc test or Welch’s unequal variances t-test. The association between HAVCR1 expression and the clinicopathological parameters in GC patients was examined by using χ² test by two-sided Fisher’s exact test. Kaplan-Meier survival curves were generated using GraphPad Prism 7.0. The Youden Index of HAVCR1 RNA expression or its DNA methylation in the Receiver operating characteristic (ROC) analysis for death and recurrence detection were identified and were used as the cutoff in Kaplan-Meier curves. Log-rank test was performed to identify the significance of the difference between the survival curves. Univariate and multivariate Cox regression models were applied to analyze the prognostic significance of HAVCR1 expression in terms of OS and RFS. Regression analysis was performed to assess the Pearson correlation coefficient between HAVCR1 expression and the methylation level of its CpG sites. p < 0.05 was considered statistically significant.

**Results**

**HAVCR1 expression was low at both mRNA and protein level in normal stomach tissues**

Using RNA-seq data and protein expression data (by IHC staining) in the HPA, we characterized HAVCR1 expression in different normal human tissues. Results showed that HAVCR1 expression varied significantly in different human tissues (Fig 1A and 1B). The RNA expression was relatively higher in colon, rectum, kidney and testis, compared to other tissues (Fig 1A), while the protein expression was consistent with its RNA expression (Fig 1B). In normal stomach tissues, HAVCR1 expression was low at both mRNA and protein level (Fig 1A and 1B, red arrows).

**HAVCR1 expression was upregulated at both mRNA and protein level in GC tissues**

Then, we checked HAVCR1 protein expression in some human tumor tissues (Fig 2A). Among 12 GC tissues examined, 6 cases had medium to high staining (5 medium and 1 high), while the remaining 6 cases had low staining (Fig 2A). Representative IHC image showed that HAVCR1 staining was low in glandular cells in normal stomach tissues (Fig 2B, left). In comparison, the upregulated HAVCR1 was distributed in both cytoplasm and cell membrane in GC tissues (Fig 2B, right). Then, we used RNA-seq data from TCGA-STAD, to examine HAVCR1 RNA expression between 415 cases of GC tissues and 35 cases of matched normal tissues. Welch’s unequal variances t-test confirmed that the GC tissues had significantly upregulated HAVCR1 expression (Fig 2C).

**Elevated HAVCR1 expression was associated with poor therapeutic responses and survival outcomes**

Then, we checked the difference in HAVCR1 expression between groups with different clinicopathological parameters and survival outcomes. Results showed that there was no significant difference in HAVCR1 expression between female and male patients (Fig 3A) or among patients in different pathological stages (Fig 3B). In comparison, the moderately differentiated (G2) tumors had substantially higher HAVCR1 expression compared to the well-differentiated tumors (G1). No significant difference was observed between G2 and G3 (Poorly differentiated) tumors (Fig 3C). Notably, the patients with responses (CR/PR) to primary therapy had significantly lower (p<0.001) HAVCR1 expression compared to the patients without therapeutic responses (SD/PD) (Fig 3D). Then, we compared HAVCR1 expression between patients
Results showed that the death cases and the cases with recurrence after primary therapy had significantly higher HAVCR1 expression compared to the control group (Fig 3E and 3F).

Elevated HAVCR1 RNA expression was an independent prognostic indicator of unfavorable survival in GC patients

To assess the prognostic value of HAVCR1 RNA expression in GC, the patients were divided into two groups according to the optimal cutoff of HAVCR1 expression. Their clinicopathological parameters and survival outcomes were compared in Table 1 (divided by the optimal cutoff in ROC analysis for death detection). Chi-square analysis showed that the high HAVCR1 expression group (N = 191) had a significantly higher proportion of patients without responses to primary therapy \((p = 0.001)\), deceased cases \((p < 0.001)\) and with recurrence \((p < 0.001)\), compared to the low HAVCR1 expression group (N = 197) (Table 1). By generating Kaplan-Meier survival curves, we found that the high HAVCR1 expression group had a significantly shorter OS and RFS (Fig 4A and 4B). By performing univariate analysis, we found that older age,
poorly differentiated tumor, advanced pathological stages (III/IV), with residual tumor and high HAVCR1 expression were risk factors of shorter OS (Table 2). Multivariate analysis confirmed that high HAVCR1 expression was an independent indicator of shorter OS (HR: 1.698, 95%CI: 1.221–2.361, \( p = 0.002 \)), after adjustment of older age, differentiation status, pathological stages and the presence of residual tumor (Table 2). By setting RFS as the survival outcome, we found that male patients, poorly differentiated tumor and high HAVCR1 expression were risk factors of shorter RFS (Table 3). Multivariate analysis confirmed that high HAVCR1 expression was an independent indicator of shorter RFS (HR: 2.577, 95%CI: 1.583–4.197, \( p < 0.001 \)), after adjustment of other two risk factors (Table 3).

Bioinformatic analysis of the epigenetic mechanism underlying HAVCR1 dysregulation in GC

Using DNA methylation data obtained from Illumina Infinium Human Methylation 450K BeadChip, we examined the methylation status of the CpG sites of HAVCR1 gene. Heatmap
Fig 3. Elevated HAVCR1 expression was associated with poor therapeutic responses and survival outcomes in GC patients. A-F: Comparison of HAVCR1 RNA expression between female and male patients (A), among patients in different pathological stages (B), different histological grade (C), with different therapeutic responses (D), and with different OS (E) and RFS (F) status.

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and the corresponding regression analysis found that the methylation level of two CpG sites (cg11188031 and cg07320595) were negatively correlated with HAVCR1 expression (Pearson’s r = -0.32 and -0.40 respectively) (Fig 5A and 5B). Then, we checked whether the methylation level of these two sites was related to OS and RFS. Kaplan-Meier curves showed that the methylation status of cg11188031 was not related to OS or RFS (Fig 5C and 5D). In comparison, high methylation level of cg07320595 was associated with significantly longer OS (p = 0.018) and RFS (p = 0.021) (Fig 5E and 5F).

Table 1. The characteristics of patients with primary GC.

| Parameters                        | HAVCR1 expression | p value |
|-----------------------------------|-------------------|---------|
|                                   | High (N = 191)    | Low (N = 197) |
| Age (Mean ± SD)                   | 64.22±10.91       | 66.35±10.28 | 0.05   |
| Gender                            |                   |          |
| Female                            | 68                | 68       | 0.83   |
| Male                              | 123               | 129      |        |
| Pathological Stage                |                   |          |
| I/II                              | 75                | 97       | 0.49   |
| III/IV                            | 110               | 93       |        |
| Discrepancy+null                  | 6                 | 7        |        |
| Histological Grade                |                   |          |
| G1/G2                             | 67                | 80       | 0.25   |
| G3                                | 121               | 111      |        |
| GX/null                           | 3                 | 6        |        |
| Reflux history                    |                   |          |
| No                                | 90                | 103      | 0.62   |
| Yes                               | 22                | 21       |        |
| null                              | 79                | 73       |        |
| Radiation therapy                 |                   |          |
| No                                | 139               | 155      | 0.35   |
| Yes                               | 37                | 31       |        |
| null                              | 15                | 11       |        |
| Targeted molecular therapy        |                   |          |
| No                                | 86                | 106      | 0.09   |
| Yes                               | 90                | 76       |        |
| Discrepancy+null                  | 15                | 15       |        |
| H. pylori infection               |                   |          |
| No                                | 81                | 73       | 0.16   |
| Yes                               | 7                 | 13       |        |
| null                              | 103               | 111      |        |
| Primary therapy outcome           |                   |          |
| CR+PR                             | 102               | 139      | 0.001  |
| SD+PD                             | 59                | 35       |        |
| Discrepancy+null                  | 30                | 23       |        |
| Residual tumor                    |                   |          |
| R0                                | 154               | 166      | 0.13   |
| R1+R2                             | 19                | 11       |        |
| RX+null                           | 18                | 20       |        |
| Recurrence status                 |                   |          |
| No                                | 106               | 146      | <0.001 |
| Yes                               | 46                | 22       |        |
| null                              | 39                | 29       |        |
| Living Status                     |                   |          |
| Living                            | 96                | 135      | <0.001 |
| Dead                              | 95                | 62       |        |

Null: data not available; G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated; R0, no residual tumor; R1, microscopic residual tumor; R2, macroscopic residual tumor; RX: The presence of residual tumor cannot be assessed. Patients were separated into two groups according to the Youden Index of HAVCR1 expression in ROC analysis for OS detection.

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Discussion

There are three HAVCR family of receptors identified in humans, including HAVCR1, HAVCR2 and HAVCR3. HAVCR2, which is also known as TIM-3, have been widely studied in GC. It is a T helper type 1 (Th1)-specific cell surface molecule that regulates Th1 responses.

Table 2. Univariate and multivariate analysis of OS.

| Parameters              | Univariate analysis | Multivariate analysis |
|-------------------------|---------------------|-----------------------|
|                         | $p$  | HR     | 95%CI lower/upper | $p$  | HR     | 95%CI lower/upper |
| Age (Continuous)        | 0.007 | 1.021 | 1.006 1.037 | <0.001 | 1.029 | 1.013 1.046 |
| Gender                  |       |       |       |       |       |                   |
| Male                    | 1.000 |       |       |       |       |                   |
| Female                  | 0.296 | 0.835 | 0.596 1.171 |      |       |                   |
| Histological grade      |       |       |       |       |       |                   |
| G1/G2                   | 1.000 |       |       |       |       |                   |
| G3                      | 0.022 | 1.479 | 1.057 2.069 | 0.025 | 0.668 | 0.469 0.950 |
| Pathological stages     |       |       |       |       |       |                   |
| I/II                    | 1.000 |       |       |       |       |                   |
| III/IV                  | <0.001 | 2.063 | 1.460 2.916 | <0.001 | 1.918 | 1.333 2.760 |
| Reflux history          |       |       |       |       |       |                   |
| Yes                     | 1.000 |       |       |       |       |                   |
| No                      | 0.124 | 1.675 | 0.868 3.232 |      |       |                   |
| Residual tumors         |       |       |       |       |       |                   |
| Yes                     | 1.000 |       |       |       |       |                   |
| No                      | <0.001 | 0.314 | 0.198 0.498 | <0.001 | 0.422 | 0.260 0.683 |
| H. pylori infection     |       |       |       |       |       |                   |
| Yes                     | 1.000 |       |       |       |       |                   |
| No                      | 0.193 | 1.753 | 0.753 4.082 |      |       |                   |
| HAVCR1 expression       |       |       |       |       |       |                   |
| Low (N = 197)           | 1.000 |       |       |       |       |                   |
| High (N = 191)          | 0.001 | 1.723 | 1.250 2.374 | 0.002 | 1.698 | 1.221 2.361 |

The cutoff of HAVCR1 expression was the Youden Index of HAVCR1 expression in ROC analysis for OS detection.

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Table 3. Univariate and multivariate analysis of RFS.

| Parameters                  | Univariate analysis | Multiivariate analysis |
|-----------------------------|---------------------|------------------------|
|                             | \( p \)  | HR       | 95%CI lower/upper | \( p \)  | HR       | 95%CI lower/upper |
| Age (Continuous)            | 0.547  | 0.994    | 0.973/1.015       |         |         |                |
| Gender                      |         |          |                   |         |         |                |
| Male                        | 1.000   |          |                   |         |         |                |
| Female                      | 0.014   | 0.504    | 0.293/0.869       | 0.003   | 0.437    | 0.253/0.756    |
| Histological grade          |         |          |                   |         |         |                |
| G3                          | 1.000   |          |                   |         |         |                |
| G1/G2                       | 0.014   | 0.522    | 0.311/0.876       | 0.010   | 0.502    | 0.298/0.845    |
| Pathological stages         |         |          |                   |         |         |                |
| I/II                        | 1.000   |          |                   |         |         |                |
| III/IV                      | 0.803   | 1.061    | 0.664/1.696       |         |         |                |
| Reflux history              |         |          |                   |         |         |                |
| Yes                         | 1.000   |          |                   |         |         |                |
| No                          | 0.207   | 1.832    | 0.716/4.689       |         |         |                |
| Residual tumors             |         |          |                   |         |         |                |
| Yes                         | 1.000   |          |                   |         |         |                |
| No                          | 0.150   | 0.538    | 0.232/1.252       |         |         |                |
| H. pylori infection         |         |          |                   |         |         |                |
| Yes                         | 1.000   |          |                   |         |         |                |
| No                          | 0.334   | 2.043    | 0.479/8.710       |         |         |                |
| HAVCR1 expression           |         |          |                   |         |         |                |
| Low (N = 177)               | 1.000   |          |                   |         |         |                |
| High (N = 147)              | <0.001  | 2.538    | 1.561/4.127       | <0.001  | 2.577    | 1.583/4.197    |

The cutoff of HAVCR1 expression was the Youden Index of HAVCR1 expression in ROC analysis for RFS detection.

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Fig 5. Bioinformatic analysis of the epigenetic mechanism underlying HAVCR1 dysregulation in GC. A-B. Heatmap (A) and the corresponding regression analysis (B) of the correlation between HAVCR1 expression and the methylation of its CpG sites. C-F. Kaplan-Meier survival curves of OS (C and E) and RFS (D and F) in GC patients. The patients were grouped according to the optimal cutoff of cg11188031 (C-D) and cg07320595 (E-F) methylation in ROC analysis for death and recurrence detection respectively.

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and induces peripheral tolerance [5]. Its expression on NK cells is induced by the pathological development of GC and might act as an independent prognostic factor [14]. Its upregulation on monocyte/macrophages might contribute to GC progression via stimulating monocyte to secrete IL-6, IL-8, and IL-10 [15]. TIM-3 also negatively regulates GC antigen-specific CD8(+) T cell [16]. CD8(+)PD-1(+)TIM-3(+) T cells were more impaired in IFN-gamma, TNF-alpha and IL-2 production compared to the PD-1(+)Tim-3(-) or PD-1(-)Tim-3(-) subsets and were less cytotoxic to GC cells [16]. However, no study examined the expression profile of HAVCR1 and its functional role in GC.

In this study, using data from the TCGA-STAD, we found that HAVCR1 was significantly upregulated at the mRNA and protein level in GC tissues compared to the adjacent normal tissues. In addition, we also demonstrated that its upregulation was an independent indicator of shorter OS (HR: 1.698, 95%CI: 1.221–2.361, \( p = 0.002 \)), after adjustment of older age, differentiation status, pathological stages and the presence of residual tumor and was also an independent indicator of shorter RFS (HR: 2.577, 95%CI: 1.583–4.197, \( p < 0.001 \)), after adjustment of gender and histological grade. These findings suggest that HAVCR1 expression might be a novel prognostic factor for gastric cancer. Previous studies HAVCR1 shedding enhances IL-6 expression in RCC, which subsequently activates the STAT-3 pathway, leading to increased HIF-1α expression [8, 17]. In GC, This pathway also mediates a reciprocal crosstalk between cancer-derived mesenchymal stem cells and neutrophils [18], resistance to trastuzumab [19] and epithelial to mesenchymal transition [20], thereby facilitating cancer progression and metastasis. These mechanisms might help to explain the association between aberrantly expressed HAVCR1 and the poor survival outcomes of GC.

Besides the specific upregulation in several human cancers, the monoclonal antibody binds to HAVCR1 can be internalized into the cells, making it an attractive target for antibody-mediated therapy, such as antibody-drug conjugates (ADCs) in certain cancers [21–23]. In this study, we observed that HAVCR1 upregulation was associated with poor responses to primary therapy. In the future, it is meaningful to further explore the potential of HAVCR1 as a target of ADCs in GC.

To explore the potential mechanism underlying its dysregulation, we examined the correlation between HAVCR1 RNA expression and the methylation level of its CpG sites. Results found two CpG sites (cg11188031 and cg07320595) that were negatively correlated with HAVCR1 expression. However, by performing survival analysis, only high methylation level of cg07320595 was associated with significantly longer OS (\( p = 0.018 \)) and RFS (\( p = 0.021 \)). Based on these findings, we infer that cg07320595 might be a critical CpG site influencing HAVCR1 expression.

**Conclusion**

HAVCR1 upregulation might serve as a valuable prognostic marker in terms of OS and RFS in GC patients. Cg07320595 might be a critical CpG site influencing HAVCR1 expression.

**Author Contributions**

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