



Original Article

HAVCR2 (TIM-3) as a favorable prognostic immune marker in seminoma: Integrative checkpoint screening and tissue validation

Ki Hong Kim^a, Dae Young Lee^a, Hee Jo Yang^a, Si Hyun Kim^a, Doo Sang Kim^a, Chang Ho Lee^a, Soon Auck Hong^b, Byung Hoon Chi^c, Ji-Hye Lee^{d,*}, Youn Soo Jeon^{a,*}

^a Department of Urology, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, Cheonan, South Korea

^b Department of Pathology, College of Medicine, Chung-Ang University, Seoul, South Korea

^c Department of Urology, Gimpo Woori Hospital, Gimpo, South Korea

^d Department of Pathology, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, Cheonan, South Korea

ARTICLE INFO

Keywords:

TIM-3
HAVCR2
Seminoma
Immune checkpoint
Prognosis
Tumor microenvironment
Testicular germ cell tumor

ABSTRACT

Background: Immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have demonstrated limited efficacy in testicular germ cell tumors (TGCTs), highlighting the need to identify alternative immune biomarkers. We evaluated the prognostic significance of HAVCR2 (TIM-3) in seminoma using an integrative approach combining transcriptomic analysis, penalized regression screen, and independent tissue-level validation.

Methods: Elastic net–penalized Cox regression was applied to a four-gene immune checkpoint panel (HAVCR2, PDCD1, LAG3, TIGIT) in the TCGA seminoma cohort. Survival analyses were performed using progression-free interval (PFI). Findings were validated in an independent tissue microarray (TMA) cohort using immunohistochemistry. A pooled analysis integrating transcriptomic and protein-level data was conducted using a standardized biomarker approach.

Results: HAVCR2 retained a relatively larger non-zero coefficient after penalization, whereas PDCD1, LAG3, and TIGIT were attenuated. In the TCGA cohort, higher HAVCR2 expression showed a trend toward improved PFI (log-rank $p = 0.120$). In the TMA cohort, high TIM-3 expression was associated with favorable relapse-free survival (log-rank $p = 0.053$), with all relapse events in the low-expression group. In the pooled cohort, HAVCR2/TIM-3 expression was associated with improved event-free survival (log-rank $p = 0.019$; HR = 0.201, $p = 0.035$); permutation-corrected $p = 0.164$, and findings are exploratory.

Conclusion: HAVCR2/TIM-3 expression demonstrates a consistent association with favorable clinical outcomes in seminoma across transcriptomic and protein-level analyses, with a lower risk of progression (HR = 0.201) in the pooled cohort. These exploratory findings support a potential prognostic role of TIM-3 reflecting an immune-active rather than exhausted microenvironment in seminoma, warranting prospective validation.

1. Introduction

Testicular germ cell tumors (TGCTs) are the most common malignancies affecting young adult men and are broadly classified into seminoma and non-seminomatous germ cell tumors [1]. Although the majority of patients achieve excellent outcomes with cisplatin-based therapy, a clinically relevant subset develops relapse or platinum-refractory disease, for whom durable treatment options

beyond established salvage strategies remain limited [2,3]. These unmet clinical needs highlight the importance of identifying biologically informative prognostic markers beyond conventional clinicopathologic factors.

Seminoma, a major histologic subtype of TGCT, is characterized by a distinctive tumor microenvironment enriched with tumor-infiltrating lymphocytes (TILs), particularly T cells [4–6]. Despite arising within the immune-privileged environment of the testis, TGCTs exhibit

* Corresponding author at: Department of Urology, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, 31 Suncheonhyang 6-gil, Dongnam-gu, Cheonan 31151, South Korea.

** Corresponding author at: Department of Pathology, Soonchunhyang University Cheonan Hospital, Soonchunhyang University College of Medicine, 31 Suncheonhyang 6-gil, Dongnam-gu, Cheonan 31151, South Korea.

E-mail addresses: whui01@schmc.ac.kr (J.-H. Lee), ysurol@schmc.ac.kr (Y.S. Jeon).

<https://doi.org/10.1016/j.ctarc.2026.101278>

substantial remodeling of immune cell composition and function, including expansion of diverse T-cell subsets and activation of immune regulatory pathways [5,7]. Recent studies have demonstrated that seminoma harbors a highly structured and dynamic immune landscape, with multiple T-cell populations, including regulatory and follicular helper T cells, contributing to tumor-immune interactions [4,5].

These observations have prompted interest in immunotherapeutic approaches for TGCT. However, in contrast to several other solid tumors, clinical trials of immune checkpoint blockade in relapsed or platinum-refractory TGCT have shown limited and inconsistent activity [8–10]. This discrepancy suggests that the immune biology of TGCT—particularly seminoma—may not be adequately captured by conventional checkpoint paradigms and that alternative immune regulatory pathways warrant further investigation [7,11].

Among emerging immune checkpoint molecules, T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), encoded by HAVCR2, is a co-inhibitory receptor that has frequently been linked to immune tolerance and dysfunctional or exhausted T-cell states in multiple malignancies [12,13]. In TGCT, transcriptome-based analyses have shown that HAVCR2 is upregulated and associated with immune-cell infiltration and checkpoint-related signaling [14]. However, the prognostic interpretation of these findings has largely relied on bulk TGCT-level analyses using overall survival as the primary endpoint. Given the limited number of death events typically observed in public TGCT datasets, such analyses have inherent limitations, and their conclusions regarding adverse prognostic significance should be interpreted with caution. Moreover, these studies did not specifically address seminoma or incorporate progression-based clinical endpoints or tissue-level validation.

Importantly, accumulating evidence indicates that the immune context of seminoma is more complex than a simple exhausted-versus-active dichotomy. Recent single-cell transcriptomic studies have identified interferon- γ -positive T-cell populations in non-metastatic seminoma and suggested that interferon- γ -related signaling may be associated with a more differentiated and less aggressive tumor state [15]. These findings imply that certain immune-related signals may reflect an active and biologically favorable immune microenvironment rather than uniform immune suppression.

In this context, preliminary tissue-level observations have suggested that higher TIM-3 expression in tumor-infiltrating lymphocytes may be associated with improved relapse outcomes in seminoma, indicating a potentially paradoxical prognostic role distinct from its conventional interpretation as an exhaustion marker. However, such observations have not been systematically validated in an integrative framework combining transcriptomic, clinical, and tissue-level data.

Therefore, we evaluated the prognostic significance of HAVCR2/TIM-3 in seminoma using an integrative framework combining TCGA-based transcriptomic discovery, exploratory ElasticNet-based penalized regression screening, independent tissue-level validation, and pooled survival analysis, with the aim of determining whether HAVCR2/TIM-3 reflects a conventional exhaustion-associated signal or a favorable immune context in seminoma.

2. Materials and methods

2.1. TCGA dataset and study population

RNA sequencing expression data and corresponding clinical information for testicular germ cell tumors (TGCT) were obtained from The Cancer Genome Atlas (TCGA) through the UCSC Xena platform. Gene expression values were derived from the TCGA TGCT RNA-sequencing dataset generated using the Illumina HiSeq platform and were analyzed as normalized log2-transformed expression values.

Among TCGA TGCT cases, patients with histologically confirmed seminoma and available progression-free interval (PFI) data were included in the analysis. Cases lacking survival information or gene

expression data were excluded.

Clinical variables including age, pathologic stage, and survival outcomes were extracted from the TCGA clinical dataset. In the TCGA cohort, progression-free interval (PFI) was used as the primary survival endpoint.

For cross-cohort integration with the validation dataset, PFI was later harmonized with relapse-free survival into an event-free survival (EFS) framework.

2.2. Immune checkpoint gene panel

Based on prior literature investigating immune checkpoint pathways in testicular germ cell tumors and other solid malignancies, four candidate immune checkpoint genes—HAVCR2 (TIM-3), PDCD1 (PD-1), LAG3, and TIGIT—were selected for analysis.

Log2-transformed gene expression values for these genes were extracted from the TCGA RNA-sequencing dataset and used as input variables for prognostic modeling. These genes were evaluated as candidate immune checkpoint biomarkers in relation to survival outcomes in seminoma.

2.3. ElasticNet-penalized Cox regression screening

To identify prognostically relevant immune checkpoint genes, ElasticNet-penalized Cox proportional hazards regression was performed as an exploratory penalized regression screen using the expression levels of the four candidate genes.

ElasticNet regularization combines L1 (lasso) and L2 (ridge) penalties, enabling simultaneous feature selection and coefficient shrinkage while reducing model overfitting. The mixing parameter (l1_ratio) was set to 0.5, equally balancing L1 and L2 penalties. Gene expression values were analyzed as log2-transformed TCGA RNA-seq data. The regularization parameter (λ) was selected along the automatically generated regularization path implemented in the ElasticNet procedure. Given the limited number of survival events ($n = 11$), this analysis was intended as an exploratory screen to identify candidate genes retaining non-zero coefficients after penalization for subsequent exploratory survival analyses, rather than as a predictive modeling approach. Formal assessment of the proportional hazards assumption was not performed, which is acknowledged as a limitation of this exploratory analysis.

Genes retaining non-zero coefficients after penalization were considered as candidate variables for subsequent survival analyses.

2.4. Survival analysis in the TCGA cohort

Survival analyses in the TCGA cohort were performed using progression-free interval (PFI) as the primary endpoint. PFI events were defined according to the TCGA clinical annotation.

Kaplan–Meier survival curves were generated to compare survival outcomes according to HAVCR2 expression levels. For group-based analyses, patients were dichotomized into high- and low-expression groups using an exploratory cutoff determined by maximizing survival separation based on the log-rank statistic, as no predefined cutoff was available for transcriptomic HAVCR2 expression in this setting.

Differences between survival curves were assessed using the log-rank test. Hazard ratios and corresponding 95% confidence intervals were estimated using Cox proportional hazards regression models. The exploratory cutoff applied for group-based analyses was used solely for survival visualization purposes and is not intended as a clinically applicable threshold.

2.5. Tissue microarray cohort

To validate transcriptomic findings at the protein level, an independent tissue microarray (TMA) cohort was analyzed. This cohort consisted of patients with histologically confirmed seminoma and was

derived from a previously established retrospective multi-institutional series involving two tertiary academic centers. All patients underwent radical orchiectomy for testicular germ cell tumors.

Formalin-fixed paraffin-embedded tumor specimens were used for TMA construction. Representative tumor areas were selected on hematoxylin- and eosin-stained slides, and the corresponding donor blocks were identified.

Duplicate cylindrical cores measuring 2.0 mm in diameter were obtained from representative tumor regions using a manual tissue arrayer (SuperBioChips Laboratories, Seoul, Korea) and assembled into recipient TMA blocks. Serial 4- μ m sections were prepared for subsequent immunohistochemical analysis.

Clinical and pathological characteristics of the TMA cohort were retrospectively collected from medical records.

2.6. Immunohistochemistry

Immunohistochemical staining was performed on TMA sections to evaluate the expression of four immune checkpoint proteins: PD-1, LAG-3, TIGIT, and TIM-3.

TMA sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval with 10 mM citrate buffer for 30 min. Immunohistochemical staining was performed using a Bond-Max automated immunostainer (Leica Microsystems GmbH, Germany) according to the manufacturer's protocol. The sections were incubated with the following primary antibodies: anti-PD-1 (clone NAT105, ready to use, Cat# 760-4895; Cell Marque, Rocklin, CA, USA), anti-LAG-3 (clone D2G40, XP Rabbit mAb; Cell Signaling Technology, Danvers, MA, USA; 1:100), anti-TIGIT (clone DIA-TG1; Dianova GmbH, Hamburg, Germany; 1:70), and anti-TIM-3 (Cat# ab185703; Abcam, Cambridge, UK; 1:400). Immunoreactivity was detected using the Bond Polymer Refine Detection Kit (Leica Microsystems GmbH, Germany), and slides were counterstained with hematoxylin. Appropriate positive and negative controls were included according to institutional staining protocols.

Expression of immune checkpoint proteins was primarily evaluated in tumor-infiltrating lymphocytes (TIL). Tumor cell staining was also assessed descriptively when present. All slides were independently reviewed by two board-certified pathologists blinded to clinical outcomes and clinicopathologic data, and discrepant cases were resolved by consensus under a multiheaded microscope. Formal interobserver agreement statistics were not prospectively calculated; however, the consensus-based review process was applied consistently across all cases.

Checkpoint expression was quantified as the proportion of positive lymphocytes, and the percentage of positive TIL was recorded. Based on prior study design, a predefined cutoff of 20% positive TIL, established based on prior tissue-level analyses in this cohort, was used to categorize cases into low- and high-expression groups for survival analyses. Based on prior TMA-based findings suggesting a potential prognostic role of TIM-3, survival analyses in the TMA cohort were primarily focused on TIM-3 expression.

2.7. Integrated pooled survival analysis

Given the limited number of events in each individual cohort, a pooled survival analysis integrating the TCGA transcriptomic cohort and the TMA validation cohort was performed as an exploratory integrative analysis to evaluate directional consistency across transcriptomic and protein-level datasets.

Because the TCGA dataset represents transcript-level expression whereas the TMA cohort reflects protein-level expression measured as the percentage of positive tumor-infiltrating lymphocytes, biomarker values from each cohort were standardized separately using cohort-specific z-score normalization to harmonize measurement scales prior to integration. It should be noted that HAVCR2 mRNA expression (TCGA cohort) and TIM-3 protein expression assessed by

immunohistochemistry in tumor-infiltrating lymphocytes (TMA cohort) are biologically related but not equivalent measurements. mRNA expression reflects transcript-level abundance across bulk tumor tissue, whereas IHC-based TIM-3 quantification captures cell-type-specific protein expression in tumor-infiltrating lymphocytes. Z-score normalization was applied to facilitate exploratory integration of the datasets across cohorts; however, this does not eliminate the underlying biological and technical heterogeneity between platforms. Accordingly, the pooled analysis should be interpreted with caution and regarded as exploratory and hypothesis-generating.

After standardization, biomarker values from the two cohorts were combined into a pooled dataset. For group-based analyses, the pooled cohort was dichotomized into biomarker-high and biomarker-low groups using an exploratory cutoff determined by maximizing survival separation based on the log-rank statistic. This cutoff was defined solely for exploratory analytical purposes and is not intended to represent a clinically validated threshold.

For the integrated analysis, event-free survival (EFS) was defined as progression-free interval in the TCGA cohort and relapse-free survival in the TMA cohort. Kaplan–Meier survival analyses and Cox proportional hazards regression were performed using EFS as the endpoint. All cutoff-based analyses were considered exploratory and interpreted in the context of continuous-variable results.

2.8. Statistical analysis

All statistical analyses were performed using Python (version 3.10). Survival analyses, including Kaplan–Meier estimation and Cox proportional hazards regression, were conducted using the lifelines library.

Continuous variables were summarized using descriptive statistics, and categorical variables were compared using the χ^2 test or Fisher's exact test when appropriate.

Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios and corresponding 95% confidence intervals were calculated using Cox proportional hazards regression models. All statistical tests were two-sided, and p values < 0.05 were considered statistically significant. This study was conducted and reported in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines.

2.9. Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of the participating institutions. Because of the retrospective nature of the study and the use of archived tissue specimens, the requirement for informed consent was waived by the institutional review boards.

3. Results

3.1. Study cohort and baseline characteristics

The study consisted of a TCGA discovery cohort and an independent TMA validation cohort. The TCGA cohort included 64 patients with pure seminoma, among whom 11 progression-free interval (PFI) events were observed during follow-up. The median follow-up duration was 26.7 months (interquartile range [IQR], 16.8–45.0 months). Most patients had early-stage disease, with 47 of 57 evaluable cases (82.5%) classified as stage I and the remaining 10 cases (17.5%) as stage II.

The independent TMA validation cohort included 34 patients with pure seminoma. During follow-up, 4 relapse or progression events were observed, and the median follow-up duration was 27.5 months (range, 0.9–166.8 months). Similar to the TCGA cohort, the TMA cohort consisted predominantly of patients with early-stage disease, with 26 patients (76.5%) classified as stage I, 6 (17.6%) as stage II, and 2 (5.9%) as

stage III.

In the TMA cohort, high TIM-3 expression on tumor-infiltrating lymphocytes ($\geq 20\%$) was observed in 18 patients (52.9%), whereas 16 patients (47.1%) were classified as having low TIM-3 expression. TIM-3 corresponds to HAVCR2 at the transcript level in the TCGA dataset.

The overall clinicopathologic characteristics of both the TCGA and TMA cohorts are summarized in Table 1.

3.2. Elastic net Cox analysis of immune checkpoint genes in the TCGA seminoma cohort

To identify prognostically relevant immune checkpoint genes in seminoma, an elastic net penalized Cox regression model was applied to a four-gene panel consisting of HAVCR2, PDCD1, LAG3, and TIGIT using progression-free interval (PFI) as the endpoint in the TCGA seminoma cohort.

Among the evaluated genes, HAVCR2 retained a relatively larger coefficient after penalization, whereas PDCD1, LAG3, and TIGIT exhibited substantially smaller coefficients following regularization

Table 1
Baseline characteristics.

Variable	TMA seminoma (n = 34)	TCGA cohort (n = 64)
N	34	64
Age, median (year, IQR)	32.5 (28.8 – 39.1)	33.0 (28.0–38.2)
Laterality		
Right	13 (38.2%)	33/64 (51.6%)
Left	21 (61.8%)	28/64 (43.8%)
Bilateral	0 (0.0%)	3/64 (4.7%)
Primary tumor (T)		
T1	21 (61.8%)	41/64 (64.1%)
T2	12 (35.3%)	20/64 (31.2%)
T3	1 (2.9%)	2/64 (3.1%)
Tx	0 (0.0%)	1/64 (1.6%)
Clinical regional lymph nodes (N)		
N0	26 (76.5%)	39/59 (66.1%)
N1	7 (20.6%)	6/59 (10.2%)
N2	1 (2.9%)	1/59 (1.7%)
N3	0 (0.0%)	2/59 (3.4%)
Nx	0 (0.0%)	11/59 (18.6%)
Clinical M stage		
M0	34 (100%)	58/59 (98.3%)
M1	0 (0.0%)	1/59 (1.7%)
Serum tumor markers (S)		
S0	17 (50.0%)	29/62 (46.8%)
S1	12 (35.3%)	14/62 (22.6%)
S2	5 (14.7%)	5/62 (8.1%)
S3	0 (0.0%)	3/62 (4.8%)
Sx	0 (0.0%)	11/62 (17.7%)
Anatomic stage/prognostic groups		
Stage I	26 (76.5%)	47/57 (82.5%)
Stage II	6 (17.6%)	10/57 (17.5%)
Stage III	2 (5.9%)	0/57 (0.0%)
Follow-up, median (months, range)	27.5 (0.9 - 166.8)	26.7 (16.8–45.0)
Relapse/progression event	4 (11.8%)	11/64 (17.2%)
TIM-3 on TIL ($\geq 20\%$)		
High	18 (52.9%)	NA
Low	16 (47.1%)	NA
HAVCR2		
High	NA	15/64 (23.4%)
Low	NA	49/64 (76.6%)
Adjuvant treatment		
Any adjuvant treatment	14 (41.2%)	17/63 (27.0%)
No adjuvant treatment	20 (58.8%)	NA
Radiation therapy	9 (26.5%)	NA
Chemo therapy	6 (17.6%)	NA

TMA, tissue microarray; TCGA, The Cancer Genome Atlas; IQR, Interquartile Range; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; TIL, tumor-infiltrating lymphocytes; HAVCR2, Hepatitis A Virus Cellular Receptor 2.

(Fig. 1). This pattern indicates that HAVCR2 showed a relatively larger non-zero coefficient within the evaluated immune checkpoint panel, while the remaining genes showed minimal independent contribution after penalization.

These results identify HAVCR2 as a prioritized candidate for subsequent survival analyses and validation in the integrated cohort.

3.3. Association between HAVCR2 expression and PFI in the TCGA seminoma cohort

Based on the elastic net screening results, we further evaluated the association between HAVCR2 expression and clinical outcomes in the TCGA seminoma cohort. HAVCR2 expression was analyzed both as a continuous variable and using dichotomized expression groups.

In continuous-variable Cox regression analysis, higher HAVCR2 expression demonstrated a consistent favorable direction of association with progression-free interval, although statistical significance was not reached, likely reflecting the limited number of events and resulting low statistical power. This directional consistency supports that the observed association is not solely dependent on dichotomization. For group-based analyses, patients were dichotomized using an exploratory cutoff determined by maximizing survival separation based on the log-rank statistic. Kaplan–Meier survival analysis demonstrated that patients with higher HAVCR2 expression tended to have more favorable PFI compared with those with lower expression levels (Fig. 2A). Although statistical significance was not reached (log-rank $p = 0.120$), the direction of association was consistent with the relative effect size observed in the elastic net model.

In the elastic net model, HAVCR2 retained a relatively larger non-zero coefficient among the evaluated immune checkpoint genes, supporting its prioritization for subsequent exploratory survival analyses.

3.4. Association between TIM-3 expression and relapse-free survival in the TMA cohort

In the independent tissue microarray (TMA) cohort comprising 34 patients with pure seminoma, TIM-3 expression on tumor-infiltrating lymphocytes was evaluated using immunohistochemistry. Patients were stratified into high and low expression groups based on a pre-defined cutoff of 20% positive cells.

Kaplan–Meier survival analysis demonstrated that patients with high TIM-3 expression had a trend toward improved relapse-free survival (RFS) compared with those with low expression (Fig. 2B). Although statistical significance was not reached (log-rank $p = 0.053$), a consistent direction of association was observed.

Notably, no relapse events were observed in the TIM-3 high-expression group across the entire follow-up period, whereas all four relapse events occurred exclusively in the low-expression group. This complete separation of events between expression groups — with 18 high-expression patients remaining event-free — represents a clear and directionally consistent pattern, particularly given the limited absolute event count. Although statistical significance was not reached (log-rank $p = 0.053$), the observed pattern is consistent with the transcriptomic findings in the TCGA cohort and supports a potential protective association of TIM-3 expression in seminoma.

3.5. Pooled survival analysis of combined TCGA and TMA cohorts

To further evaluate the prognostic significance of HAVCR2 (TIM-3), a pooled survival analysis was performed by integrating the TCGA seminoma cohort with the independent TMA cohort. The combined dataset included 98 patients with pure seminoma (64 from TCGA and 34 from the TMA cohort).

Across individual cohorts, HAVCR2/TIM-3 expression demonstrated a consistent direction of association with clinical outcomes, although statistical significance was not reached in the TCGA cohort and was

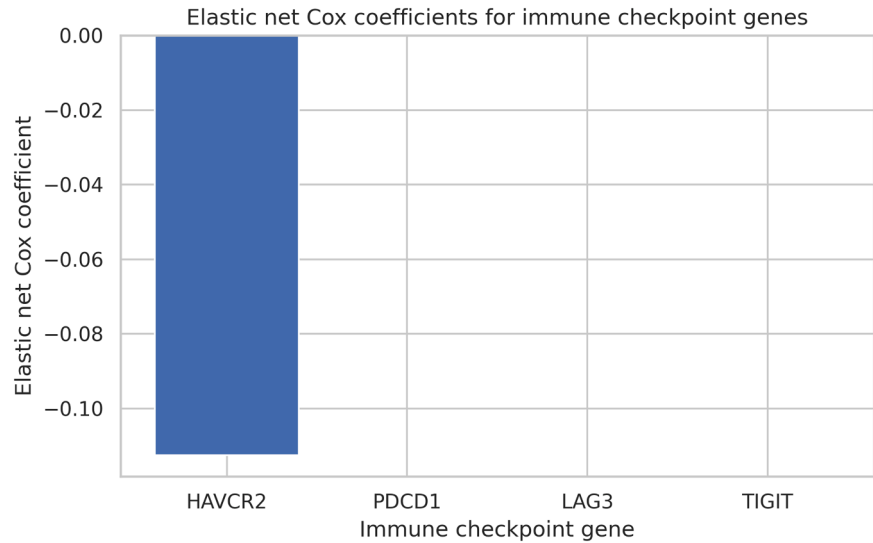


Fig. 1. Elastic net Cox regression coefficients for immune checkpoint genes in the TCGA seminoma cohort. Elastic net regression was applied to a four-gene immune checkpoint panel (HAVCR2, PDCD1, LAG3, TIGIT) using progression-free interval as the outcome. HAVCR2 retained a relatively larger non-zero coefficient after penalization, whereas PDCD1, LAG3, and TIGIT showed substantially attenuated coefficients following regularization.

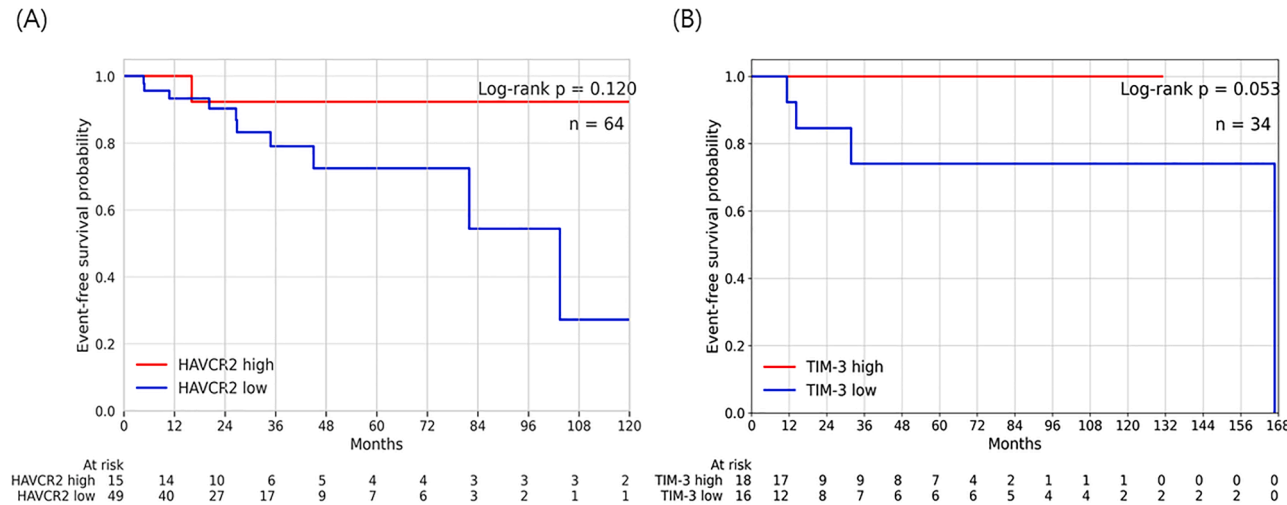


Fig. 2. Association between HAVCR2/TIM-3 expression and event-free survival in seminoma. (A) Kaplan–Meier analysis of progression-free interval (PFI) according to HAVCR2 expression in the TCGA seminoma cohort. Patients with higher HAVCR2 expression showed a trend toward improved PFI compared with those with lower expression levels, although the difference did not reach statistical significance (log-rank $p = 0.120$). (B) Kaplan–Meier analysis of relapse-free survival (RFS) according to TIM-3 expression on tumor-infiltrating lymphocytes in the independent tissue microarray (TMA) cohort ($n = 34$). Patients with high TIM-3 expression ($\geq 20\%$, $n = 18$) remained entirely event-free throughout follow-up, whereas all four relapse events occurred exclusively in the low-expression group ($n = 16$), representing complete separation of events between groups (log-rank $p = 0.053$). Although the small number of events limited statistical power, the observed separation demonstrated a directionally consistent pattern across expression groups.

borderline in the TMA cohort. The direction of effect was concordant between transcriptomic and protein-level analyses.

For group-based analyses, patients were dichotomized using an exploratory cutoff determined by maximizing survival separation based on the log-rank statistic. In the pooled cohort, patients with high HAVCR2/TIM-3 expression showed improved event-free survival compared with those with low expression (log-rank $p = 0.019$; Fig. 3). Cox proportional hazards analysis demonstrated that higher HAVCR2 expression was associated with a reduced risk of progression (HR = 0.201, 95% CI: 0.045–0.891, $p = 0.035$).

To account for potential bias related to cutoff optimization, permutation testing was performed, yielding a corrected p value of 0.164. While this indicates that the cutoff-based grouping should be regarded as exploratory, the concordant direction of association across cohorts

remained consistent. Nevertheless, these findings should be interpreted cautiously given the exploratory nature of the pooled analysis and the non-significant permutation-corrected result.

4. Discussion

In this study, we identified HAVCR2 (TIM-3) as having a relatively larger non-zero coefficient among the evaluated immune checkpoint genes, with a consistent favorable direction of association across cohorts, using an integrative analytical framework combining transcriptomic discovery, an exploratory penalized regression screen, independent tissue-level validation, and pooled survival analysis. Among the evaluated checkpoint genes, HAVCR2 retained a relatively larger non-zero coefficient after ElasticNet penalization, whereas the

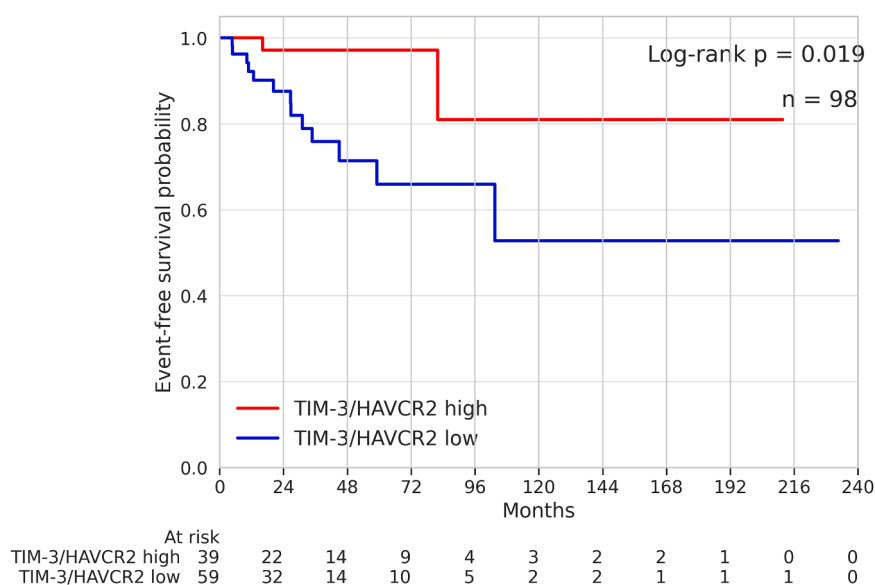


Fig. 3. Pooled survival analysis of HAVCR2/TIM-3 expression in combined TCGA and TMA seminoma cohorts. Kaplan–Meier analysis of event-free survival in the pooled cohort integrating the TCGA transcriptomic dataset and the independent TMA cohort (n = 98). Patients with high HAVCR2/TIM-3 expression demonstrated improved event-free survival compared with those with low expression (log-rank p = 0.019; HR = 0.201, 95% CI: 0.045–0.891, p = 0.035). Biomarker values were standardized within each cohort using z-score normalization prior to integration. Permutation-corrected p value = 0.164.

coefficients of PDCD1, LAG3, and TIGIT were substantially reduced through regularization. The favorable association of HAVCR2 was consistently observed across independent cohorts and measurement platforms; however, given the limited number of events in both cohorts, these findings should be interpreted as hypothesis-generating rather than confirmatory, supporting further investigation of TIM-3 as a context-dependent immune biomarker within the seminoma immune microenvironment.

A central finding of this study is the context-dependent association between TIM-3 expression and improved clinical outcomes, which contrasts with its conventional interpretation as a marker of T-cell exhaustion [12,16]. TIM-3 has been widely characterized as a co-inhibitory receptor associated with dysfunctional T-cell states and immune tolerance [12,17–19]. However, this interpretation may not be directly applicable to seminoma. Although the testis is a well-recognized immune-privileged organ characterized by tightly regulated immune tolerance [5], seminoma exhibits substantial remodeling of the immune microenvironment, including increased TIL infiltration and activation of immune-related pathways [4–7], and accumulating evidence indicates that it represents an immune-inflamed tumor with active immune engagement rather than a uniformly immunosuppressed phenotype [2, 4–7]. Multiple studies have demonstrated that immune checkpoint molecules including PD-1, programmed death-ligand 1 (PD-L1), and TIGIT are highly expressed in TILs in seminoma, reflecting an antigen-experienced and immune-engaged microenvironment [20–23], and notably, PD-L1 expression on TILs has been associated with favorable clinical outcomes [22], suggesting that checkpoint expression in seminoma may reflect immune activation rather than exhaustion.

This interpretation is further supported by the biology of TIM-3 itself. TIM-3 expression has been observed on interferon- γ -producing Th1 cells, indicating that it can be expressed in functionally active rather than exhausted T-cell states [24]. Furthermore, TIM-3 signaling is ligand-dependent and context-specific, involving interactions with galectin-9, CEACAM1, and HMGB1, which fine-tune immune responses in a tissue- and tumor-specific manner [18,19]. Taken together, these findings provide a biological basis for the favorable prognostic association of TIM-3 in seminoma, and suggest that its expression in this context may reflect an immune-active microenvironment rather than conventional T-cell dysfunction.

Despite the high expression of immune checkpoint molecules, clinical trials of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have demonstrated limited and inconsistent efficacy in TGCT [8–10]. Phase II studies of pembrolizumab, avelumab, and durvalumab-based regimens have shown low response rates and limited survival benefit in patients with refractory disease [8–10]. This discrepancy between checkpoint expression and therapeutic response represents a key biological observation, suggesting that checkpoint molecules in TGCT may not primarily function as direct mediators of immune escape. Instead, they may serve as indicators of an underlying immune-active tumor microenvironment, consistent with the findings of the present study.

Within this framework, our results indicate that HAVCR2 showed a relatively larger non-zero coefficient among the evaluated checkpoint genes, with a favorable direction of association across cohorts. The consistency of this association across multiple analytical levels supports its biological plausibility. In the TCGA cohort, HAVCR2 expression showed a favorable trend despite limited statistical power. In the independent TMA cohort, all relapse events occurred exclusively in the TIM-3 low-expression group, indicating clear biological separation at the protein level. The pooled analysis demonstrated a similar direction of association, although these findings should be interpreted in the context of limited event numbers, exploratory cutoff selection, and the non-significant permutation test. The consistent direction of association observed in continuous-variable analysis further supports that the findings are not purely driven by threshold selection.

Several limitations of this study should be acknowledged. First, the number of events in each individual cohort was limited (4 in the TMA cohort and 11 in the TCGA cohort), which constrained statistical power and precluded multivariable adjustment; residual confounding by pathologic stage cannot be excluded. Accordingly, all prognostic associations observed in this study should be interpreted as exploratory and hypothesis-generating, pending validation in larger independent cohorts. Second, the cutoff used for dichotomizing biomarker expression in the pooled analysis was determined exploratorily by maximizing log-rank separation and is not intended for direct clinical application. Third, the pooled analysis integrated transcriptomic and immunohistochemical data from heterogeneous platforms — specifically, HAVCR2 mRNA expression from TCGA and TIM-3 protein expression from TMA

immunohistochemistry — which are biologically related but not equivalent measurements; the non-significant permutation-corrected p value (0.164) further supports interpreting these findings as hypothesis-generating rather than confirmatory. Fourth, formal assessment of the proportional hazards assumption was not performed, which is acknowledged as a limitation of the exploratory penalized regression analysis. Finally, the retrospective design limits causal inference, and the absence of functional validation in vitro or in vivo represents an additional limitation; however, the consistent direction of association across independent cohorts and measurement platforms supports the biological plausibility of the findings.

Despite these limitations, this study has several strengths, including the use of an exploratory penalized regression approach for candidate gene screening, validation across independent cohorts, and integration of RNA and protein-level data. These features support the potential relevance of HAVCR2 as a candidate biomarker.

From a clinical perspective, our findings support HAVCR2/TIM-3 as a potential prognostic biomarker in seminoma. However, these results should not be overinterpreted as evidence against TIM-3-directed therapy, but rather suggest that its biological role in seminoma may differ from conventional expectations. Although TIM-3 has been proposed as a therapeutic target in various malignancies [13,16,25], its relevance in TGCT remains uncertain, underscoring the need for tumor-specific interpretation of immune checkpoint biology.

5. Conclusion

HAVCR2 (TIM-3) may represent a favorable prognostic immune marker in seminoma, reflecting an active and biologically favorable immune microenvironment rather than conventional T-cell exhaustion.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used Claude (Anthropic) and ChatGPT (OpenAI) in order to support language editing and manuscript refinement. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Funding

This work was supported by the Soonchunhyang University Research Fund.

CRediT authorship contribution statement

Ki Hong Kim: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dae Young Lee:** Investigation, Data curation. **Hee Jo Yang:** Methodology, Investigation, Formal analysis. **Si Hyun Kim:** Methodology, Data curation. **Doo Sang Kim:** Resources, Project administration. **Chang Ho Lee:** Investigation, Data curation. **Soon Auck Hong:** Resources, Methodology, Investigation, Formal analysis. **Byung Hoon Chi:** Resources, Data curation. **Ji-Hye Lee:** Validation, Supervision, Resources, Investigation, Formal analysis. **Youn Soo Jeon:** Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ki

Hong Kim reports financial support was provided by Soonchunhyang University Hospital Cheonan. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The transcriptomic data and clinical information used in this study were obtained from The Cancer Genome Atlas (TCGA) through the UCSC Xena platform (<https://xenabrowser.net/>), which is publicly available. The tissue microarray cohort data are not publicly available due to patient privacy and institutional review board restrictions, but are available from the corresponding author on reasonable request.

References

- [1] J.S. Park, J. Kim, A. Elghiaty, et al., Recent global trends in testicular cancer incidence and mortality, *Medicine* 97 (2018) e12390.
- [2] K. Evmoropoulos, K. Marsitopoulos, R. Karachalios, et al., The immune landscape and immunotherapeutic strategies in platinum-refractory testicular germ cell tumors, *Cancers (Basel)* 16 (2024) 428.
- [3] D.R. Feldman, S. Patil, M.J. Trinos, et al., Progression-free and overall survival in patients with relapsed/refractory germ cell tumors treated with single-agent chemotherapy: endpoints for clinical trial design, *Cancer* 118 (2012) 981–986.
- [4] R. Islam, J. Heyer, M. Figura, et al., T cells in testicular germ cell tumors: new evidence of fundamental contributions by rare subsets, *Br. J. Cancer* 130 (2024) 1893–1903.
- [5] H. Farahani, R. Darvishvand, A. Khademolhosseini, et al., Unwrapping the immunological alterations in testicular germ cell tumors: from immune homeostasis to malignancy and emerging immunotherapies, *Andrology* 13 (2025) 747–762.
- [6] L. Wang, Y. Peng, D. Hou, et al., Tumor mutational burden, gene expression patterns, and immune-cell deconvolution in testicular germ cell tumors, *Transl. Androl. Urol.* 14 (2025) 2663–2679.
- [7] R. Islam, M. Figura, J. Heyer, et al., T cell microenvironmental impacts and therapeutic opportunities: application to Human testicular germ cell tumors, *Andrology* (2026), <https://doi.org/10.1111/andr.70200>.e70200.
- [8] M. Mego, D. Svetlovskaya, M. Chovanec, et al., Phase II study of avelumab in multiple relapsed/refractory germ cell cancer, *Invest. New Drugs* 37 (2019) 748–754.
- [9] N. Adra, L.H. Einhorn, S.K. Althouse, et al., Phase II trial of pembrolizumab in patients with platinum refractory germ-cell tumors: a Hoosier cancer research network study GU14-206, *Ann. Oncol.* 29 (2018) 209–214.
- [10] A. Necchi, P. Giannatempo, D. Raggi, et al., An open-label randomized phase 2 study of Durvalumab alone or in combination with Tremelimumab in patients with advanced germ cell tumors (APACHE): results from the first planned interim analysis, *Eur. Urol.* 75 (2019) 201–203.
- [11] F.K. Mehr, N. Emtiazi, E. Zolfi, Advances in cell therapy for testicular cancer: a comprehensive overview of immunotherapy and stem cell therapy, *Tissue Cell* 98 (2026) 103169.
- [12] A. Schnell, L. Bod, A. Madi, et al., The yin and yang of co-inhibitory receptors: toward anti-tumor immunity without autoimmunity, *Cell Res.* 30 (2020) 285–299.
- [13] B. Xu, L. Yuan, Q. Gao, et al., Circulating and tumor-infiltrating Tim-3 in patients with colorectal cancer, *Oncotarget* 6 (2015) 20592–20603.
- [14] L. Xue, X. Zhao, H. Jia, et al., Upregulated HAVCR2: a prognostic and immune-related marker in testicular germ cell tumors, *Andrologia* 2023 (2023) 5524200.
- [15] Z. Bian, B. Chen, J. Guo, et al., Single-cell analysis unravels divergent gene signatures shaping seminoma stemness and metastasis, *Cell Death. Discov.* 11 (2025) 514.
- [16] Y. Zhang, P. Cai, T. Liang, et al., TIM-3 is a potential prognostic marker for patients with solid tumors: a systematic review and meta-analysis, *Oncotarget* 8 (2017) 31705–31713.
- [17] V. Dardalhon, A.C. Anderson, J. Karman, et al., Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b+ly-6G+ myeloid cells, *J. Immunol.* 185 (2010) 1383–1392.
- [18] J.S. Dolina, T.J. Braciale, Y.S. Hahn, Liver-primed CD8+ T cells suppress antiviral adaptive immunity through galectin-9-independent T-cell immunoglobulin and mucin 3 engagement of high-mobility group box 1 in mice, *Hepatology* 59 (2014) 1351–1365.
- [19] Y.H. Huang, C. Zhu, Y. Kondo, et al., CEACAM1 regulates TIM-3-mediated tolerance and exhaustion, *Nature* 517 (2015) 386–390.
- [20] Z. Cierna, M. Mego, V. Miskovska, et al., Prognostic value of programmed-death-1 receptor (PD-1) and its ligand 1 (PD-L1) in testicular germ cell tumors, *Ann. Oncol.* 27 (2016) 300–305.
- [21] C.D. Fankhauser, A. Curioni-Fontecedro, V. Allmann, et al., Frequent PD-L1 expression in testicular germ cell tumors, *Br. J. Cancer* 113 (2015) 411–413.

- [22] M. Chovanec, Z. Cierna, V. Miskovska, et al., Prognostic role of programmed-death ligand 1 (PD-L1) expressing tumor infiltrating lymphocytes in testicular germ cell tumors, *Oncotarget* 8 (2017) 21794–21805.
- [23] A. Hinsch, N.C. Blessin, R. Simon, et al., Expression of the immune checkpoint receptor TIGIT in seminoma, *Oncol. Lett.* 18 (2019) 1497–1502.
- [24] L. Monney, C.A. Sabatos, J.L. Gaglia, et al., Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease, *Nature* 415 (2002) 536–541.
- [25] S. Qin, B. Dong, M. Yi, et al., Prognostic values of TIM-3 expression in patients with solid tumors: a meta-analysis and database evaluation, *Front. Oncol.* 10 (2020) 1288.