# Expression of the immune checkpoint receptor TIGIT in seminoma

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**Abstract.** A characteristic feature of testicular seminoma is the abundance of immune cells in the tumor microenvironment, raising the possibility that immune checkpoint inhibitors may serve as a therapeutic option in these types of tumors. T cell immunoreceptor with Ig and ITIM domains (TIGIT) is an inhibitory immune checkpoint receptor in analogy to PD-1, and drugs targeting TIGIT are currently being investigated in clinical trials. Little is known about the expression of these proteins in testicular seminomas. Therefore the present study performed immunohistochemical analysis to determine the relative abundance of TIGIT and PD-1 in relation to the total $CD3^+$ immune cell infiltration in a tissue microarray (TMA) constructed from 78 seminoma patients. The fraction of $TIGIT^+$ and $PD-1^+$ lymphocytes was highly variable in individual cancers and ranged from 2.3 to 69.4% (mean: 32.2±14.7%) for TIGIT and from 0.8 to 56.5% (mean: 21.6±13.2%) for PD-1. The same high degree of variability was also identified for the ratio of PD-1 to TIGIT positive cells, which varied from a dominance of TIGIT (PD-1: TIGIT ratio=0.02) in 74% of patients, to a predominance of PD-1 (PD-1: TIGIT ratio=12.5) in 23% of patients. In summary, the immune checkpoint receptors TIGIT and PD-1 are abundantly expressed in human seminomas. Once available, anti-TIGIT antibodies, possibly in combination with anti-PD-1 drugs, may be a reasonable therapeutic strategy for this type of cancer.

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## Introduction

Testicular seminoma is one of the most common solid cancer types in young men and accounts for almost half of testicular germ cell tumors (1). The vast majority of seminoma patients are cured by current therapy concepts, including orchiectomy, radiotherapy and/or platinum-based chemotherapy (2-4). However, survival is poor in these patients with platinum refractory disease relapsing after high-dose chemotherapy (5). Given the high density of immune cells typically seen in seminomas, it is intuitive, that immune checkpoint inhibitors may represent a therapeutic option in these tumors. Clinical response and non-response of advanced germ cell cancers after therapy with Nivolumab or Pembrolizumab have indeed been reported and clinical trials systematically evaluating these drugs in platinum resistant germ cell tumors are ongoing (6,7).

The clinical success of checkpoint inhibitors targeting the PD-1/PD-L1 system is stunning in many cancers (8-10), and earlier studies reported a prognostic role of PD-L1 expressing tumor infiltrating lymphocytes in seminoma samples (11,12). Moreover, there is much hope that the use of combinatorial drugs targeting not only a single, but several immune checkpoint receptors, might further improve therapeutic results. T cell immunoreceptor with Ig and ITIM domain (TIGIT), a co-inhibitory transmembrane glycoprotein of the poliovirus receptor (PVR)/nectin superfamily, is another interesting candidate for novel checkpoint therapies (13,14). In mouse models and ongoing clinical studies, blockade or ablation of TIGIT, alone or in combination with blockade of PD-1, can restore tumor suppressive effects (15-19). These findings indicate that TIGIT, similar to PD-1, has a crucial role in inhibiting the tumor-directed immune response and, thus, might be a suitable and relevant target for novel immune-modulating therapies. Several drugs targeting TIGIT are currently under development (20). TIGIT expressing lymphocytes have so far been demonstrated in acute myeloid leukemia, non-small cell lung cancer, colorectal carcinoma and melanoma (16,17,21).
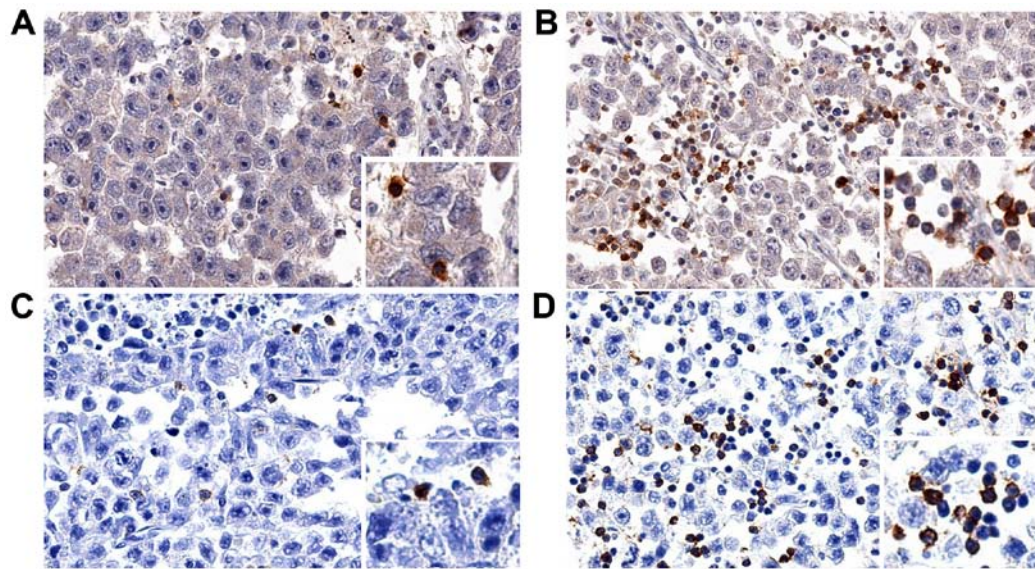
Figure 1. (A and B) TIGIT and (C and D) PD-1 staining in seminomas. Examples of cases with low-level infiltration of (A) TIGIT+ and (C) PD-1+ immune cells and cases with high level of infiltration of (B) TIGIT+ and (D) PD-1+ immune cells. TIGIT, T cell immunoreceptor with Ig and ITIM domains; PD-1, Programmed Cell Death 1.

While it appears possible that the selection of the optimal immune checkpoint inhibitor may depend on the role of the respective target in a cancer's associated immune cells, we were interested in the expression of TIGIT and PD-1 on lymphocytes in seminomas. In this study, the patterns of TIGIT and PD-1 were analyzed by immunohistochemistry in 78 seminomas in a tissue microarray (TMA) format.

**Materials and methods**

*Patients and tissues*. Formalin-fixed paraffin embedded tissue samples from 78 anonymized patients with seminoma were retrieved from the archives of the Institute of Pathology of the University Medical Center, Hamburg Eppendorf. On average, the mean age was 38±9 years and the median and mean tumor sizes were 30 mm (range:10 to 70 mm) and 33±14 mm. This patient cohort contained two 0.6 mm tumor punches per patient were assembled in a TMA. The TMA manufacturing process has been described earlier (22).

*Immunohistochemistry*. Three freshly cut consecutive TMA sections were immunoassayed for CD3, TIGIT and PD-1. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121˚C in pH 6 buffer for PD-1, pH 7.8 buffer for TIGIT and pH 9 buffer for CD3. Primary antibody specific for CD3 (rabbit polyclonal antibody, undiluted, cat. no. IR503; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), TIGIT (mouse monoclonal antibody, Dianova GmbH, Hamburg, Germany; cat. no. DIA-TG1; 1:70) and PD-1 [mouse monoclonal (NAT105) antibody, Abcam, Cambridge, UK; cat. no. ab52587; 1:50] was applied at 37˚C for 60 min. Bound antibody was then visualized using the EnVision Kit (Dako; Agilent Technologies, Inc.) according to the manufacturer's directions.

*Scoring of CD3, TIGIT and PD-1 immunostaining*. To assure that the quantity of tissue analyzed per patient was identical, only the first spot per patient that was interpretable for all three markers was analyzed. The total number of CD3+, TIGIT+ and PD-1+ cells was manually counted in each TMA slide and tissue spot. The TIGIT:CD3 and PD-1:CD3 ratio was calculated for each tissue spot to determine the fraction of TIGIT and PD-1 positive T lymphocytes.

*Statistical analysis*. The JMP 12.0 software package (23) (SAS Institute Inc., NC, USA) was used to calculate the mean and standard deviation of the fraction of TIGIT and PD-1 positive cells.

**Results**

All 78 seminomas included in this study harbored tumor infiltrating CD3+ T lymphocytes. Their number was variable between individual cancers and ranged from 16 to 2,113 (mean: 623, standard deviation: 509). All tumors also showed TIGIT and PD-1 staining in a variable number of immune cells. This number was overall somewhat higher for TIGIT than for PD-1: The number of TIGIT+ lymphocytes per 0.6 mm tissue spot ranged from 2 to 1,147 (mean: 194, standard deviation: 185), the number of PD-1+ lymphocytes ranged from 2 to 424 (mean: 107, standard deviation: 93). Representative images of immunostainings are shown in Fig. 1.

To compare the relative abundance of TIGIT and PD-1 expressing T cells in individual tumor samples, we used the number of CD3+ T cells per tissue spot as a reference. It showed that both the fraction of TIGIT+ T cells and of PD-1+ T cells was variable among the 78 seminoma patients. The fraction of TIGIT+ T cells (mean: 32.2±14.7%) ranged from 2.3 to 69.4% and that of PD-1 (mean: 21.6±13.2%) from 0.8 to 56.5% (Fig. 2).

In individual cancers, TIGIT expression largely but not fully paralleled that of PD-1. However, the relative importance of PD-1 and TIGIT appeared to be rather variable. TIGIT expression exceeded that of PD-1 in 58 cancers while 18
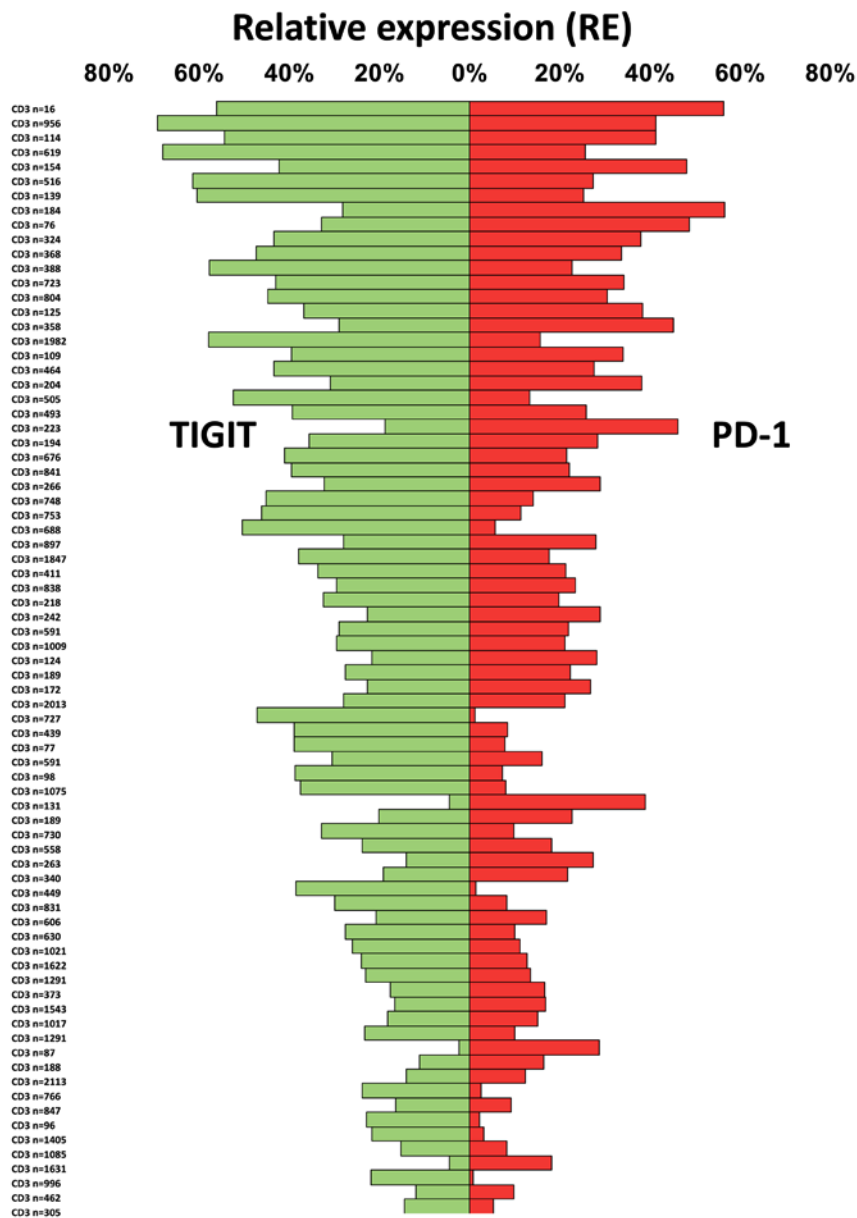
Figure 2. The inter-individual fraction of TIGIT (green) and PD-1 (red) positive CD3+ immune cells in 78 seminoma patients. The absolute number of CD3 positive cells is indicated at the left-hand side. TIGIT, T cell immunoreceptor with Ig and ITIM domains.

tumors had PD-1 levels beyond that of TIGIT. In these cancers, the PD-1: TIGIT ratio ranged from 0.02 to 12.5 (Fig. 3).

## Discussion

The data from the present study demonstrate that not only the fraction of TIGIT+ and/or PD-1+ T cells, but also their relative abundance, is highly variable between individual seminomas.

TIGIT+ T lymphocytes were detected in all tumors in our study. This was expected because in an earlier study using a comparable experimental set-up, we had also regularly found TIGIT expression in a considerable fraction of lymphocytic cells in healthy lymphatic organs, various inflammatory diseases and in samples of lung and colorectal cancers (19). Taken together, these findings strongly support the concept that TIGIT expression is an inherent feature of T cell lymphocytic infiltrations in normal, inflammatory

and cancerous tissues (19). Earlier studies using flow cytometry have also described regular presence of TIGIT+ T cells in these immune microenvironments (13,17,21,24,25). For example, Johnston et al (16), detected TIGIT expression among CD8+ cytotoxic T cells in colon and breast cancer. Josefsson et al (24), described TIGIT expressing cells in follicular lymphomas. Luo et al (25), showed increased TIGIT expression in the autoimmune environment of rheumatoid arthritis (26). Drugs targeting TIGIT are currently developed by various companies (15,20). Although there is some evidence for a lack of response to PD-L1 inhibitory drugs in more than 90% of the treated patients (27), further therapy attempts using combined or single anti-TIGIT and/or anti-PD-1 therapies are still lacking in testicular seminoma.

Overall, the existing data on the prevalence of TIGIT expression seems to suggest that such drugs may potentially be applicable to a very broad range of different tumor types.
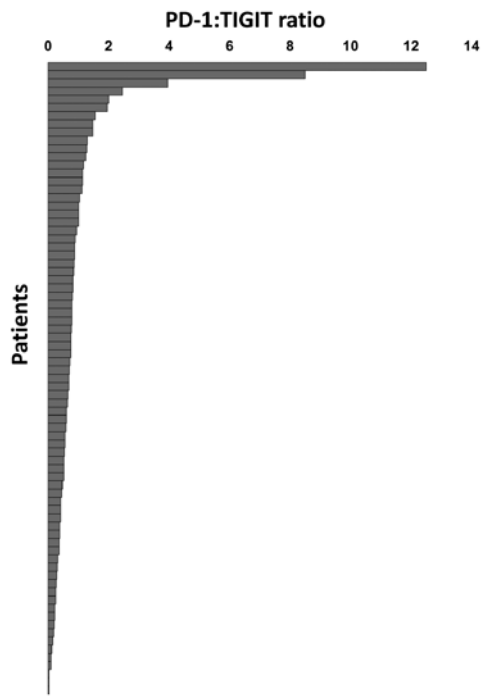
Figure 3. Distribution of the PD-1: TIGIT ratio. Each bar represents a single patient. TIGIT, T cell immunoreceptor with Ig and ITIM domains; PD-1, Programmed Cell Death 1.

The high interest in TIGIT emanates from its analogy to PD-1, which has become a major therapeutic 'host target' in a multitude of human tumor types (8,28,29). That PD-1 expression was seen in a fraction of T lymphocytes in all analyzed seminomas is in line with a recent study using multiplex fluorescence immunohistochemistry (30). In this study, Siska *et al* found a variable T cell infiltration and immune checkpoint expression in almost all analyzed large sections of seminomas and non-seminomas. That comparable absolute and relative numbers were found in our study using brightfield immunohistochemistry represents an indirect validation of our experimental approach.

The high numbers of intratumoral CD3^{+}, TIGIT^{+} and PD-1^{+} cells per 0.6 mm tissue spot (0.28 mm[2]) demonstrate that immune cells play a particularly strong role in seminoma. Adjusted numbers per square millimeter (CD3: Average $2,203\pm1,799$ per mm[2]) are higher than what we found in urinary bladder cancer (CD3: $625\pm800$, cells/mm^{2}) (31) or what was earlier described in breast (150 to 300 CD3^{+} cells/mm^{2}) (32) or colorectal cancer (400 to 700 CD3^{+} cells/mm^{2}) (33). The potential significance of these immune cells for anti-tumor activity is best demonstrated by cases of 'burned out seminomas' (34). In these patients-sometimes extensive-metastatic seminoma spread occurs in the absence of vital tumor tissue in the testis. Instead, circumscribed scar formation indicates the location of a 'self-healed' testicular seminoma. Based on this, it is tempting to speculate that treatment with immune checkpoint inhibitors-perhaps even first line-might be particularly successful in testicular germ cell tumors. Currently used platinum-based therapies are highly efficient (35) but there are only inadequate treatment options available for chemotherapy refractory or relapsed metastatic testicular seminomas (36). However, because of the young age, patients often develop long-term

sequelae of treatment, such as cardiovascular disease, renal insufficiency or secondary malignancies (35,37,38). Therapies targeting immune checkpoint receptors may exert comparable little long-term side effects (39,40).

The most striking observation in our study was the high variability of the relative fraction of TIGIT[+] and PD-1^{+} lymphocytes in seminomas. We earlier reported a similar diversity of the relative role of TIGIT and PD-1 in a cohort of 40 Hodgkin's lymphomas (41). If it holds true that the different checkpoint receptors are so variably expressed in individual cancer patients, the analysis of the inflammatory cells may proof instrumental to select the optimal immune checkpoint inhibitor for a given patient.

In conclusion, the results of our study demonstrate frequent expression of immune checkpoints receptors in human seminomas. This argues for a potential benefit of drugs targeting immune checkpoint molecules in these tumors. The high variability of the relative prevalence of TIGIT[+] and PD-1^{+} cells between patients raises the hypothesis that a thorough analysis of checkpoint proteins in tumor infiltrating lymphocytes may in the future assist the choice of therapy.

## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

WW, GS, RS, NB and KF undertook study conception and design. AH, NB, WL, RS, GS, KF were responsible for development of the methodology. Acquisition of data, including providing animals, acquiring and managing patients, providing facilities, was the responsibility of AH, WL, NB, TM, BW, ND, WW, DH, ML, JI, SM, FB, RU, DD, TK, AL, CW, FJ, EB and SS. Analysis and interpretation of data, including statistical analysis, biostatistics, computational analysis was performed by AH, WW, WL, NB, RS and GS. Writing, review, and/or revision of the manuscript was performed by RS, GS, WW and NB. Administrative, technical, or material support (i.e., reporting or organizing data, tissue processing, antibody development) was performed by MK, CHM, GMF, DH, ML, JI, SM, FB, RU, DD, TK, AL, CW, FJ, EB, SS and WW. GS, RS, WW and AH supervised the study.

## Ethics approval and consent to participate

Archived diagnostic leftover tissues for manufacturing of TMAs was pseudo-anonymized and used without informed

consent according to the local law (HmbKHG, §12a). The study was approved by the local ethic committee (Ethics commission of the Ärztekammer Hamburg no. WF-049/09) and in line with the Declaration of Helsinki.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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