ORIGINAL ARTICLE

TNFSF4 (OX40L) expression and survival in locally advanced and metastatic melanoma

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Abstract

Immunotherapy with checkpoint inhibitors revolutionized melanoma treatment in both the adjuvant and metastatic setting, yet not all metastatic patients respond, and metastatic disease still often recurs among immunotherapy-treated patients with locally advanced disease. TNFSF4 is a co-stimulatory checkpoint protein expressed by several types of immune and non-immune cells, and was shown in the past to enhance the anti-neoplastic activity of T cells. Here, we assessed its expression in melanoma and its association with outcome in locally advanced and metastatic disease. We used publicly available data from The Cancer Genome Atlas (TCGA) and the Cancer Cell Line Encyclopedia (CCLE), and RNA sequencing data from anti-PD1-treated patients at Sheba medical center. TNFSF4 mRNA is expressed in melanoma cell lines and melanoma samples, including those with low lymphocytic infiltrates, and is not associated with the ulceration status of the primary tumor. Low expression of TNFSF4 mRNA is associated with worse prognosis in all melanoma patients and in the cohorts of stage III and stage IIIc–IV patients. Low expression of TNFSF4 mRNAs is also associated with worse prognosis in the subgroup of patients with low lymphocytic infiltrates, suggesting that tumoral TNFSF4 is associated with outcome. TNFSF4 expression was not correlated with the expression of other known checkpoint mRNAs. Last, metastatic patients with TNFSF4 mRNA expression within the lowest quartile have significantly worse outcome on anti-PD1 treatment, and a significantly lower response rate to these agents. Our current work points to TNFSF4 expression in melanoma as a potential determinant of prognosis, and warrants further translational and clinical research.

Keywords Melanoma · Immunotherapy · Immune-oncology · TNFSF4 · Survival · OX40L

Abbreviations

CAF	Cancer-associated fibroblasts
CCLE	Cancer Cell Line Encyclopedia
LS	Lymphocyte score
OS	Overall survival
PFS	Progression-free survival
RPKM	Reads per kilo-base per million
TCGA	The Cancer Genome Atlas
TIL	Tumor infiltrating lymphocytes
TPM	Transcripts per million

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Introduction

In the last few years, immunotherapy has revolutionized the treatment of metastatic melanoma by dramatically improving patient outcome [1–3]. In the adjuvant setting, immunotherapy with ipilimumab or pembrolizumab was also shown to improve overall survival [4] and relapse free survival [5], respectively, when compared to placebo, proving the concept that potentiation of anti-cancer immunity can prevent micro-metastatic disease from developing into a clinically

significant, and potentially fatal, disease. Nivolumab was also shown to be superior to ipilimumab in improving disease-free survival in stage III melanoma at a lower toxicity cost [6], and results of the overall survival are awaited. Notwithstanding these major advancements, many metastatic patients do not respond to, or progress after responding to the currently available immunotherapeutic agents (reviewed in [7]), and a significant percentage of patients with stage III disease develop metastatic disease. Unfortunately, there are still no predictive biomarkers to guide clinical decision-making, and there is still urgent need to further enhance the ability of the immune system to attack and eliminate melanoma.

The immunological synapse—namely, the interface between immune cells and cancer or antigen presenting cells—is multi-faceted and complex, comprising of many pairs of both co-inhibitory and co-stimulatory protein pairs, collectively dubbed 'checkpoint proteins'. There is now extensive research effort aimed at finding novel targets and treatment approaches (reviewed in [8]). One such co-stimulatory pair is TNFSF4 and TNFRSF4 (also known as OX40L-OX40), expressed on antigen presenting cells and T cells, respectively. There are currently several clinical trials assessing the activity of agonistic TNFRSF4 in potentiating T cell anti-neoplastic activity (www.clinicaltrials.gov).

More than a decade ago, Dannul et al. showed that transfection of dendritic cells with TNFSF4 mRNA effectively enhanced the immune-stimulatory function of these cells at multiple levels, and that vaccination of melanoma-bearing mice using OX40L-transfected dendritic cells resulted in significant enhancement of therapeutic antitumor immunity [9]. A few years later, TNFSF4 was shown to be expressed on airway smooth muscle cells [10], demonstrating its expression on cells outside the immune system. We, therefore, asked whether TNFSF4 is also expressed on melanoma cells, and whether its expression is associated with outcome in locally advanced and metastatic melanoma.

Methods

Single-cell and cell line expression analysis

Gene expression data of single melanoma and non-malignant T cells, B cells, NK cells, cancer-associated fibroblasts (CAFs), endothelial cells, and macrophages were obtained from [11]. CCLE cell line expressions were downloaded from the website of the CCLE project [12].

The Cancer Genome Atlas (TCGA) data analysis

TCGA gene expression data from RNA-sequencing and clinical characteristics including survival time and stage were downloaded from public TCGA repositories. Kaplan–Meier analyses were performed in R using the 'survival' package. Lymphocyte score (LS) from the TCGA was used to define the LS low (score 0–3) and LS high (score 4–6) cohorts. For correlation analysis, Spearman correlation coefficients were calculated for the expression of each possible checkpoint mRNA pair and a q value was calculated for each correlation using the false detection rate (FDR) correction for multiple comparison (using a cutoff of $q < 0.1$ as statistically significant).

RNA sequencing and survival analysis of patients receiving anti-PD1 therapy

RNA was extracted from 38 tumor biopsies of melanoma patients at the Sheba medical center from 2015 to 2018 prior to the start of the treatment with PD1 blockade (Pembrolizumab or Nivolumab), following their informed consent and following ethical approval by the institutional review board. All patients had metastatic melanoma, none received prior systemic therapy, and all agreed to provide a fresh biopsy prior to the start of the treatment, with a median time between biopsy and treatment of 2.2 months, in which period the patients did not receive any other systemic treatments. Biopsies were taken from lymph nodes metastases ($n = 12$), dermal or subcutaneous metastases ($n = 12$), visceral metastases ($n = 6$), and lung, bone and mucosal metastases ($n = 8$). RNA was extracted with RNeasy FFPE Kit (Qiagen, USA). Libraries were prepared with Illumina's Ribo Zero Gold and TruSeq stranded library prep kit and sequenced using paired-end sequencing with read length of 2×100–125 bps, based on the Illumina HiSeq 2500 platform. Transcriptome reads were aligned to the UCSC hg19 reference genome using Tophat2 and raw count matrix was produced with HTseq-count. Raw counts were filtered and normalized using the R package LIMMA pipeline. Progression-free survival (PFS) and overall survival (OS) comparison between TNFSF4-high patients (upper three quartiles) and TNFSF4-low patients (lower quartile) was performed in Python using 'Lifelines' package.

Results

Single-cell RNA sequencing analysis shows that TNFSF4 mRNA is expressed on melanoma, T and B cells, and almost completely absent in NK cells, CAFs, endothelial cells and macrophages (Fig. 1a). Similarly, TNFSF4 mRNA is expressed in melanoma cell lines with a mean of 5.5 log2 RMA (Fig. 1b). For comparison, it is expressed in significantly higher levels in B-cell ALL cell lines, as expected, and not expressed in soft tissue sarcoma cell lines (Fig. 1b).
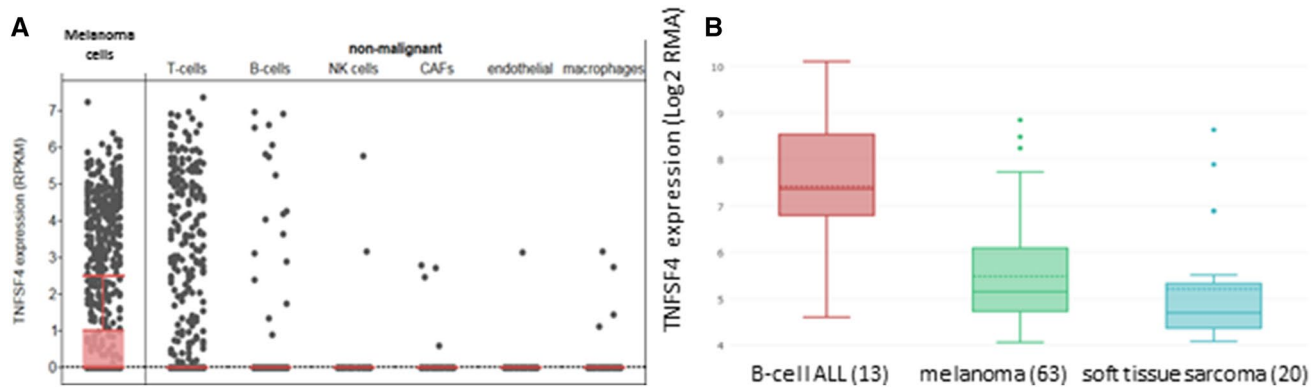
Fig. 1 TNFSF4 mRNA expression in melanoma. **a** The expression of TNFSF4 mRNA [in reads per kilo-base per million (RPKM)] in single melanoma cells (left) and in T cells, B cells, NK cells, cancer-associated fibroblasts (CAFs), endothelial cells and macrophages using expression data published earlier in [11]. **b** TNFSF4 mRNA expression (based on affymetrix mRNA arrays) in melanoma cell lines, B-cell ALL cell lines and soft tissue sarcoma cell lines from the Cancer Cell Line Encyclopedia (CCLE). The y axis represents the log2 of the robust multi-array average, and the number of cell lines from each cell type is given in parenthesis

The median expression of TNFSF4 across all 472 samples of the melanoma TCGA was 2 transcripts per million (TPM) (interquartile range 1–4, maximal level of 85 TPM). The median TNFSF4 was above the cutoff of 1 TPM in both the lymphocyte-score-(LS-) low and high cohorts, indicating that the majority of the transcripts in the sample originate from the tumor and not from the tumor infiltrating lymphocytes (TILs; Fig. 2a, left panel). This is in contrast to the median expression of PD1 that was below the cutoff of 1 TPM in the LS-low cohort, and above this cutoff in the LS-high cohort, in keeping with its known expression on T cells (Fig. 2a, right panel). The median expression of TNFSF4 mRNAs was slightly higher in metastatic lesions than in primary tumors but was not associated with the ulceration status of the primary tumor (Fig. 2b).

There was a significant association between the expression of TNFSF4 mRNA (either above or below the median) and survival in all melanoma patients with survival data in the TCGA ($n = 459$; $p = 0.00044$), in stage III patients ($n = 168$; $p = 0.004$) and in stage IIIc–IV patients ($n = 89$; $p = 0.00002$) (Fig. 2c). Patients with low TNFSF4 consistently had the worse prognosis. Similar significant associations were seen in the cohort of patients with LS-low ($n = 164, 57, 30$ for all patients, stage III and stage IIIc–IV patients, respectively; Fig. 2d), verifying that this association is driven by TNFSF4 expression in the tumor and not in the TILs. Patients with ulcerated primary tumors and lower than median expression of TNFSF4 had significantly worse outcome than patients with higher than median TNFSF4 (either with a non-ulcerated or ulcerated primary) in the entire cohort (Fig. 2e; left panel; $p = 0.007$) and in the cohort of stage III patients (Fig. 2e; right panel; $p = 0.003$).

A comprehensive literature search revealed 23 gene products suggested to potentially serve as checkpoints at the melanoma side of the synapse. Of these, 17 has a median mRNA expression that was higher than 1 TPM in the TCGA database—CD274 (PD-L1), PDCD1L2 (PD-L2), BTLA (CD272), TNFRSF9 (4-1BB), CD40 (TNFRSF5), CD48 (BLAST-1 or BCM-1), CD86 (B7-2), C10orf54 (VISTA or VSIR), LGALS9 (Galectin-9), ICOSLG (B7-H2), CD276 (B7-H3), TNFRSF14 (HVEM or CD270), PVR (CD155 or NECL-5), PVRL2 (CD112 or Nectin-2), TNFSF4 (OX40L or CD134), CD70 (TNFSF7), CD200 (MOX1 or MOX2). Of these, the expression of the first 10 was correlated with one another with a Spearman correlation coefficient of above 0.5 (corresponding to a corrected q value ≤ 0.1). The expression of TNFSF4 was not significantly correlated with the expression of any of these 10 mRNAs or with any of the other 6 checkpoint mRNAs (Fig. 3 and supplementary Fig. 1).

To corroborate our TCGA results in a different cohort, we analyzed the association between TNFSF4 mRNA expression and survival of patients with metastatic disease following monotherapy with anti-PD1 monoclonal antibodies (for patient characteristics see "Methods" section and Table 1). Patients with TNFSF4 expression within the lowest quartile had significantly worse progression-free survival (median progression-free survival of 4.9 months vs not reached; $p = 0.04$) and a trend toward worse overall survival (median survival of 23 months vs not reached, $p = 0.12$). The objective response rate in patients with TNFSF4 expression within the lowest quartile was 11% (1/9 patients) vs 76% (22/29 patients) in patients with higher TNFSF4 ($p = 0.005$ using Chi square) (Fig. 4).
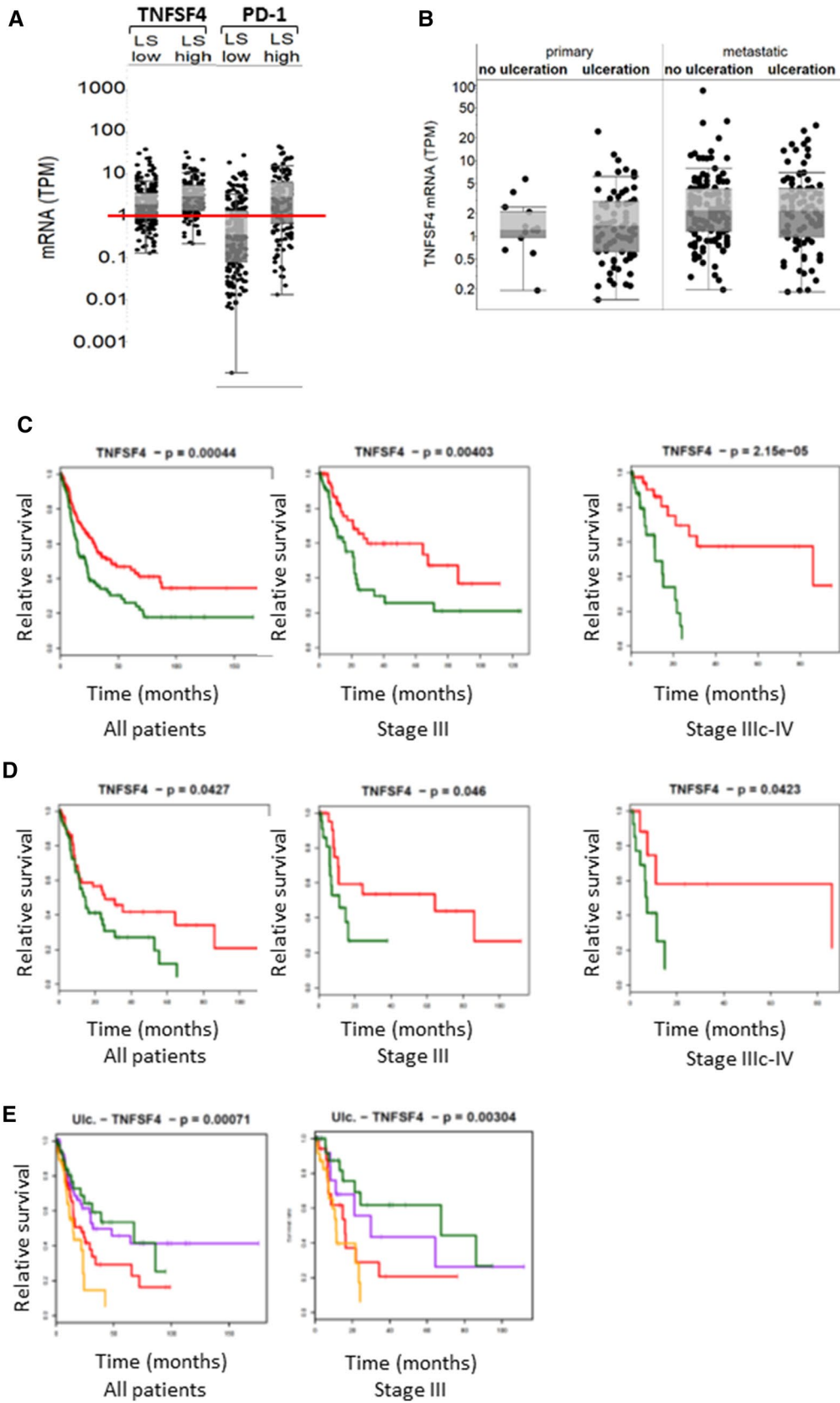
◀**Fig. 2** TNFSF4 and PD-1 mRNA expression, tumor ulceration and survival in melanoma. **a** The expression of TNFSF4 and PD1 mRNA [in transcripts per million (TPM)] in the lymphocyte-score- (LS) low and high cohorts of the melanoma TCGA database. **b** The expression of TNFSF4 in primary and metastatic melanoma samples (left) and in samples from ulcerated and non-ulcerated melanoma (right). **c** The association between TNFSF4 mRNA expression and survival in the melanoma TCGA database. Red: TNFSF4 mRNA > median, green: TNFSF4 mRNA < median. **d** The association between TNFSF4 mRNA expression and survival in the LS-poor cohort of the melanoma TCGA database. Red: TNFSF4 mRNA > median, green: TNFSF4 mRNA < median. **e** The association between the ulceration status and TNFSF4 mRNA expression and prognosis in the TCGA database. Red: no ulceration—TNFSF4 low; purple: no ulceration—TNFSF4 high; orange: ulceration—TNFSF4 low; green: ulceration—TNFSF4 high

Discussion

We show here that TNFSF4 mRNAs is expressed in melanoma cell lines and in melanoma samples from the TCGA database, and that its expression is not dependent on the extent of lymphocyte infiltration within the tumor or on the ulceration status of the primary tumor. High TNFSF4 mRNA is associated with significantly better prognosis in all melanoma patients, in patients with locally advanced disease and in patients with metastatic disease. This observation holds true in the sub-groups of LS-low samples, suggesting that the survival difference is driven by TNFSF4 expression within the tumor and not the TILs. Patients with low TNFSF4 expression and an ulcerated primary tumor had significantly worse outcome than all other patients, suggesting that TNFSF4 expression carries additional prognostic information than the ulceration status of the primary tumor alone. TNFSF4 expression was not correlated with the expression of other checkpoint mRNAs suggested to be expressed on the melanoma side of the synapse. Last, metastatic melanoma patients with low TNFSF4 mRNA expression have significantly worse response rates and outcome following treatment with anti-PD1 antibodies.

TNFSF4 (OX40L or CD134L) is the only known ligand of TNFRSF4 (OX40 or CD134). It is a type II transmembrane protein that contains the conserved tumor necrosis factor (TNF) homology domain that enables trimerization [13]. Upon activation, three TNFRSF4 (OX40) molecules bind to the TNFSF4 (OX40L) trimer [14]. TNFSF4 has long been known to be expressed on antigen presenting cells and to be inducible in T cells [15]. Expression of TNFSF4 by
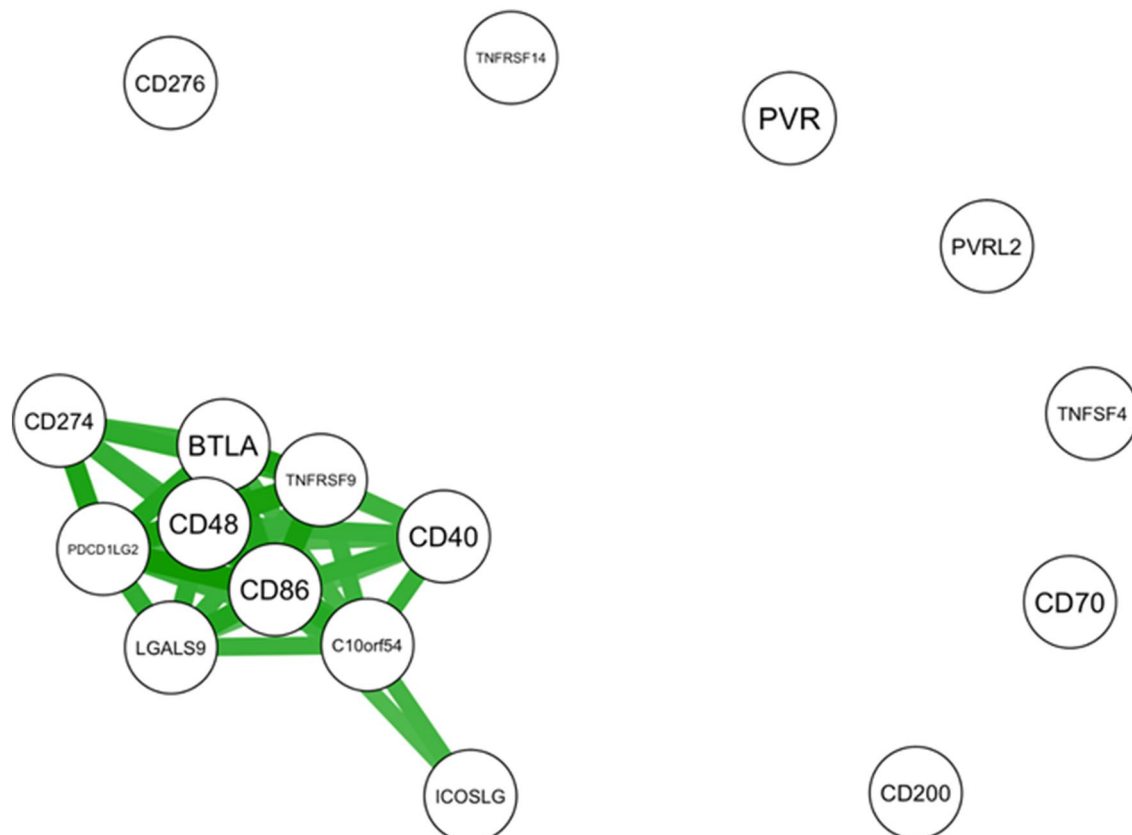

Fig. 3 Correlations between checkpoint mRNAs expressed at the cancer side of the immunological synapse. Correlations with a Spearman rho coefficient > 0.5 (green lines), corresponding to a corrected *q* value of 0.1 or lower are shown

Table 1 Patient characteristics

	TNFSF4 high	TNFSF4 low
Number of patients	$N = 29$	$N = 9$
Sex		
Male (%)	17 (59%)	3 (33%)
Female (%)	12 (41%)	6 (67%)
Mean age (range)	60.3 (31–85)	60.2 (41–85)
BRAF mutation status		
Mutated	12 (41%)	1 (11%)
Wild type	12 (41%)	7 (78%)
Unknown	5 (17%)	1 (11%)
Biopsy site:		
Lymph nodes	9	3
Subcutaneous/dermal	11	1
Visceral	4	2
Lung	3	0
Mucosal	1	3
Bone	1	0
LDH high (%)	7 (24%)	3 (33%)
PD1 blockade outcome		
Objective responses (%)	22 (76%)	1 (11%)
Median PFS (months)	Not reached	4.9
Median OS (months)	Not reached	23

monocytes was shown to promote T follicular helper cell polarization and pathogenesis in human lupus [16], suggesting that it has a role in potentiating adaptive immune responses in normal and pathological conditions.

The role of TNFSF4 signaling in cancer has been studied both in vitro and in vivo. In vitro studies have shown that stimulation with TNFSF4 enhances proliferation and expression of effector molecules and cytokines by human T cells (reviewed in [17]). In a mouse model of sub-cutaneous melanoma, treatment with intratumoral injection of a recombinant adenovirus vector expressing mouse TNFSF4 induced a significant suppression of tumor growth along with survival advantages in the treated mice. The in vivo adenoviral modification of tumors evoked tumor-specific cytotoxic T lymphocytes in the treated host correlated with in vivo priming of T helper 1 immune responses in a tumor-specific manner [18]. Similarly, B cells co-expressing CD40L with either CD70, OX40L, or 4-1BBL induced potent therapeutic antitumor effects in a B16 mouse melanoma model [19]. These two studies suggest that TNFSF4 potentiates the anti-neoplastic effects of the adaptive immune system in melanoma.

The association between high TNFSF4 mRNA expression and improved outcome is in keeping with these previous works, and in line with its function as a co-stimulatory molecule. We hypothesize that its increased expression augments the activation of an existing subpopulation of tumor-specific lymphocytes, perhaps by activating TNFRSF4 (OX40) on these cells. Others have shown that there is a synergistic anti-neoplastic effect of OX40-agonism with TGF-beta inhibition [20, 21], but it is still unknown whether in melanoma, high TNFSF4 expression decreases a TGF-beta-induced immune-suppressory microenvironment.

Our analysis cannot delineate which component of the tumor sample—the melanoma cells themselves, the stromal cells, or other non-lymphocyte types of infiltrating immune cells—contributes most to the expression of TNFSF4, and more work is needed to clarify this. Nonetheless, our work points to a potentially novel prognostic biomarker within melanoma sample that may serve—if further validated—to
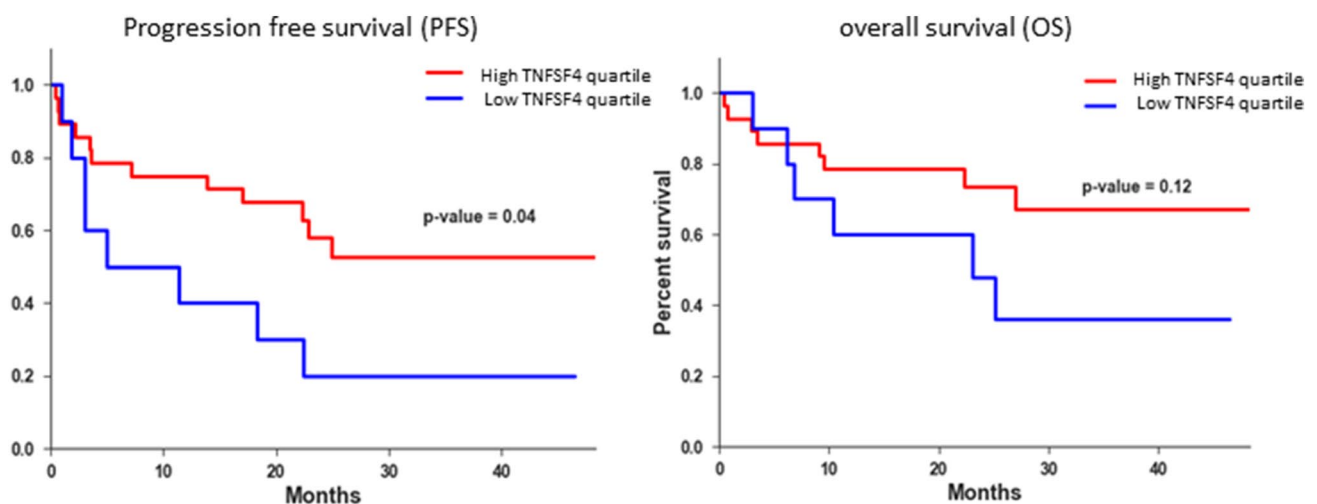

Fig. 4 Survival of patients with metastatic melanoma treated with anti-PD1 antibodies. Progression-free survival (PFS; left) and overall survival (OS; right) of patients with metastatic melanoma treated with anti-PD1 antibodies; Red: upper three quartiles of TNFSF4 expression. Blue: lowest quartile of TNFSF4 expression

stratify locally advanced and metastatic melanoma patients to additional risk groups. Many checkpoint genes are known to be co-expressed together at the immunological synapse [22], but whereas 10 different checkpoint mRNAs were suggested to be co-expressed, at least at the mRNA level in melanoma, TNFSF4 did not exhibit such co-expression.

The decreased response rate and survival observed in metastatic melanoma patients with the lowest quartile of TNFSF4 expression following anti-PD1 treatment may suggest that such monotherapy is not sufficient to achieve disease control. Inversely, the improved survival in patients with the highest quartiles of TNFSF4 mRNA expression (with the median OS not reached in our cohort) may suggest that monotherapy with anti-PD1 may suffice in these cases. If a similar observation will be detected for patients with stage III melanoma following complete resection of their tumors, then patients with high TNFSF4 mRNA within their tumor may need adjuvant monotherapy; whereas, patients with low TNFSF4 within their tumor may need more potent therapeutic approaches. Clearly, both retrospective analysis of existing clinical trial data and prospective clinical trials are needed to prove these hypotheses.

There are currently several ongoing trials with agonist anti-TNFRSF4 (anti-OX40) antibodies, aimed at potentiating TNFRSF4 at the T cell membrane. The relative pro-immunogenic contributions of local vaccine-secreted–agonists versus–systemic T-cell co-stimulation of the TNFSF4-TNFRSF4 axis was investigated. In an elegant work, the relative immune and antitumor activity of vaccine cell-secreted costimulatory molecules Fc-OX40L was compared with systemically administered agonist OX40 antibodies, with the former showing superior activity. In addition, complete tumor rejection was seen in 11% of mice treated with OX40L-expressing vaccine cells [23]. Our results and these suggest that potentiating tumor-expressed TNFSF4 (OX40L) may be a more potent approach to increase the costimulatory TNFSF4–TNFRSF4 signaling pathway than by agonist systemic monoclonal anti-TNFRSF4 antibodies. We are currently studying potential ways to increase the expression of TNFSF4 in melanoma in vitro. Alternatively, our work may suggest that melanoma patients with low TNFSF4 expression may benefit from a combination of anti-PD1 and agonistic anti-TNFRSF4. Clearly, this hypothesis warrants more translational research, followed by a formal prospective clinical trial.

Our work has several limitations. First, it is retrospective in nature, precluding the ability to determine a causal relationship between the level of TNFSF4 and disease outcome, nor does it provide molecular insights on how such expression affects outcome. Yet, the cohort of metastatic patients treated with anti-PD1 antibodies corroborates our main retrospective findings from the TCGA database, and thus strengthens our correlative observations. Second, our current work does not provide data on the expression of TNFSF4 protein within melanoma samples. With that in mind, it is arguable that for the purpose of establishing a biomarker, calculating mRNA levels in a sample (by means of next-generation sequencing) may not be inferior to assessing a protein biomarker by immunohistochemistry. Last, our current data are non-informative in regards to the role of TNFSF4 in predicting response to mono or combination immunotherapy in either the locally advanced or metastatic setting. Clinical trial data from the pivotal adjuvant and metastatic trials are needed to establish, or refute, a role for TNFSF4 mRNA in predicting response or resistance to mono or combination immunotherapy.

In summary, our current work points to TNFSF4 expression in melanoma as a potential determinant of prognosis and warrants further translational and clinical research to establish whether it has a predictive, or even therapeutic, role in this disease. No doubt that a better understanding of the intricate regulation and crosstalk between checkpoint proteins on both sides of the immunological synapse is mandatory to enhance the potential of immunotherapy to prevent or cure metastatic disease.

Compliance with ethical standards

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