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ORIGINAL ARTICLE

Evaluation of CTLA-4 expression and relevance as a novel prognostic factor in patients with non-small cell lung cancer

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Abstract The role of CTLA-4 in negative regulation of T-cell mediated immune response is particularly well established. Much less is known about its expression and function in tumour cells, and to our knowledge, no data are available on its possible impact on prognosis of NSCLC patients. We investigated CTLA-4 expression and prognostic role in 81 patients with radically resected stage I–III NSCLC. The analysis was performed by tissue microarray immunohistochemistry, and the median *H-score* of 20 was used as a threshold to define CTLA-4 overexpressing tumours. Correlation with standard prognostic factors was performed by using absolute and relative fold change indexes. Hazard ratios (HR) and corresponding 95% confidence limits (95% CL) were computed through the Cox model. A higher frequency of CTLA-4 overexpression (>20) was found in non-squamous than in squamous NSCLC (52.8 vs. 35.7%) and in Ki67 $\leq$ 15 expressing tumours, as compared to those with Ki67 > 15 (51.5 vs. 38.7%). A reduced death rate was found in CTLA-4 overexpressing tumours (HR = 0.60, 95% CL = 0.28/1.23), and a further decrease was observed when considering tumours with CTLA-4 > 20 and Ki67 $\leq$ 15, in comparison with tumours with CTLA-4 $\leq$ 20 and Ki67 > 15 (HR = 0.41; 95% CL = 0.15/1.13). Our observational and exploratory study provides a first and promising indication for an independent prognostic effect of CTLA-4 overexpression in radically resected NSCLC. We presume that this effect relies on modulation of the interaction of microscopic disease with CTLA-4-ligands expressing cells leading to NSCLC cell death.

Keywords CTLA-4 · Immunohistochemistry · Non-small cell lung cancer · Overall survival · Prognostic factor

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Introduction

CTLA-4 glycoprotein is traditionally regarded as an inhibitory regulator of T-cell activation and effector functions. CTLA-4 is expressed on the surface of T cells upon activation, and its interaction with the B7 ligands (CD80/CD86), expressed on antigen presenting cells (APC), inhibits cell proliferation, cytokine (IL-2, IFN-γ) production and cell cycle progression [1, 2].

The ability of CTLA-4 to downregulate T-cell activation has been well established in multiple experimental systems including knock-out mouse models and T-cell lines [Reviewed in Ref. 3]. CTLA-4 may exert its inhibitory function through multiple mechanisms including competition with CD28-positive costimulation for binding to their shared B7 ligands, as well as direct inhibitory effects

through the cytoplasmic tail which associates with signalling molecules [4]. CTLA-4 signalling interferes with TCR function, inhibits the AKT/ERK pathway, cyclin D3, cyclin-dependent kinases (cdk4/cdk6) and nuclear transcription factors (NF-κB, NF-AT, AP-1) [Reviewed in Ref. 3].

The majority of CTLA-4 is usually found intracellularly, even after activation, with transient expression on the surface of conventional effector T cells and constitutive expression on the surface of regulatory T cells (Tregs). In Tregs, CTLA-4 signalling plays an essential role in their immune-suppressive function controlling T-cell self-tolerance [5].

CTLA-4 is also known to be expressed on different types of non-T cells, either normal [6–10] or neoplastic, from both haematological [11–13] and solid tumours [14–16], in which its function has not been clearly defined.

The blockade of CTLA-4 physiological function in T cells, by means of CTLA-4-specific monoclonal antibodies (mAbs), is currently used as a therapeutic approach in a variety of human malignancies, particularly in advanced melanoma [17, 18], with the aim of promoting the activation and expansion of tumour-specific T cells [19]. Two human anti-CTLA-4 immunomodulating mAbs, ipilimumab (MDX-010; Medarex/Bristol-Myers Squibb, Princeton, NJ) and tremelimumab (CP675,206; Pfizer, New York, NY), are used, either alone or in combination with chemotherapy, vaccine or interleukin (IL)-2, in metastatic melanoma [17–20] and in ongoing phase II/III trials of other tumours including NSCLC [18, 21].

Our group has previously reported that CTLA-4 is constitutively expressed on NSCLC cell lines in which it is able to induce apoptotic cell death upon engagement with soluble CD80/CD86 recombinant ligands [15]. These findings suggest a functional role of CTLA-4 in NSCLC cell biology but whether CTLA-4 can mediate negative regulatory effects in NSCLC cells, comparable with those currently observed in T cells, has not been assessed yet.

Although CTLA-4 expression has been recently reported in NSCLC tissues [22], no data are available on its significance as a possible prognostic factor in this tumour. The only report, to our knowledge, suggesting a prognostic role of CTLA-4 expression in tumours refers to breast cancer in a Chinese patient population [14]. High levels of CTLA-4 mRNA and protein expression were found in the tumour tissues of those patients showing axillary lymph node metastases [14], highlighting the importance of CTLA-4 in breast cancer progression.

We present here the results of a descriptive and exploratory study undertaken to investigate CTLA-4 protein expression in tumour tissues derived from radically resected stage I–III NSCLC patients. CTLA-4 expression pattern was analysed by immunohistochemistry in relation to well-known clinicopathological factors, along with β-catenin and Ki67 proteins, as these markers may be functionally related to CTLA-4. We also investigated the prognostic significance of CTLA-4 expression on overall survival of NSCLC patients.

Materials and methods

Patients and clinical samples

A search of the prospective Pathology database, at the IRCCS A.O.U. San Martino-IST (Genoa, Italy), identified 81 patients with stage I–III NSCLC who underwent radical surgery at the Thoracic Surgery Division, between July 2005 and March 2007, that satisfied the following selection criteria: (1) at least 4 weeks survival after surgery; (2) no additional radiotherapy or chemotherapy; (3) microscopically negative resection margins (R0); and (4) availability of archival formalin-fixed paraffin-embedded tumour tissue suitable for tissue microarray (TMA) preparation. Regional lymph node staging was performed by systematic mediastinal lymph node sampling; nodal stations were classified according to Naruke's map [23]. Demographic, clinical, and pathologic characteristics are listed in Table 1. Formalin-fixed, paraffin-embedded primary tumour tissue samples were retrieved from the archives of the local pathology department. To confirm the diagnosis of NSCLC, histological slides from all patients were independently reviewed. Tumour size and nodal status were obtained from the original pathology reports. The tumours were staged according to the International Union Against Cancer's tumour node metastasis classification [24]. Histological subtypes were classified according to the World Health Organization guidelines [25]. Survival data were available for all patients from hospital records or local registries. Ethics approval was obtained according to local practice.

TMA construction

Sections of 4 μm thickness were prepared from formalin-fixed paraffin-embedded NSCLC blocks and stained with haematoxylin and eosin to select the most representative tumour areas.

Duplicate tumour tissue cylinders with a diameter of 1.0 mm were punched from these areas using a tissue-arraying precision instrument (Tissue-Tek Quick-Ray Tissue Microarray System, Sakura Finetek USA, Inc., Torrance, CA, USA) and arrayed into a recipient paraffin block to produce TMA. TMA consisted of two cores (diameter 1 mm) from each of the 81 NSCLC tumours and from three benign tissues adjacent to tumours, for a total of 42 cores/slide (84 punches). Sections of 3–4 μm thickness were then cut from array blocks and transferred to positively charged glass slides that were used for immunohistochemical analysis.

Table 1 Patient characteristics

Characteristics	Number (%)
Number of patients	81 (100)
Median age, years (range)	67 (47–82)
Sex	
Female	19 (23.4)
Male	62 (76.6)
Smoking habit	
Current smoker	53 (65.4)
Ex-smoker	22 (27.2)
Never smoker	6 (7.4)
Histology	
Adenocarcinoma	35 (43.2)
Bronchoalveolar carcinoma	14 (17.3)
Squamous cell carcinoma	28 (34.6)
Large cell carcinoma	3 (3.7)
Other	1 (1.2)
Pathologic stage	
IA	23 (28.4)
IB	21 (25.9)
IIA	4 (4.9)
IIB	10 (12.3)
IIIA	16 (19.8)
IIIB	7 (8.7)
Surgery	
Bilobectomy	10 (12.3)
Lobectomy	70 (86.4)
Pneumectomy	1 (1.3)

Immunohistochemistry (IHC)

CTLA-4

TMA immunohistochemistry (IHC) for CTLA-4 expression was performed using the automatic immunostainer Benchmark XT (Ventana Medical Systems SA, Strasbourg, France). Antigen retrieval was performed utilizing citrate buffer (pH 6) at 90°C for 30 min followed by staining with 10 μg/ml (final concentration) of the murine anti-CTLA-4 mAb (14D3 clone, from eBioscence, San Diego, CA, USA) incubated for 40 min at 37°C. Reactivity detection was performed by addition of the peroxidase-based polymeric system ultraView Universal DAB Detection Kit (Ventana Medical Systems). Negative control was obtained by staining with murine anti-CD20 IgGγ2a (L26 clone, from Ventana Medical Systems) as isotype-matched irrelevant mAb. CTLA-4 staining with 14D3 mAb was detected at the surface and cytoplasm in tumour cells as well as in infiltrating lymphocytes (internal positive control).

Validation of 14D3 mAb was obtained by testing in IHC different concentrations of antibody on formalin-fixed, paraffin-embedded, non-neoplastic lymph node and melanoma tissues. These melanoma tissues had previously been positive in IHC with the therapeutic anti-CTLA-4 ipilimumab antibody (S. Laurent et al., submitted manuscript).

β-Catenin and Ki67

TMA were also automatically immunostained with prediluted anti-β-catenin mAb (14 clone) and anti-Ki67 mAb (30-9 clone, both from Ventana Medical Systems) incubated for 30 min at 37°C. Reactivity was developed using the same detection kit used for CTLA-4.

All the sections were counter-stained with Mayer's haematoxylin and then cover-slipped.

β-catenin staining was detected mainly at the surface and cytoplasm, whereas Ki67 was detected in the nuclei of tumour cells. The percentage of Ki67-positive cells was calculated, on all the neoplastic area, through the use of an image analysis system consisting of a Leica DMLA computerized microscope (Leica, Italy).

Stained slides were analysed by two independent observers under an optical microscope (Olympus BX41) using 10× ocular lens and 40× objectives. Image acquisition was performed with a Leica DMD1.08 microscope.

Evaluation of IHC staining

CTLA-4 and β-catenin stainings were evaluated by taking into account both the percentage and intensity of positive cells. Scores for percentage of stained cells were 0 (0%), 1 (1–30%), 2 (31–60%) or 3 (61–100%). Scores for staining intensity were 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). A final histochemical score (*H-score*) was obtained by multiplying both intensity and percentage values using the formula: $(1 + I) \times Pc$, where I represents the intensity score; Pc, the percentage of stained cells at each intensity; and 1 is a correction for optical density [26, 27]. *H-scores* ranged from 0 to 380 for CTLA-4 and from 0 to 400 for β-catenin.

Statistical analysis

Data analysis was oriented towards two different but complementary aims.

1. Evaluation of CTLA-4 overexpression across the categories of well-known or potential prognostic factors of lung cancer survival, namely stage, histology, Ki67, β-catenin, age at diagnosis, sex and smoking habit. Quantitative measurements (CTLA-4, β-catenin, age at diagnosis) were dichotomized according to median values (20, 160, 70, respectively), in order to obtain numerically balanced

subgroups. Values of CTLA-4 over 20 and β-catenin over 160 were considered as an indication of over-expression. A cut-off value of 15%, most commonly used in literature, was assumed to define Ki67 overexpression.

Two indexes were used to measure the variation of CTLA-4 overexpression between the categories of each dichotomous prognostic factor: absolute fold change (AFC), which was the ratio of the CTLA-4 overexpressed percentage in a category to the same percentage of the other category; relative fold change (RFC), namely the relative difference (excess or reduction) between two overexpression frequencies. For each index, approximate 95% confidence limits (95% CL) were also calculated.

2. Assessment of prognostic significance of the study characteristics on overall survival (OS), with particular reference to CTLA-4 expression. In this case, simple (Kaplan–Meier method) and multivariate (Cox model) analyses were, respectively, applied to estimate survival probabilities stratified by each study characteristic and to assess the joint effect of all factors on OS. Hazard ratio (HR) point and interval (95% CL) estimates were computed through the Cox modelling. Each HR represents a relative index of lung cancer death risk due to the related prognostic factor, adjusted for the influence (confounding effect) of the other characteristics included in the same regression equation. Comparisons between univariate (one-factor-based modelling) and multivariate Cox regressions were used to give a measure of such an influence.

Three patients with missing data on β-catenin and Ki67 were excluded from survival analyses.

The two-sided log-rank and likelihood ratio statistics were respectively computed to test differences between Kaplan–Meier survival probabilities and death rates derived from the Cox regression [28].

All analyses were performed using Stata 11.2 statistical software (StataCorp).

Results

CTLA-4 expression in primary NSCLC tissues

We investigated the expression of CTLA-4 in tumour tissues from 81 radically resected stage I–III NSCLC patients. The analysis was performed through the immunohistochemical (IHC) staining of TMA, derived from formalin-fixed, paraffin-embedded tumour blocks, with the murine anti-CTLA-4 mAb 14D3 (pre-validated as specified in "Materials and methods"). CTLA-4 positivity was found, at both the cell surface and cytoplasm, in 41/81 (50.6%) tumours with a heterogeneous pattern, ranging from weak (*H-score* 20–60) in 10/41 (24.4%) tumours to moderate (*H-score* 120–180) in 20/41 (48.8%) tumours and intense (*H-score* 240–380) in 11/41 (26.8%) tumours (representative staining patterns are shown in Fig. 1, panels a, b, c, respectively). The remaining 40/81 (49.4%) tumours showed negative CTLA-4 staining, as well as the benign lung tissue adjacent to tumours (Fig. 1, panels d, f, respectively).

As expected from CTLA-4 expression pattern in T [1, 2, 4], as well as in B cells [6], CTLA-4 positive staining with 14D3 mAb was also observed in tumour infiltrating cells with lymphoid morphology (TILs), and this staining was used as an internal positive control (Fig. 1, panel a). As an irrelevant control mAb, having the same isotype (IgGγ2a) of the anti-CTLA-4 14D3 mAb, we used the murine L26 mAb against the B-cell marker CD20. Because tumour cells did not express CD20, L26 staining of those cells resulted completely negative (Fig. 1, panel e).

On the basis of the *H-score* cut-off level of 20, it was possible to differentiate between 38/81 (46.9%) CTLA-4 overexpressing tumours (>20) and 43/81 (53.1%) CTLA-4 negative or low-expressing tumours ($\leq$20). For further analysis, these two groups were evaluated according to prognostic factors and patient survival.

Analysis of CTLA-4 over-expression according to prognostic factors

By considering an a priori chosen RFC of at least 20% as clinically and biologically relevant, we observed differences in the frequency of CTLA-4 overexpression just in relation to histology and Ki67. In particular, 52.8% of non-squamous tumours showed overexpression, as compared to 35.7% of squamous tumours, corresponding to an RFC of −32.0% (95% CL = −61.3/+18.2). Similar results were also found for Ki67. In this case, the RFC was −24.2% (95% CL = −55.1/+28.1), resulting from the comparison between CTLA-4 overexpression percentage of 51.1% in patients with Ki67 $\leq$ 15 and 38.7% in patients with Ki67 > 15 (Table 2).

Impact of CTLA-4 expression on patient survival

During the five-year study period, 44.4% (36/81) of patients died. This percentage corresponded to a lung cancer death rate of 10.9×10^{-3} (95% CL = 7.8×10^{-3}–15.1×10^{-3}) patient-months of observation. Also, the median follow-up time was 48.5 (range, 1.4–60.0) months.

The influence of each study characteristic, in particular CTLA-4 expression, on OS was analysed in terms of 1-, 3- and 5-year survival probabilities estimated through the Kaplan–Meier method (Table 3). As expected, stage I patients had a better prognosis than stage II and III patients (5-year probability: 71.9 vs. 34.7%; *P* value <0.001). In addition, Ki67 and smoking habit showed a remarkable prognostic significance. In particular, after 5 years of

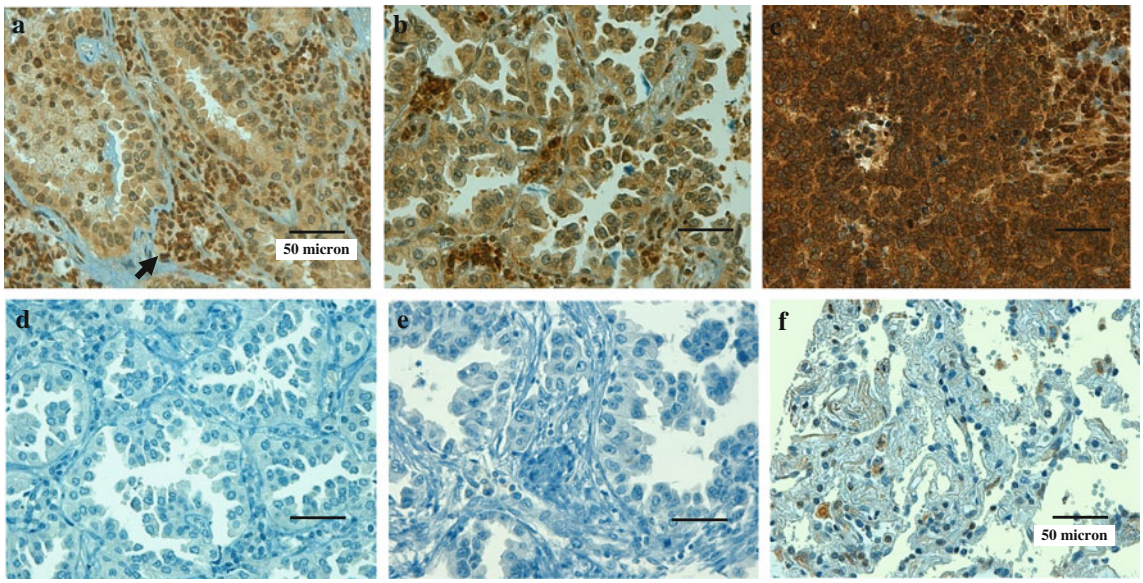


Fig. 1 Immunohistochemical (IHC) staining patterns of CTLA-4 in representative NSCLC tissue sections. IHC staining was performed with the murine anti-CTLA-4 mAb 14D3 and detected with peroxidase-based DAB Detection Kit. CTLA-4 positive expression ranged from weak (**a**) to moderate (**b**) and intense (**c**). A representative NSCLC tissue section with negative CTLA-4 staining is also shown (**d**). CTLA-4 staining observed in tumour infiltrating lymphocytes (*black arrow*, **a**) was used as an internal positive control. Negative staining of tumour cells with the murine anti-CD20 mAb used as an isotype (IgGγ2a)-matched irrelevant mAb for 14D3 (**e**). Negative staining of the benign lung tissue adjacent to tumour with 14D3 mAb (**f**). Images were taken using magnification 400×. *Scale bar*, 50 μm

follow-up, patients with Ki67 ≤ 15 showed a better OS than those with Ki67 > 15 (5-year probability: 61.1 vs. 44.9%; *P* value = 0.069). Moreover, current smokers at cancer onset had a worse life expectancy than non-smokers (5-year probability: 48.6 vs. 67.2%; *P* value = 0.079).

CTLA-4 overexpressing tumours tended to have a better prognostic impact than did those with lower CTLA-4 expression (5-year probability: 64.8 vs. 45.9%; *P* value = 0.078) (Table 3; Fig. 2, panel a).

The joint effect of all factors estimated through the Cox modelling (Table 4) confirmed stage as the main prognostic characteristic (HR = 3.57, 95% CL = 1.68–7.59; *P* value <0.001). By comparing the multivariate to univariate HR estimates (Table 4), discernable attenuation effects (HR towards 1.00) were found, in particular for smoking habit (2.29–1.90), Ki67 (1.79–1.46), β-catenin (1.65–1.57) and CTLA-4 (0.54–0.60), which indicate a reciprocal, although moderate, confounding effect.

Given this last finding, in order to evaluate the degree of independence of CTLA-4 from all other factors in predicting survival, several models were fitted by inserting and removing factors in the regression equation. In this framework, using a model which included CTLA-4 alone as the reference model, the absolute (ΔHR) and the percent (ΔHR%) change in relative risk related to CTLA-4 was used as an index of prognostic independence: the smaller the change, the greater the independence. This simple sensitivity analysis pointed out a substantially independent prognostic behaviour of CTLA-4 on OS. In fact, the overexpressing patients' subgroup showed a decreased death risk ranging from 0.54 to 0.60, corresponding to a ΔHR% of about 12% (data not shown). Ultimately, this subgroup experienced a death rate which was approximately 45% less than that expected for the other subgroup, although such a difference in OS resulted not to be statistically significant.

Discussion

In this study, we carried out a descriptive and exploratory investigation of CTLA-4 expression in tissue specimens from an Italian cohort of NSCLC patients. This investigation was mainly based on both the correlation between CTLA-4 and some clinic-pathological features and its capability of predicting OS.

To our knowledge, this retrospective study represents the first investigation of CTLA-4 expression as a possible prognostic factor for OS of patients with radically resected I–III stage NSCLC. So far, there is only a single report on CTLA-4 expression in lung cancer, referring to Chinese patients [22], in which CTLA-4 overexpression was related to patient age and histological differentiation but not to prognosis, nor to β-catenin and Ki67, that have been investigated in the present study.

We evaluated CTLA-4 expression in NSCLC by immunohistochemical staining that allowed us to differentiate

Table 2 Frequency of CTLA-4 overexpression according to selected prognostic factors

Factors and levels	*T*	CTLA-4 > 20		AFC	95% CL	RFC	95% CL
		N	%				
Stage							
I	44	22	50.0	1.00	(Ref.)	0.0	(Ref.)
II and III	37	16	43.2	0.87	0.54–0.39	−13.1	−46.1/+38.8
Histology							
Non-squamous	53	28	52.8	1.00	(Ref.)	0.0	(Ref.)
Squamous	28	10	35.7	0.68	0.37–1.18	−32.0	−61.3/+18.2
Ki67							
≤15	47	24	51.1	1.00	(Ref.)	0.0	(Ref.)
>15	31	12	38.7	0.76	0.45–1.28	−24.2	−55.1/+28.1
Missing	3	2	–				
β-Catenin							
≤160	47	21	44.7	1.00	(Ref.)	0.0	(Ref.)
>160	32	16	50.0	1.12	0.70–1.79	+11.9	−30.1/+79.1
Missing	2	1	–				
Age							
≤70	46	21	45.7	1.00	(Ref.)	0.0	(Ref.)
>70	35	17	48.6	1.06	0.67–1.69	+6.4	−33.1/+69.3
Sex							
Female	19	9	47.7	1.00	(Ref.)	0.0	(Ref.)
Male	62	29	42.0	0.99	0.57–1.70	−1.3	−42.7/+70.0
Smoking habit							
Ex/never	28	13	46.4	1.00	(Ref.)	0.0	(Ref.)
Current	53	28	52.8	1.02	0.62–1.66	2.0	−37.7/+65.7
Whole sample	81	38	46.9	–	–	–	–

T total number of patients, *N*, % number, percent of patients with CTLA-4 overexpressing tumour, *AFC* absolute fold change, *RFC* relative fold change, *95% CL* 95% confidence limits for AFC/RFC, *Ref.* reference category

between CTLA-4-overexpressing tumours (46.9%) and low/negative CTLA-4-expressing tumours (53.1%). We found about 1.5-fold increase in CTLA-4 overexpression in non-squamous as compared to squamous histological type.

We also assessed the association between CTLA-4 expression and two additional biomarkers, β-catenin and Ki67, as they might be functionally related to CTLA-4 protein. In particular, β-catenin levels might directly control CTLA-4 levels in NSCLC as reported in melanoma [16]. Indeed, in melanoma cells, nuclear β-catenin strongly upregulates CTLA-4 gene promoter activity by binding to the T-cell factor-1/lymphoid enhancing factor-1 (TCF/LEF) transcription factors [16]. On the other hand, Ki67 might be regulated by CTLA-4 expression levels as CTLA-4 signalling inhibits cell cycle progression in T cells and tumour cells [1, 2, 13, 15] through the induction of cell cycle block in G0/G1 phase or inhibition of cyclin D3- and cyclin-dependent kinase (cdk4/cdk6) production.

β-Catenin plays a central role in cell proliferation, differentiation, tumour development and invasiveness [29–31]. In NSCLC, reduced expression of β-catenin significantly correlates with a poor prognosis [32, 33], but such an observation was not confirmed in our study.

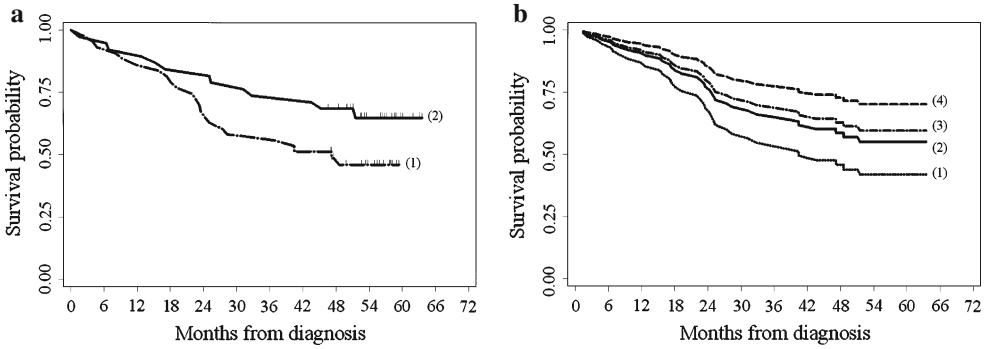
We did not find any remarkable correlation between CTLA-4 and β-catenin expression levels on the basis of the RFC threshold value (±20%) a priori chosen for CTLA-4 overexpression frequency. However, it was noteworthy that patients with higher β-catenin levels (>160) showed higher CTLA-4 overexpression. This finding suggests that in NSCLC, as in melanoma [16], the β-catenin pathway might affect CTLA-4 expression, although this observation needs further investigation.

Ki67 nuclear antigen, regarded as an indicator of cell proliferation rate, is expressed in all cell cycle phases (in the mid-G1, S, G2 and the entire M phase) except in the resting cells (G0 phase) [34]. In NSCLC, intense immunohistochemical staining for Ki67 is correlated with poor prognosis [35], observation that was confirmed in our study, although not statistically significant. We also found an inverse correlation between CTLA-4 and Ki67 as patients with Ki67 ≤ 15 displayed an AFC for CTLA-4 overexpression which was about 1.3-fold higher than that of patients with a Ki67 > 15. Indeed, CTLA-4 could exert an inhibitory effect on Ki67 by inducing a cell cycle block in G0/early G1 phases in which Ki67 should not be expressed [34]. This inhibitory effect might potentially

Table 3 Survival probabilities estimated through the Kaplan–Meier method according to categories of the study prognostic factors

Factors and levels	*N*	*D*	*D%*	1-year survival		3-year survival		5-year survival		*P* value
				Prob%	95% CL	Prob%	95% CL	Prob%	95% CL	
CTLA-4										0.078
≤20	43	23	53.5	86.1	71.6–93.5	58.1	42.1–71.2	45.9	30.5–60.1	
>20	38	13	34.2	92.1	77.5–97.4	73.7	56.6–84.9	64.8	47.0–78.0	
Stage										<0.001
I	44	12	27.3	95.5	83.0–98.8	86.4	72.1–93.6	71.9	55.6–83.0	
II and III	37	24	64.9	81.1	64.4–90.5	40.5	24.9–55.7	34.7	19.9–49.9	
Histology										0.998
Non-squamous	53	24	45.3	90.6	78.8–96.0	66.0	51.6–77.1	53.9	39.4–66.4	
Squamous	28	12	42.9	85.7	66.3–94.4	64.3	43.8–78.9	56.9	36.8–72.8	
Ki67										0.069
≤15	47	18	38.3	95.7	84.0–98.9	72.3	57.2–82.9	61.1	45.4–73.5	
>15	31	17	54.8	77.4	58.4–88.5	51.6	33.0–67.4	44.9	27.1–61.2	
Missing	3	1	33.3	–	–	–	–	–	–	
β-Catenin										0.150
≤160	23	9	39.1	91.5	78.9–96.7	68.1	52.8–79.4	61.3	45.7–73.6	
>160	56	27	48.2	84.4	66.5–93.2	59.4	40.5–74.0	43.1	25.7–59.4	
Missing	2	0	0.0	–	–	–	–	–	–	
Age										0.972
≤70	46	20	43.5	91.3	78.5–96.6	63.0	47.5–75.2	56.5	41.1–69.4	
>70	35	16	45.7	85.7	69.0–93.8	68.6	50.5–81.2	52.1	33.6–67.7	
Sex										0.773
Female	19	8	42.1	84.2	58.7–94.6	68.4	42.8–84.4	57.4	32.6–76.0	
Male	62	28	45.2	90.3	79.7–95.5	64.5	51.3–75.0	54.2	40.9–65.8	
Smoking habit										0.079
Ex/never	28	9	32.1	92.9	74.4–98.2	78.6	58.4–89.8	67.2	46.3–81.5	
Current	53	27	50.9	86.8	74.3–93.5	58.5	44.1–70.4	48.6	34.5–61.3	
Whole sample	81	36	44.4	88.9	79.7–94.1	65.4	54.0–74.7	54.9	43.3–65.1	–

N number of patients entered the study, *D* number of patients died during the study period, *Prob%* percent survival probability estimates, *95% CL* 95% confidence limits for Prob%, *P value* significance level of the log-rank test



(1) CTLA-4 expression ≤ 20
(2) CTLA-4 expression > 20

(1) CTLA4 ≤ 20, *Ki67* > 15: *HR* = 1.00 (*Ref.*)
(2) CTLA4 ≤ 20, *Ki67* ≤ 15: *HR* = 0.68 (95% *CL* = 0.33/1.44)
(3) CTLA4 > 20, *Ki67* > 15: *HR* = 0.60 (95% *CL* = 0.29/1.23)
(4) CTLA4 > 20, Ki67 ≤ 15: *HR* = 0.41 (95% *CL* = 0.15/1.13)

Fig. 2 Survival probability curves estimated via Kaplan–Meier method by CTLA-4 (**a**) and by CTLA-4 and Ki67 (**b**) expression categories, estimated using the Cox modelling adjusted for stage, *β*-catenin, age at diagnosis, sex and smoking habit

occur through the interaction between CTLA-4 expressed by the NSCLC cells and B7 ligands expressed by the tumour micro-environment cells including the APC, such as dendritic cells (DCs) [36], or antitumour activated T cells [37]. In this case, this interaction might result in delivering CTLA-4-mediated negative signals into NSCLC cells leading to inhibition of their proliferation rate [15].

In contrast to what reported in breast cancer [14], the multivariate Cox analysis showed a very interesting tendency towards a favourable independent role of CTLA-4 overexpression on OS. Patients with higher expression

Table 4 Cox regression analysis of overall survival

Factors and levels	Univariate analysis		Multivariate analysis		*P* value
	HR	95% CL	HR	95% CL	
CTLA-4					0.155
≤20	1.00	(Ref.)	1.00	(Ref.)	
>20	0.54	0.27/1.09	0.60	0.28/1.23	
Histology					0.886
Non-squamous	1.00	(Ref.)	1.00	(Ref.)	
Squamous	1.11	0.55/2.22	1.06	0.47/2.41	
Stage					<0.001
I	1.00	(Ref.)	1.00	(Ref.)	
II and III	3.73	1.82/7.66	3.57	1.68/7.59	
Ki67					0.320
≤15	1.00	(Ref.)	1.00	(Ref.)	
>15	1.79	0.92/3.47	1.46	0.70/3.06	
β-Catenin					0.224
≤160	1.00	(Ref.)	1.00	(Ref.)	
>160	1.65	0.85/3.21	1.57	0.76/3.23	
Age					0.671
≤70	1.00	(Ref.)	1.00	(Ref.)	
>70	1.01	0.52/1.97	1.17	0.56/2.45	
Sex					0.830
Female	1.00	(Ref.)	1.00	(Ref.)	
Male	1.17	0.51/2.69	1.10	0.44/2.74	
Smoking habit					0.117
Ex/never	1.00	(Ref.)	1.00	(Ref.)	
Current	2.29	1.04/5.04	1.90	0.83/4.36	

Results of regression analyses performed through the Cox model. In univariate analysis, just one factor entered the regression equation. Multivariate analysis result represents the join effect of all factors. *HR* hazard ratio (relative risk) point estimate, *95% CL* 95% confidence limits, *P value* significance level of likelihood ratio test

tended to have a better prognosis than did those with the lower expression. This favourable trend was also strengthened whenever we estimated, through the Cox modelling, the death rate due to CTLA-4 over-expression by Ki67 categories. Thanks to this analysis, we identified the patient subgroup with CTLA-4 > 20 and Ki67 ≤ 15 as having the best OS when compared with the subgroup with CTLA-4 ≤ 20 and Ki67 > 15 (Fig. 2, panel b).

In addition, through survival analysis, we confirmed that the a priori posed 20% RFC cut-off point was a reasonable and biologically significant, although arbitrary, choice. In fact, the results of the Cox regression pointed out that Ki67 and histology were important covariates (i.e. confounding factors) which influenced the prognostic performance of CTLA-4 more than the other variables available for analysis (data not shown). Therefore, although CTLA-4 did not show a statistically significant prognostic value, patients with CTLA-4 > 20 experienced a 40% reduction in death risk, which can be considered a clinically relevant finding. Definitely, the precision of measures of effect (i.e. HR) is an important issue in any investigation. *P* values and confidence intervals reflect such a characteristic which is mainly due to the available sample size, in the sense that the larger the study sample, the narrower the confidence intervals, the smaller the *P* value. However, in a small-sized, observational and exploratory study like the current one, statistical inference and precision are characteristics which can be considered ancillary to other analytical criteria (i.e. biological plausibility, clinical consistency and strength of effect), which can really give evidence of a causal relationship.

In our study, we observed a favourable effect of CTLA-4 overexpression on OS, a finding which might appear in contrast with the commonly accepted notion that CTLA-4 is an important inhibitory molecule in T cells; its overexpression would lead to a worse prognosis due to an increased downregulation of T-cell activation. Indeed, we suggest that CTLA-4 can mediate negative signals into tumour cells, comparable with the ones currently observed in T cells. In particular, CTLA-4 expressed by early disseminated NSCLC cells (microscopic disease) might interact with B7 ligands expressed by cells of the tumour microenvironment [36, 37], thus leading to inhibition of lung cancer cell proliferation and/or induction of apoptotic cell death. This hypothesis is in accordance with our previous finding that established NSCLC cell lines undergo apoptotic

death upon CTLA-4 engagement with soluble B7 (CD80/CD86) ligands [15].

As this apoptosis induction was associated with activation of the extrinsic caspase (caspase-8 and -3) pathway [15] and we recently highlighted in melanoma cells that CTLA-4 ligation with anti-CTLA-4 mAbs can lead to direct antiproliferative and proapoptotic effects (S. Laurent et al. manuscript submitted), our findings may support a role for CTLA-4 as a negative regulator of proliferation, important for cancer biology. Therefore, different CTLA-4 expression levels in tumours, such as in our NSCLC specimens, might influence clinical outcome.

In this regard, a relationship between CTLA-4 expression and clinical outcome has been reported in B-cell chronic lymphocytic leukaemia (CLL) cells. In this study, high CTLA-4 mRNA levels were associated with good clinical outcome and longer period of time to treatment onset, indicating a CTLA-4 inhibitory role on the growth of B-CLL cells [12].

In conclusion, the results of the current study provide a promising indication that, in addition to its classical role as a major attenuator of the immune cell activation, CTLA-4 may behave as an independent prognostic factor in NSCLC. These results, although exploratory, may serve as a basis for further CTLA-4 studies in larger NSCLC patient cohorts which can allow the researchers to obtain more statistically reliable results.

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Conflict of interest The authors declare that they have no conflict of interest.

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