IMMUNO-ONCOLOGY INSIGHTS

HUNTING FOR BETTER BIOMARKERS OF RESPONSE

SPOTLIGHT

REVIEW

CTLA-4 expression by human tumor cells and its impact on immunotherapeutic strategies: a systematic review

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Background: Cancer is a leading cause of death worldwide and its development is closely related to immune dysfunction. Immune checkpoint (IC) receptors maintain immune homeostasis to protect normal tissues, but cancers use several immune escape mechanisms including altered IC expression to evade destruction by the immune system. Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) is one such IC, which downregulates T-cell activation. There are at least two isoforms of CTLA-4 in humans; the full-length receptor isoform and an alternatively spliced soluble CTLA-4 (sCTLA-4) isoform. The aim of this systematic review is to investigate whether or not human tumor cells express CTLA-4, and to examine if there are any consistent retrospective correlates of increased CTLA-4 expression with disease outcome.

Methods: We searched Medline, Scopus, Embase and Web of science for original research articles that investigated CTLA-4 expression by human primary tumor cells or tumor cell lines, from 1987 to April 2020. Forty-five records were deemed eligible and data describing tumor site and stage, CTLA-4 isoform studied, test sample and control groups involved, methods and level (mRNA or protein) of detection, location and any retrospective association with disease outcome were extracted.

Results: Of the forty-five eligible manuscripts, thirty-eight studies focused on the full-length isoform, one study focused on the soluble isoform and six studies investigated both. Forty-two studies reported an increase in CTLA-4 detection by cancer cells. Twenty-one manuscripts performed a retrospective comparison of patient outcomes in CTLA-4 high and low groups in terms of overall survival; eleven studies found that high tumor CTLA-4 expression correlated with poor outcome while seven studies found an opposite correlation. Three studies, however, reported no association.

Conclusions: This review provides strong evidence that a variety of cancer cells express both CTLA-4 transcripts and functional CTLA-4, detectable in the cytoplasm or on the cell surface. Overall, the data suggest that CTLA-4 expression levels in cancer cells are an important but variable feature of the disease phenotype, which will be both increasingly important to evaluate in the context of immune CI therapeutics, and may also be a useful response biomarker.

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INTRODUCTION

Cancer is one of the leading causes of death worldwide, causing an estimated 9.96 million deaths in 2020 [1]. The most common types of cancer include lung, breast, colorectal, prostate, leukemia, lymphoma and skin cancers (carcinomas and melanomas). Limitations of both cancer diagnosis and effective treatment place a colossal strain on those affected, as well as healthcare budgets for middle- and low-income countries [2].

Our current understanding of how cancers develop points to an initial failure of immune surveillance and elimination of transforming cells, followed by an equilibrium period in which nascent cancer cells are kept in check by the immune system, and finally the evolution of molecular mechanisms that allow the cancer to evade the immune system to proliferate and metastasize uncontrollably [3,4]. Cancer cells can escape detection by the immune system through a number of potential mechanisms that can model the tumor microenvironment to tolerate growth of the tumor. They can secrete immunosuppressive factors, such as TGF-β and IL-10 [5,6] or promote recruitment of immunosuppressive cells, such as regulatory CD4 T cells (Treg) [7] and myeloid-derived suppressor cells (MDSC) [8] to the tumor microenvironment. Intrinsically low or loss of MHC class I molecules also allow escape from detection [9]. Moreover, cancer cells can take advantage of immune checkpoints by usurping either directly or indirectly their function, including CTLA-4 on regulatory T cells and programmed cell death-ligand 1 (PD-L1) on tumor cells, leading to dampening of the anti-tumor immune response [10,11]. Maintained high exposure to antigens in the tumor microenvironment, induces a state of dysfunction in anti-tumor effector T cells, called T cell exhaustion [12]. Exhausted T cells are terminally differentiated T Cells that lose their functionality and consequently fail to effectively eliminate cancer cells. They increasingly and sustainably express multiple inhibitory receptors, including CTLA-4 and programmed cell death-1 (PD-1) [13], which suppress their effector function.

The emergence of effective immunotherapy by antibody-mediated checkpoint blockade now offers new opportunities for improving patient outcomes in a range of cancers [14]. Immune checkpoints are typically surface receptors on T cells that aid in maintaining homeostasis, particularly during resolution of an immune response [15]. Unlike traditional cancer therapies that exhibit direct cytotoxic effects, e.g., chemotherapy and radiotherapy, blockade of immune checkpoints functions indirectly by boosting anti-tumor immunity [16].

Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) or CD152 is a well-known immune cell checkpoint receptor. This full-length receptor isoform, also called transmembrane CTLA-4 (tmCTLA-4), is constitutively expressed in homodimeric form on the surface of regulatory T cells and activated effector T cells [17]. A second less well-known

isoform, soluble CTLA-4 (sCTLA-4), is secretable and produced by alternative mRNA splicing of the *CTLA-4* gene [18,19].

Ipilimumab, a monoclonal anti-CTLA-4 antibody and the first approved checkpoint inhibitor (CI), was approved for the treatment of malignant melanoma in 2011 by the FDA [20]. Immunotherapy with anti-CTLA-4 CI antibodies has been somewhat overshadowed by the emergence of anti-PD-1 and anti-PD-L1 antibodies that have seen much greater clinical and commercial success [21,22]. These antibodies, first introduced in 2014, target PD-1 on anti-tumor effector T cells or PD-L1 on tumor cells. Patient response frequency and stratification are aided by PD-L1 staining levels on tumor biopsies [23]. Since their inception, the use of antibodies to inhibit the PD-1: PD-L1 axis has been approved for the treatment of over 20 cancers including non-small cell lung cancer [24]. Anti-CTLA-4 antibodies, in comparison to the anti-PD-1/PD-L1 antibodies have received fewer FDA approvals despite their potential to completely eradicate disease and provide an enduring remission from disease. Ipilimumab is currently approved as a monotherapy solely for melanoma but has also been partnered with nivolumab (anti-PD-1) for several cancers including advanced renal cell carcinoma [25], metastatic colorectal cancer, non-small cell lung cancer [26] and malignant pleural mesothelioma [27]. This has resulted in a significant increase in the number of patients receiving long term survival benefits [28,29] compared with monotherapy. Therefore, it is now imperative to understand the role of anti-CTLA-4 therapy as well as CI therapy more broadly, particularly its effects on the tumor microenvironment including effector immune cell activation or regulatory T cell depletion in order to optimize treatment. Indeed, a combination of tumor intrinsic, immune cell specific and even tissue contextual biomarkers may need to be combined in future bioassays to both stratify responsive patients and refine dosing strategies for an optimum outcome [30].

Although CTLA-4 is generally associated with immune cells, particularly T cells, it is also expressed by a number of non-immune cells including pituitary gland cells [31] and cancer cells [32]. The aim here was to survey and review systematically which tumors have been reported to express increased tumor cell levels of tmCTLA-4 or sCTLA-4 and further to determine whether patient outcome was influenced by the level of CTLA-4 expression by tumor cells.

METHODS

We conducted and reported this systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations [33].

Search strategy

A systematic search of Medline, Scopus, Embase, and Web of science biomedical and pharmacological databases of published literature from 1987 (discovery of CTLA-4 [17]) to April 2020 (date when search performed) was conducted. The search was restricted to studies published in the English language and studies conducted on humans and for studies related to the expression of CTLA-4 and/or sCTLA-4 by cancer cells. The following keywords were used in our search strategy: (CTLA-4 OR sCTLA-4 OR "soluble CTLA-4" OR CD152 OR tm?CTLA-4 OR "transmembrane CTLA-4" OR CTLA-4delTM OR "cytotoxic T lymphocyte associated protein?4" OR "soluble cytotoxic T lymphocyte associated protein?4" OR "cytotoxic T lymphocyte associated antigen?4" OR "soluble cytotoxic T lymphocyte associated antigen?4") AND (cancer* OR malignan* OR tumor* OR tumor* OR neoplasm* OR "cell line"). The final search was performed on 11 April 2020.

Our inclusion criteria were:

1. Original research articles

2. Articles published in English
3. Studies assessing the expression of human full-length and/or soluble CTLA-4 by cancer cells
4. Studies conducted on human samples or human cell lines

Our exclusion criteria were:

1. Case reports, case studies, letters to the editor, conference abstracts, comments, review and systemic review articles
2. Studies conducted on animals
3. Studies assessing the expression of transmembrane and/or soluble CTLA-4 in the tumor microenvironment including infiltrating lymphocytes

Duplicates were removed (based on authors, title, journal, volume, issue and page numbers), using the referencing software Mendeley. Titles and abstracts were screened for potential relevance. 101 records were passed to the second stage (full-text screening) for further screening and data extraction.

For these 101 entries, the full text of the articles was obtained. In case of articles without full text, we searched for the relevant full-text articles using the authors' names and/or combinations of the title words or requested a full-text from the authors. Full texts were subjected to the inclusion and exclusion criteria listed above. All entries and full texts were evaluated independently by members of the study team; the senior author (FW) checked for accuracy and settled any cases of disagreement.

Data extraction

The studies which met the inclusion criteria were summarized and data extraction was performed independently by three investigators, using a pre-defined form and accuracy checks were performed by FA, RAE and FW. Data extracted included: First author, year of publication, sample size, control group, tumor site, clinical stage, study design, method of sample analysis, CTLA-4 isoform analyzed and association of CTLA-4 expression with tumor progression.

RESULTS

Manuscripts included in the systematic review

Of 4911 identified citations from the search results, we identified 101 articles which met the inclusion criteria by title and abstract screening. Most of the identified studies did not discriminate whether CTLA-4 was expressed/produced by the tumor cells or the microenvironment (immune cells), or if the studies only focused on CTLA-4 in immune cells. These studies therefore, had to be excluded as they did not meet the inclusion criteria. It was not possible to refine the search strategy to address this as this can be only identified upon screening the manuscripts. Following full text screening, 45 articles were deemed to be eligible for inclusion in this study. Figure 1 shows the flow diagram of the studies retrieved for this systematic review. The characteristics of these studies are listed in Table 1 [32,34–77].

Manuscripts excluded from the systematic review

As illustrated in Figure 1, a total of 12,174 results were obtained from the search from different databases. Following removing the duplicate, of the 4911 identified citations, we excluded 4810 articles that did not meet our inclusion criteria by title and abstract screening. Following full-text screening, 56 articles were excluded due to the reasons listed in Figure 1.

Data summary

The full characteristics of the study populations in the included manuscripts are displayed in Table 2.

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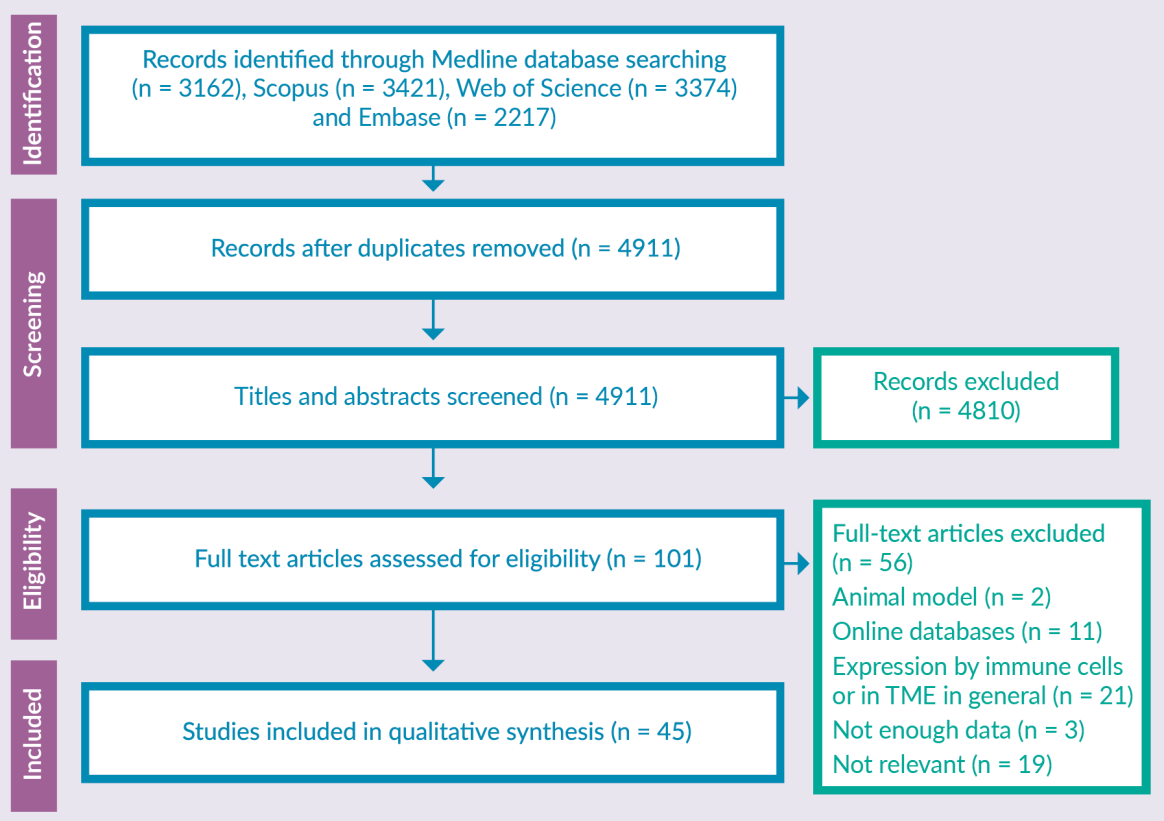
FIGURE 1

Flow diagram of the studies retrieved for the review.



Samples & controls

All studies were conducted on human samples, either by extracting tumor cells and tissues by surgery from patients (n = 34), by using commercially available cancer cell lines (n = 5) or by both (n=6). 23 out of 45 studies included control groups, either tissues or cells from healthy volunteers or normal tissues adjacent to tumors from the same patients. However, the remaining 22 studies did not mention any information about including controls. Table 2 summarizes the study population and the control group.

Tumors expressing CTLA-4

The studies assessed CTLA-4 expression mainly in leukemia/lymphoma (n = 12) (two of the studies assessed the same cohort of CLL patients [36,37]), breast cancer (n = 7), lung cancer (n = 7) and melanoma (n = 6) while the remaining articles were about gastric cancer (n = 3), esophageal (n = 2), uterine (n = 1), cervical (n = 2), ovarian (n = 1) and nasopharyngeal cancers (n = 2), thymoma (n = 1), mesothelioma (n = 1), testicular cancer (n = 1), salivary cystic carcinoma (n = 1), osteosarcoma (n = 1), rhabdomyosarcoma (n = 1), neuroblastoma (n = 1), renal (n = 1), colorectal (n = 1), bladder (n = 2) and bile duct cancers (n = 1). Figure 2 summarizes the different types of cancers that express CTLA-4 which were reported in the manuscripts included in our study.

Twelve included articles discussed the expression of CTLA-4 in leukemia/lymphoma. The subtypes of leukemia/lymphoma studied were: CML (n=1) [34], ALL (n=2) [34,42], AML (n=2) [34, 36], CLL (n=5) [34,45,47,48,67] with two studies assessing the same cohort ([47,48]), ATL (n=3) [35,39,77], CTCL (n=1) [37] and mantle cell lymphoma (n=1) [69]. All these studies showed that malignant cells express CTLA-4, apart from

TABLE 1

Main characteristics of eligible studies.

Author	Year	Cancer subtype	CTLA-4 isoform	Studies conducted on mRNA or protein	Method for CTLA-4 detection	CTLA-4 expression
Pistillo *et al.* [34]	2003	AML CML B-ALL T-ALL B-CLL T-CLL	Tm & s	mRNA and protein	IHC, Flow cytometry, RT-PCR, Western blot	Expressed in 25–85% of AMLs and CMLs; positive expression in B-ALL, T-ALL and B-CLL; few negative cases in T-CLL
Contardi *et al.* [32]	2005	Colorectal adenocarcinoma Breast carcinoma Lung carcinoma Ovarian carcinoma Uterine carcinoma Renal carcinoma Bladder carcinoma Neuroblastoma Rhabdomyosarcoma Melanoma Osteosarcoma	Tm & s	mRNA and protein	Flow cytometry, RT-PCR IHC, flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR IHC, Flow cytometry, RT-PCR, Western blot	Expressed in high levels in all the tested cell lines
Matsubara *et al.* [35]	2006	ATL	Tm	Protein	Flow cytometry	ATL cells from Foxp3-high cases expressed considerable levels, while those of Foxp3-low cases expressed no or very little CTLA-4
Laurent *et al.* [36]	2007	AML (M0-M7 subtypes)	Tm & s	mRNA and protein	Flow cytometry, nested RT-PCR (semi quantitative)	Consistently expressed by leukemic blasts (M0, M1, M2 and M5 subtypes), although at different levels by flow cytometry, Extracellular domain detected while no full-length CTLA-4 detected by nested RT-PCR
Capriotti *et al.* [37]	2008	CTCL	Tm	mRNA	qPCR	Expressed in 21% of the samples
Shah *et al.* [38]	2008	Melanoma	Tm & s	mRNA & protein	RT-PCR, RT- qPCR, Western blot, Flow cytometry	Positive expression
Shimauchi *et al.* [39]	2008	ATL	Tm	Protein	IHC, Flow cytometry	Elevated expression on 13.33% of the patients
Mao *et al.* [40]	2010	Breast cancer	Tm	mRNA & protein	IHC and RT-PCR	Strong expression in 100% of all the samples at both the protein and mRNA levels
Salvi *et al.* [41]	2012	NSCLC	Tm	Protein	IHC	Expression increased in 52.8% (non-squamous) and 35.7% squamous NSCLC
Simone *et al.* [42]	2012	ALL	s	mRNA and protein	Flow cytometry, ELISA, Western blot, RT-PCR	Positive expression in 70% of B-ALL patients
Antczak *et al.* [43]	2013	NSCLC	Tm	mRNA	q PCR	Expression increased in 74.65% of the patients
Laurent *et al.* [44]	2013	Melanoma	Tm & s	mRNA & protein	IHC, flow cytometry, ELISA, RT-PCR, qPCR	Positively expressed in all the tested cell lines; sCTLA-4 transcript was expressed at lower levels than the full-length, in all cell lines except MECO
Mittal *et al.* [45]	2013	CLL	Tm	mRNA and protein	Flow cytometry, RT-PCR (semi-quantitative), qPCR, Western blot	Positively expressed; with CLL cells having different levels of expression (high CTLA-4 and low CTLA-4 expression)
Yu *et al.* [46]	2015	Breast cancer	Tm & s	Protein	IHC	Positively expressed
Ciszak *et al.* [47]	2016	CLL	Tm	Protein	Flow cytometry	Significantly higher levels expressed in patients compared to the controls
Ciszak *et al.* [48]	2016	CLL	Tm	Protein	Flow cytometry	Patients expressed significantly higher levels in comparison to the controls
Huang *et al.* [49]	2016	Nasopharyngeal carcinoma	Tm	Protein	IHC	Expressed with different intensities in 97.4% of the patients
Kim *et al.* [50]	2016	Gastric cancer	Tm	Protein	IHC	Positive expression in 65.8% of the patients
Roncella *et al.* [51]	2016	Mesothelioma	Tm	Protein	IHC	Expressed in 56% of the samples with variable intensity
Schloβer *et al.* [52]	2016	Gastric adenocarcinoma	Tm	Protein	IHC, fluorescence microscopy, targeted sequence	Positive expression in 86% of the sample
Zhang *et al.* [53]	2016	Esophageal carcinoma	Tm	Protein	IHC	Expressed in 87% of the patients. Elevated CTLA-4 expression (“+” and “++”) was detected in 52.6% of the samples expressing CLTA-4
Chakravarti *et al.* [54]	2017	Melanoma	Tm	Protein	IHC	Highly expressed

Tm, Transmembrane (Full length) CTLA-4; s: Soluble CTLA-4; AML, Acute myeloid leukemia; ATL, Adult T cell leukemia; B-ALL, Acute lymphoblastic leukemia of B cell lineage; T-ALL, Acute lymphoblastic leukemia of T cell lineage; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; EHBD, Extrahepatic bile duct cancer; ESCC, Esophageal squamous cell carcinoma; TGCTs, Testicular germ cell tumors; MCL, Mantle cell lymphoma; NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancer; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer; N/A, Not available; IHC, Immunohistochemistry; PCR, Polymerase chain reaction; RT-PCR: reverse transcription PCR; qPCR: quantitative real-time PCR; RT-qPCR: reverse transcription quantitative real-time PCR; ELISA, Enzyme-linked immunosorbent assay.

TABLE 1 (CONT.)

Main characteristics of eligible studies.

Author	Year	Cancer subtype	CTLA-4 isoform	Studies conducted on mRNA or protein	Method for CTLA-4 detection	CTLA-4 expression
Chen *et al.* [55]	2017	Breast cancer	Tm	Protein	Flow cytometry	Expressed by breast cancer cell lines, especially MDA-MB-231 and MCF-7
Le Goux *et al.* [56]	2017	Bladder urothelial carcinoma	Tm	mRNA	Real-time RT-qPCR	CTLA-4 over-expressed in 84.5% in MIBC and in 35.2% in NMIBC samples
Karpathiou *et al.* [57]	2017	Laryngeal and pharyngeal squamous cell carcinoma	Tm	Protein	IHC	Positive expression
Kim *et al.* [58]	2017	Breast cancer	Tm	mRNA	Whole exome sequence, RNA-Seq, gene enrichment analysis	Positive expression
Lafuente-Sanchis *et al.* [59]	2017	NSCLC	Tm	mRNA	IHC, RT-qPCR	Expression is detected in all the samples (100%)
Lim *et al.* [60]	2017	EHBD	Tm	Protein	IHC	Positive expression in 95% of the patients
Paulsen *et al.* [61]	2017	NSCLC	Tm	Protein	IHC	Over-expression in 50% stromal-CTLA-4 and 43% epithelial-CTLA-4
Yang *et al.* [62]	2017	Gastric cancer	Tm	Protein	IHC, Western blot	Positive expression in 43.7% of the sample by IHC
Kassardjian *et al.* [63]	2018	Breast cancer (ductal carcinoma in situ, invasive ductal carcinoma, invasive lobular carcinoma and invasive tubular carcinoma)	Tm	Protein	IHC	Over expressed in 52.7% of the all the samples with variation depending on tumor type and grade
Lan *et al.* [64]	2018	Breast cancer	Tm	Protein	IHC	Expressed in 41.2% of the samples
Mo *et al.* [65]	2018	Melanoma	Tm	mRNA and protein	Confocal microscopy, flow cytometry, RT-qPCR, Western blot	Highly expressed by most human melanoma cell lines
Santoni *et al.* [66]	2018	Thymoma	Tm	mRNA and protein	IHC, RT-qPCR, confocal microscopy	CTLA-4 expression was statistically found to progressively increase in A, B1, B2, AB and it was maximal in B3 thymomas
Do *et al.* [67]	2019	CLL	Tm	mRNA and protein	qPCR, flow cytometry, confocal microscopy	CTLA-4 expression in CLL B-cells was one of the most differentially expressed genes, average 19-fold change over normal B-cells (microarray); constitutive expression in CLL B cells compared to control (qPCR and confocal microscopy); constitutive intracellular expression in 61% patients (flow cytometry)
Gutiérrez-Hoya *et al.* [68]	2019	Cervical cancer	Tm	Protein	Flow cytometry	Positive expression
Harrington *et al.* [69]	2019	MCL	Tm	mRNA and protein	qPCR, flow cytometry	Very low mRNA expression No Surface protein expression
Inozume *et al.* [70]	2019	Melanoma	Tm	mRNA and protein	IHC, flow cytometry, confocal microscopy, RT-PCR	Expressed in 50% of tested the cell lines
Lobo *et al.* [71]	2019	TGCTs	Tm	Protein	IHC	Positive expression
Mosconi *et al.* [72]	2019	ACC of salivary gland	Tm	Protein	IHC	No expression
Regzedmaa *et al.* [73]	2019	SCLC	Tm	Protein	IHC	Expressed in 89.5% of the samples
Zhang *et al.* [74]	2019	ESCC	Tm	Protein	IHC	Elevated expression in 48.8% of the patients
Zhang *et al.* [75]	2019	NSCLC	Tm	Protein	IHC, Western blot	Expressed in high levels in A549, H460, HCC827 and H1975; very low levels in H661 and no detectable expression in H1650
Karpathiou *et al.* [76]	2020	uterine cervix cancer	Tm	Protein	IHC	Expression was found in 61.5 % of the invasive cases; CTLA-4 tumor cell expression was more often found in squamous cell carcinomas than in adenocarcinomas
Takeuchi *et al.* [77]	2020	ATL	Tm	Protein	IHC	No IHC stains with greater than 50% staining detected

Tm, Transmembrane (Full length) CTLA-4; s: Soluble CTLA-4; AML, Acute myeloid leukemia; ATL, Adult T cell leukemia; B-ALL, Acute lymphoblastic leukemia of B cell lineage; T-ALL, Acute lymphoblastic leukemia of T cell lineage; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; EHBD, Extrahepatic bile duct cancer; ESCC, Esophageal squamous cell carcinoma; TGCTs, Testicular germ cell tumors; MCL, Mantle cell lymphoma; NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancer; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer; N/A, Not available; IHC, Immunohistochemistry; PCR, Polymerase chain reaction; RT-PCR: reverse transcription PCR; qPCR: quantitative real-time PCR; RT-qPCR: reverse transcription quantitative real-time PCR; ELISA, Enzyme-linked immunosorbent assay.

TABLE 2

Study population and control groups used in the included studies.

Author	Cancer subtype	Stage or grade	Sample		Control	
			Type	Size (n)	Type	Size (n)
Pistillo *et al.* (2003) [34]	AML CML B-ALL T-ALL B-CLL T-CLL	N/A	Donor patients (Primary samples) and cell lines (CEM, Jurkat, Molt-4, Daudi, Raji, HOM-2, HL60, KG1a, K562)	100 patients and 9 cell lines	Healthy donors	10
Contardi *et al.* (2005) [32]	Colorectal adenocarcinoma, breast carcinoma, lung carcinoma, ovarian carcinoma, uterine carcinoma, renal carcinoma, bladder carcinoma, neuroblastoma, rhabdomyosarcoma, melanoma, osteosarcoma	Grade 1 and grade 2 (breast carcinoma), grade 4 (osteosarcoma), N/A (colorectal adenocarcinoma, lung, ovarian, uterine, renal and bladder carcinoma, neuroblastoma, rabdomyosarcoma and melanoma)	Donor patients (primary samples from osteosarcoma and breast cancer) and cell lines (4 colorectal adenocarcinoma cell lines: HCT-8, HT-29, COLO 205 and CACO-2; 4 breast carcinoma cell lines: MCF-7, MDA-MB-231, T-47D, BT-20; 3 lung carcinoma cell lines: CALU-1, CALU-6, A549; 2 ovarian carcinoma cell lines: SKOV-3 and A2780; 1 uterine carcinoma cell line; 5 neuroblastoma cell lines: NB100, SJNKP, CHP212, SY5Y, SKNBE-2C; 3 renal carcinoma cell lines: SKRC-10, SKRC-52, SKRC-59; 2 uterine carcinoma cell lines: TG, HELA; 1 bladder carcinoma cell line: T24; 2 rabdomyosarcoma cell lines: RD/18, TE671; 4 osteosarcoma cell lines, HOS, MG-63, U2-OS, SaOS-2; 3 melanoma cell lines, MEL-1, ALO-39, F0-1; 2 nontumorigenic human breast epithelial cell lines: MCF10A, HC11)	6 Osteosarcoma samples 5 breast cancer samples and 34 cell lines	PBMCs from healthy donors; for osteosarcoma cell lines, HSSCs from healthy donors stimulated to differentiate toward the osteogenic lineage; for breast tissue, non-malignant tissue adjacent to tumor	10 HSSC; 5 non-malignant breast cancer tissue adjacent to tumor
Matsubara *et al.* (2006) [35]	ATL	I-IV	Donor patients (primary samples) and cell lines (ATL-T, ATL-2, ATL-43T, ATL-48T^{+}, ATL-55T^{+}, ED-40515^{+}, MT-1)	20 patients (9 patients of the acute type, 10 of the chronic type, and 1 of the lymphoma type) and 7 ATL derived cells lines	CD4^{+} and CDD4^{+}CD25^{+} T cells purified from PBMCs from healthy donors	N/A
Laurent *et al.* (2007) [36]	AML (M0-M7 subtypes)	N/A	Donor patients (primary samples)	25 (15 untreated and 10 chemoresistant patients)	PBMCs from healthy donors	N/A
Capriotti *et al.* (2008) [37]	CTCL	I-III	Donor patients (primary samples)	28	PBMCs from healthy donors	6
Shah *et al.* (2008) [38]	Melanoma	N/A	Donor patients (primary samples) and cell lines (UACC 1273, A2058)	N/A (patients) and 2 cell lines	N/A	N/A
Shimauchi *et al.* (2008) [39]	ATL	N/A	Donor patients (primary samples)	21	PBMCs from healthy donors	8
Mao *et al.* (2010) [40]	Breast cancer	N/A	Donor patients (primary samples)	60	Normal breast tissue from patients with benign breast disease or external breast injury	30
Salvi *et al.* (2012) [41]	NSCLC	I–III	Donor patients (primary samples)	81	Tumor-adjacent normal tissues	N/A
Simone *et al.* (2012) [42]	ALL	N/A	Donor pediatric patients (primary samples)	80	Age-matched normal serum samples from healthy donors	45
Antczak *et al.* (2013) [43]	NSCLC	N/A	Donor patients (primary samples)	71 (23 adenocarcinoma, 41 squamous cell carcinoma and 7 large cell carcinoma)	N/A	N/A
Laurent *et al.* (2013) [44]	Melanoma	N/A	Donor patients (primary cell lines from metastatic lesions of cutaneous melanoma and melanoma tissue sections) and long term cell lines (C23, MeWo, FO-1)	14 primary cell lines, 3 long-term cell lines and 33 tissue sections	N/A	N/A
Mittal *et al.* (2013) [45]	CLL	N/A	Donor patients (primary samples including peripheral blood, bone marrow and lymph node samples)	105	N/A	N/A
Yu *et al.* (2015) [46]	Breast cancer	I-III	Donor patients (Primary samples)	130	N/A	N/A
Ciszak *et al.* (2016) [47]	CLL	N/A	Donor patients (primary samples)	38	B cells purified from healthy donor PBMCs	15
Ciszak *et al.* (2016) [48]	CLL	I–IV	Donor patients (primary samples)	38	B cells purified from healthy donor PBMCs	6
Huang *et al.* (2016) [49]	Nasopharyngeal carcinoma	UICC I–IVc; WHO II & III	Donor patients (primary samples)	191	N/A	N/A
Kim *et al.* (2016) [50]	Gastric cancer	I–III	Tissue microarrays from donor patients (primary samples)	243	Non-neoplastic gastric mucosa specimens	N/A

n, number; N/A, not available; CLL, Chronic lymphocytic leukemia; ATL, Adult T-cell leukemia/lymphoma; PBMCs, Peripheral blood mononuclear cells; HSSCs, human stromal stem cells; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer .

TABLE 2 (CONT.)

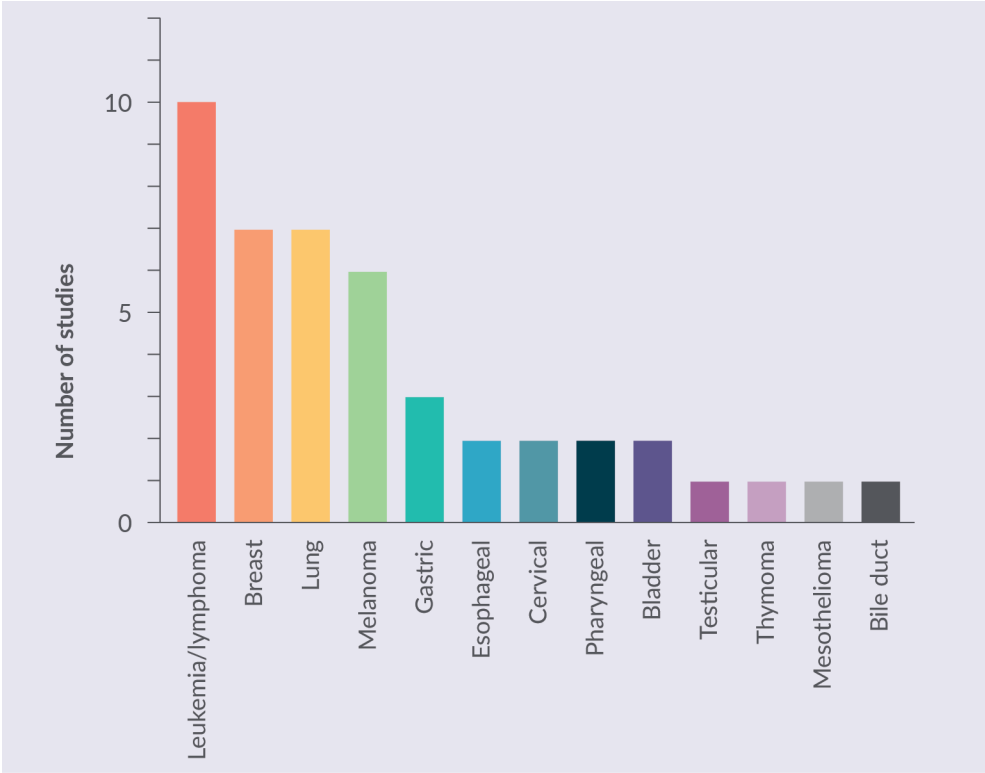
Study population and control groups used in the included studies.

Author	Cancer subtype	Stage or grade	Sample		Control	
			Type	Size (n)	Type	Size (n)
Schloβer *et al.* (2016) [52]	Gastric adenocarcinoma	I-IV	Donor patients (primary samples)	127	N/A	N/A
Zhang *et al.* (2016) [53]	Esophageal carcinoma	I-IV	Donor patients (primary samples)	158	N/A	N/A
Chakravarti *et al.* (2017) [54]	Melanoma	N/A	Donor patients (primary samples)	81	N/A	N/A
Chen *et al.* (2017) [55]	Breast cancer	N/A	Cell lines (MDA-MB-231, SKBR3, MCF-7, T47D)	4 cell lines	N/A	N/A
Le Goux *et al.* (2017) [56]	Bladder urothelial carcinoma	Ta-T3, low grade and high grade	Donor patients (primary samples)	155 (84 with MIBC and 71 with NMIBC)	Normal bladder tissues from surgery unrelated to bladder tumors	15
Karpathiou *et al.* (2017) [57]	Laryngeal and pharyngeal squamous cell carcinoma	I–IV	Donor patients (primary samples)	152	N/A	N/A
Kim *et al.*(2017) [58]	Breast cancer	Stage IV or recurrent after curative treatment	Donor patients (primary samples)	37	N/A	N/A
Lafuente-Sanchis *et al.* (2017) [59]	NSCLC	I–III	Donor patients (primary samples)	78	Tumor-adjacent lung tissues	78
Lim *et al.* (2017) [60]	EHBD	T1–T4	Donor patients (primary samples)	77	N/A	N/A
Paulsen *et al.* (2017) [61]	NSCLC	I–IIIA	Donor patients (primary samples)	536	N/A	N/A
Yang *et al.* (2017) [62]	Gastric cancer	N/A	Donor patients (primary samples)	48	Tumor-adjacent normal tissues	48
Kassardjian *et al.* (2018) [63]	Breast cancer (ductal carcinoma *in situ*, invasive ductal carcinoma, invasive lobular carcinoma and invasive tubular carcinoma)	I–IV	Commercially obtained breast tissue microarray sections	93 (73 invasive ductal, 10 invasive lobular, 2 invasive tubular, 8 ductal carcinoma *in situ*)	Normal breast tissues from the same tissue microarrays	6 (2 normal and 4 with fibrocystic changes)
Lan *et al.* (2018) [64]	Breast cancer	I–III	Donor patients (primary samples)	102	N/A	N/A
Mo *et al.* (2018) [65]	Melanoma	N/A	Cell lines (Hs 936.T, A2058, COLO679, WM983(B), 451 Lu, WM3918 and WM3912)	7 cell lines (in addition to 61 melanoma cell lines from the cancer cell encyclopedia database)	Human primary neonatal foreskin melanocytes	N/A
Santoni *et al.* (2018) [66]	Thymoma	N/A	Donor patients (primary samples)	68	PBMCs from healthy donors	N/A
Do *et al.* (2019) [67]	CLL	0–IV	Donor patients (primary samples) and cell lines (Mec1, OSU-CLL)	28 N/A	B cells and T cells purified from blood from healthy donors	N/A
Gutiérrez-Hoya *et al.* (2019) [68]	Cervical cancer	N/A	Cell lines (HeLa (HPV 18), CaSki (HPV 16), C33A (HPV-), INBL)	4 cell lines	N/A	N/A
Harrington *et al.* (2019) [69]	MCL	N/A	Donor patients (primary samples)	16	PBMCs from healthy donors	N/A
Inozume *et al.* (2019) [70]	Melanoma	N/A	Donor patients (primary samples) Melanoma cell lines	13 melanoma tissue sections (5 shown in manuscript) 10 cell lines	N/A	N/A
Lobo *et al.* (2019) [71]	TGCTs	I–III	Donor patients (primary cells)	271 tumour samples from 162 patients	N/A	N/A
Mosconi *et al.* (2019) [72]	ACC of salivary glands	I–III	Donor patients (primary samples)	36	N/A	N/A
Regzedmaa *et al.* (2019) [73]	SCLC	I-IV	Donor patients (primary samples)	38	N/A	N/A
Zhang *et al.* (2019) [74]	ESCC	I–IV	Donor patients (primary samples)	84	N/A	N/A
Zhang *et al.* (2019) [75]	NSCLC	N/A	Cell lines (A549, H460, HCC827, H1975, H1650, H661)	N/A	N/A	N/A
Karpathiou *et al.* (2020) [76]	Uterine cervix cancer	0–IV	Donor patients (primary samples)	63 lesions from 52 patients	N/A	N/A
Takeuchi *et al.* (2020) [77]	ATL	I–IV	Donor patients (primary samples)	69	N/A	N/A

n, number; N/A, not available; CLL, Chronic lymphocytic leukemia; ATL, Adult T-cell leukemia/lymphoma; PBMCs, Peripheral blood mononuclear cells; HSSCs, human stromal stem cells; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer .

FIGURE 2

Studies that reported detectable CTLA-4 in tumor cells.



2 studies, which stated that mantle cell lymphoma [69] and ATL [77] do not express CTLA-4, and this might be due to the small sample size [69] or the method used (only IHC was used) [77], in addition to the lack of control group [77].

A study that investigated the expression levels of CTLA-4 in adenoid cystic carcinoma of salivary gland founds that CTLA-4 expression in tumor cells is negative [72]. It is worth noting that only one method was used to assess protein expression (IHC).

On the other hand, we included seven studies about lung cancer which have clearly demonstrated positive expression of CTLA-4 by cancer cells; the majority were focused on NSCLC (n=6) [32,41,43,59,61,75] with a single study on SCLC (n=1) [73].

Breast (n=7) [32,40,46,55,58,63,64], gastric (n=3) [50,52,62] and melanoma (n=6) [32,38,44,54,65,70] cancer cells were confirmed for positive CTLA-4 expression by all the included manuscripts.

All the remaining types of cancers included in this systematic review were positive for CTLA-4 expression. Expression patterns are summarized in Table 1.

In terms of cytoplasmic vs surface expression, twenty-two out of the forty-five studies looked at the intracellular localization of CTLA-4. One study examined only the cytoplasmic CTLA-4 [63] while the other twenty-one studies investigated both cytoplasmic and surface CTLA-4 levels, sixteen of them observed higher CTLA-4 levels in the cytoplasm than on the cell membrane [32,34,44,46–48,51,55,60–62,65,67,70,71,76], which is consistent with what we know about the endosomal/lysosomal vesicular localization within cytoplasm previously reported in T cells, where CTLA-4 is rarely expressed on the membrane and is rapidly internalized into the cytoplasm by means of endocytosis [65]. The other five studies, however, did not specify where the highest levels of CTLA-4 are localized [40,41,53,64,66].

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CTLA-4 isoform studied

With the exception of seven studies that investigated the soluble isoform of CTLA-4, either alone [42] or together with the full-length isoform [32,34,36,38,44,46], the majority of the studies focused on the full-length isoform (n = 44).

Methodologies used to detect CTLA-4 expression by cancer cells

As summarized in Table 1, CTLA-4 was detected either at the mRNA level (by real-time PCR and/or RT-PCR) and/or at the protein level (by Western blotting, immunohistochemistry, flow cytometry, ELISA and/or fluorescence microscopy). Most studies measured CTLA-4 at the protein level (n = 27) [35,39,41,46–55,57,60–62,68,71–77] with two examining the same cohort [47,48] while four studies measured CTLA-4 only at the mRNA level [37,43,58,59]. Fourteen studies, however, measured CTLA-4 at both levels [32,34,36,38,40,42,44,45,56,65–67,69,70].

Correlation of CTLA-4 expression by tumor cells with clinical outcome

The outcomes of the studies analyzing the potentially prognostic role of CTLA-4 in cancers are varied, especially with regards to whether increased expression signifies a better or poorer outcome for the patient cohort (Figure 3). Out of the forty-five papers included in this study, twenty-one papers looked retrospectively at cancer progression including overall survival. In general, eleven studies found that high tumor CTLA-4 expression correlated with poorer outcome compared with lower CTLA-4 expression [42,46,49,52–54,57,64,66,71,74]. Conversely, seven studies found an opposite correlation [42,45,47,51,60,61,73]. In mesothelioma, only the sCTLA-4 in the pleural effusion, rather than serum, was found to be a statistically significant positive predictive factor [51]. Three studies, however, reported no association between tumor expression levels of CTLA-4 and tumor progression [56,59,76]. Table 3 illustrates the correlation between CTLA-4 expression levels in cancer cells and disease outcome, in the twenty-one articles which reported that.

These observations led to the obvious question of whether or not any associations between patient outcome and CTLA-4 expression were specific to particular tumor types. Out of the twelve leukemia/lymphoma articles included in our review, only three investigated the association of tumor CTLA-4 expression with patient clinical outcome. Two studies found that high tumor CTLA-4 expression in CLL is a good prognostic factor [45,47]. Another study suggests that increased tumor sCTLA-4 expression in ALL correlates with poor outcome [42]. Two studies by Ciszak *et al.* assessed the same cohort for CTLA-4 expression in CLL [47,48] and only one of them studied the correlation with disease progression [47].

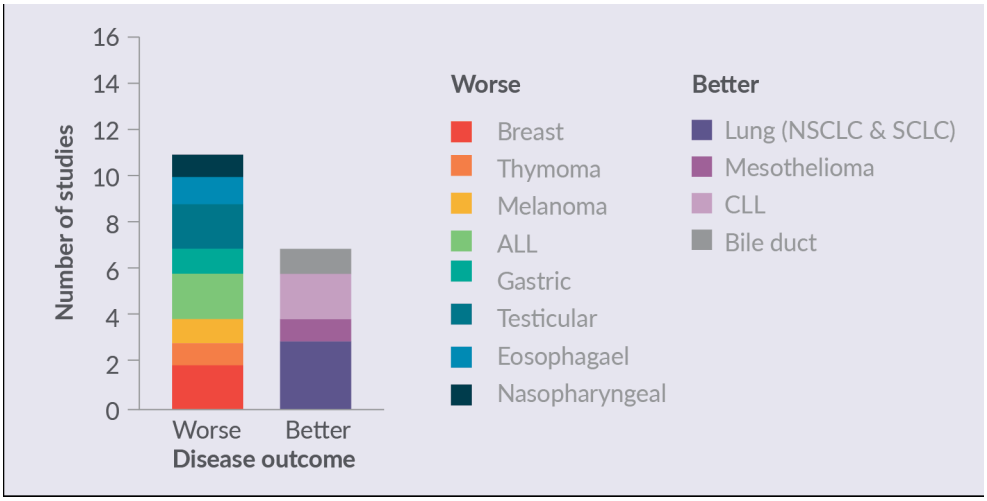
In lung cancer, increased tumor CTLA-4 expression was associated with better patient outcomes in three studies [61,73,78], including one small cell lung cancer study [73]. One study found a diverging prognostic impact of CTLA-4 expression in metastatic NSCLC lymph nodes versus primary tumor; while high stromal CTLA-4 was a positive prognostic factor in the squamous cell carcinoma (SCC) subgroup, no association with survival was found in the adenocarcinoma (ADC) and large cell carcinoma subgroups [61]. However, a study by Lafuente-Sanchis *et al.* demonstrated no association between tumor CTLA-4 expression levels and patient clinical outcomes [59].

Conversely, the two studies that examined the effect of increased CTLA-4 expression in breast cancer found a negative correlation with patient clinical outcome, suggesting that CTLA-4 might be a negative prognostic factor in breast cancer [46,64].

In esophageal carcinoma, increased tumor CTLA-4 expression is an independent predictor of shorter overall survival [53,74].

FIGURE 3

Cancers in which relatively high expression levels of CTLA-4 correlate with disease outcome.



Regarding gastric cancer, the prognostic effect of CTLA-4 was only studied in one article, which found a negative association between tumor CTLA-4 levels and overall survival [52].

In uterine cervix and bladder urothelial carcinoma, researchers could not find any correlation between CTLA-4 expression levels and clinical outcome [56,76]. On the other hand, increased CTLA-4 tumor expression predicted longer overall survival in patients with mesothelioma [51] and EHDC [60], and shorter overall survival in patients with melanoma [54], thymoma [66], nasopharyngeal carcinoma [49], testicular germ cell tumors [71] and laryngeal and pharyngeal squamous cell carcinoma [57]. However, we cannot build strong evidence collectively from these studies and more should be conducted to ascertain the relationship between CTLA-4 expression in tumor cells, disease progression and patient outcomes. Furthermore, different methodologies were applied in the analysis of CTLA-4 in these studies, and at different levels (gene and/or protein) further complicating a generalized conclusion.

DISCUSSION

One of the most important recent advances in cancer treatment has been the emergence of cancer immunotherapy, which is based on boosting the anti-tumor immune response rather than directly targeting tumor cells. Despite its impressive successes over the last decade, in some patients the response is limited or short-lived and indeed, protocols that consistently identify and stratify patients that will respond well to this type of therapy remain a high priority. These limited responses are mainly due to multiple tumor-mediated immune escape mechanisms which tumor cells use to suppress anti-tumor immunity. One of the major and most important immune escape mechanisms is by expressing co-inhibitory molecules, called immune checkpoints (IC). CTLA-4 in the context of the tumor microenvironment has typically been associated with infiltrating T cells, not least increased recruitment of regulatory T cells [79], but less attention has been paid to any role CTLA-4 may have when expressed by tumor cells directly. The clinical significance of the existence of this immunosuppressive molecule in both tumor and immune cells within the tumor microenvironment remains to be fully elucidated, and its potential as a prognostic marker or a therapeutic biomarker, in addition to any functional role it might have, needs to be further examined.

In this systematic review, we assessed the body of available peer-reviewed literature

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regarding CTLA-4 expression, both tmCTLA-4 and sCTLA-4, by a wide variety of cancer subtypes with the aim of understanding its expression by tumors and its correlation with disease progression and clinical outcome.

We found that the vast majority of studies demonstrated CTLA-4 expression was detectable, at the mRNA and/or protein levels, in tumor cells compared to its counterpart healthy cells. Three studies, however, observed no CTLA-4 expression, although this might be because they only investigated its expression at the protein level using only one methodology, IHC [72,77], because of the small sample size [69] or because the type of the tumor cells they investigated might not express CTLA-4. In contrast, sCTLA-4 was not studied as thoroughly as its counterpart receptor; only seven studies investigated sCTLA-4 expression by cancer cells, but these studies confirmed the possibility that cancer cells secrete this naturally immunosuppressive protein, perhaps as an immune evasion strategy [32,48–50].

Overall, this survey of CTLA-4 expression in tumor cells points to an area, which could yield a useful biomarker for CI therapy as part of the ongoing drive to generate predictable bioresponse profiles to treatment, but it also demands further comprehensive

TABLE 3

The correlation of CTLA-4 expression levels in tumor cells (mRNA and/or protein) with the disease outcome.

Author	Cancer subtype	Studies conducted on mRNA or protein?	Correlation of higher levels of tumor CTLA-4 with outcome
Salvi *et al.* (2012) [41]	NSCLC	Protein	Good outcome
Simone *et al.* (2012) [42]	ALL	mRNA and protein	Poor outcome
Mittal *et al.* (2013) [45]	CLL	mRNA and protein	Good outcome (Low-CTLA-4 CLL was associated with poor outcome, while high-CTLA-4 CLL was associated with good outcome)
Yu *et al.* (2015) [46]	Breast cancer	Protein	Poor outcome
Ciszak *et al.* (2016) [47]	CLL	Protein	Good outcome
Huang *et al.* (2016) [49]	Nasopharyngeal carcinoma	Protein	Poor outcome
Roncella *et al.* (2016) [51]	Mesothelioma	Protein	Good outcome
Schloβer *et al.* (2016) [52]	Gastric adenocarcinoma	Protein	Poor outcome
Zhang *et al.* (2016) [53]	Esophageal carcinoma	Protein	Poor outcome
Chakravarti *et al.* (2017) [54]	Melanoma	Protein	Poor outcome
Le Goux *et al.* (2017) [56]	Bladder urothelial carcinoma	mRNA and protein	No correlation
Karpathiou *et al.* (2017) [57]	Laryngeal and pharyngeal squamous cell carcinoma	Protein	Poor outcome
Lafuente-Sanchis *et al.* (2017) [59]	NSCLC	mRNA	No correlation
Lim *et al.* (2017) [60]	EHBD	Protein	Good outcome
Paulsen *et al.* (2017) [61]	NSCLC	Protein	Good outcome
Lan *et al.* (2018) [64]	Breast cancer	Protein	Poor outcome
Santoni *et al.* (2018) [66]	Thymoma	mRNA and protein	Poor outcome
Lobo *et al.* (2019) [71]	TGCTs	Protein	Poor outcome
Regzedmaa *et al.* (2019) [73]	SCLC	Protein	Good outcome
Zhang *et al.* (2019) [74]	ESCC	Protein	Poor outcome
Karpathiou *et al.* (2020) [76]	Uterine cervix cancer	Protein	No correlation

study. In particular, it will be useful to definitively resolve the impact of high CTLA-4 tumor cell levels both on patient outcome for each type of cancer and whether or not it affects CI therapy performance. Soluble CTLA-4 for instance, is bound by anti-CTLA-4 antibodies such that high serum levels of this immunosuppressive molecule could affect the amount of antibody engaging with tmCTLA-4. Moreover, our data suggest that antibodies specific for CTLA-4 expressed by T cells could also target cancer cells directly.

We looked for any correlation between CTLA-4 levels, disease progression and patient outcome in this study. Eleven studies found that high tumor CTLA-4 expression correlated with disease progression while lower CTLA-4 expression correlated with better outcomes [42,46,49,52–54,57,64,66,71,73]. Conversely, seven studies found an opposite correlation, where high CTLA-4 expression correlated with better clinical outcomes [45,47,51,60,61,73,78]. Three studies, however, reported no association between tumor expression levels of CTLA-4 and tumor progression [56,59,76]. The data from these studies are not robust enough to define clearly why these differences in outcome exist, but it is interesting to note that the cancers in which a worse outcome was observed do not overlap with those with a better outcome (Figure 3). This suggests that increased CTLA-4 expression has different, yet to be determined, effects in different types of cancer. Other reasons might be differences in methods used for CTLA-4 detection and whether it was at an mRNA or protein level. Additionally, there is a significant variation in the assessment of different CTLA-4 isoforms with sCTLA-4 being understudied.

Accordingly, we suggest a more robust streamlined protocol to assess CTLA-4 expression in tumors and its correlation with disease progression and clinical outcome.

Another possible biomarker could be the secretable sCTLA-4, which has not received the same level of examination in terms of immune regulation that its receptor counterpart has over the years and any role it might play particularly with regard to cancer progression is still unclear. Interestingly, it has been previously shown that selective blockade of sCTLA-4 exhibited a stronger and more consistent, significant enhancing effect on Ag-driven PBMC responses than pan-specific blockade of total CTLA-4 [80]. However, most of the studies included in this review which investigated sCTLA-4 expression used the ELISA assay method to measure serum levels [40,42,44,51] or pleural effusion [51], which does not discriminate whether it is produced and secreted by cancer cells or immune cells. This emphasizes the need to further study the expression of the soluble isoform by different tumor cell types with selective antibodies, as well as the need to use more than one method to detect its expression and to study its role in cancer and how cancer cells potentially use it to escape the immune system.

TRANSLATIONAL INSIGHT

Taken together, data from this systematic review provide evidence that CTLA-4 is expressed not only by immune cells but also by many types of cancer cells. Further, the data emphasize the importance of assessing the correlation between CTLA-4 levels and a patient's clinical outcome by using a more robust streamlined protocol to assess CTLA-4 levels in cancer cells, together with correlating both mRNA and protein levels with the disease progression. Moreover, there are only few studies which investigated the expression of the soluble CTLA-4 isoform by cancer cells, which means that the role of this key molecule might be underestimated, and further studies should be conducted to understand its role and function in cancer. Therefore, our findings suggest the need to define better and more robust methods to detect soluble CTLA-4 expression by tumor cells, in a wide variety of tumor types, and to deeply study its role in immune cells as well as in cancer cells.

Checkpoint inhibitor antibodies represent a novel type of cancer immunotherapy that

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has proven obvious success in the treatment of different cancers. As one of the major targets of checkpoint inhibitors, CTLA-4 needs to be studied more thoroughly in regards of its expression by cancer cells to assess its full potential, not only as a therapeutic target, but also as a biomarker for patient stratification, predicting prognosis and response to therapy within a broader set of biomarkers, which help to delineate the tumor microenvironment as a prelude to CI therapy. Despite the huge clinical benefits that CTLA-4 offers in both cancer and autoimmune disease immunotherapy, its role and function especially in non-immune cells remains largely unexplored.

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