CTLA-4 Immunohistochemistry and Quantitative Image Analysis for Profiling of Human Cancers

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Supplementary Data

Tissues and Cells

The non-clinical FFPE tissues included full sections of tonsil and lymph node tissue and numerous resected tumor specimens of triple-negative breast cancer, colorectal carcinoma, head and neck squamous cell carcinoma, squamous and non-squamous NSCLC, pancreatic carcinoma, and urothelial carcinoma. The commercially-obtained tumor specimens were purchased as previously described[24] or were obtained separately (Avaden Biosciences, Seattle, WA). In addition, tumor and normal tissue microarray (TMA) specimens (TA1962, TA1788/02; TriStar Technology Group, Rockville, MD) were used to provide samples of additional major human tissues and a variety of solid human cancers.

Tissues contained in TMAs of normal human tissues (blocks from which sections were prepared contained 3 or more 1-mm cores of the following): Adrenal gland; brain, cerebrum; brain, cerebellum; breast, glandular; cervix, endo; cervix, exo; colon, descending; fallopian tube; gallbladder; heart; intestine, ileum; kidney, cortex; kidney, medulla; liver; lung, parenchyma; lymph node; muscle, skeletal; ovary; pancreas; parotid gland; prostate gland; placenta, early; seminal vesicle; skin; spleen; stomach, fundus; stomach, muscular; testis; thymus; thyroid gland; tonsil; ureter; urinary bladder; uterus, endometrium; uterus, myometrium.

Tumors contained in TMAs of human cancers (blocks from which sections were prepared contained 12 or more 1-mm cores of the following): Breast carcinoma, invasive, ductal; colorectal adenocarcinoma; head and neck, squamous cell carcinomas of oral cavity, tongue, larynx; kidney, renal cell carcinoma; lung, non–small-cell carcinoma (NSCLC), squamous and non-squamous; melanoma, cutaneous; ovarian adenocarcinoma; pancreatic adenocarcinoma; prostatic adenocarcinoma; thyroid gland carcinoma; urinary bladder, invasive urothelial carcinoma.

Excisional (whole slide) tumors and numbers of samples with both CTLA-4 and FoxP3 immunostains assessed microscopically for image analysis (IA) performance (the numbers of these samples assessed by systematic IA is described in the body of the manuscript):
Breast cancer, triple-negative (99); colorectal carcinoma (94); head/neck carcinoma (99); NSCLC, non-squamous (92), squamous (88); pancreatic carcinoma (81); urothelial carcinoma (89).

Clinical trial baseline archival or fresh NSCLC samples used for staining with the CTLA-4/FoxP3 dual assay and validation by IA were obtained as previously described.[25] For fresh samples, trial sites were directed to obtain 2 or more core-needle biopsy specimens, 18g or larger, and then fix in 10% NBF for 24-48 hours. Routine histological processing was performed to prepare FFPE blocks. Decalcified specimens were excluded. Samples were assessed microscopically by a pathologist to ensure the presence of neoplastic cells.

Activated human T lymphocytes to assess CTLA-4 expression were generated as follows. A human Leukopak was received from StemCell Technologies (Cambridge, MA). Red blood cells were lysed by adding ACK lysing solution (Thermo Fisher Scientific, Waltham, MA), and then pan T cells were isolated with a T-cell enrichment kit with magnetic-bead separation (StemCell Technologies, Vancouver, Canada). Isolated cells were incubated on anti-CD3/CD28 (clones OKT3/CD28.2; BioLegend, San Diego, CA)–coated plates (Greiner Bio-One, Monroe, NC) and harvested as separate aliquots at 6 and 24 hr later. Stimulated and unstimulated cells were fixed in formalin, pelletized, and processed to paraffin blocks.

Transfected cell lines to serve as CTLA-4 positive and negative controls were generated as follows. The CHO-CTLA4 cell line was created by transducing the CHO-K1 cell line with Lentivirus containing the human CTLA4 cDNA. CHO-K1 were plated in a 6 well plate at 4x10e5 cells/well and placed in the incubator overnight. The next morning cells were transduced with Lenti-CTLA4 virus. Three days post-transduction, cells were placed in selection medium containing 10ug/ml blasticidin. The CHO-PD1 cell line was created by transducing the CHO-K1 cell line with Lentivirus containing the human PD1 cDNA. CHO-K1 were plated in a 6 well plate at 4x10e5 cells/well and placed in the incubator overnight. The next morning cells were transduced with Lenti-PD1 virus. Three days post-transduction, cells were placed in selection medium containing 10ug/ml puromycin. The HEK-CD28 cell line was created by transducing 293X cells with lentivirus containing human CD28 cDNA (OriGene Technologies Inc, Rockville, MD; cat# RC211318L1).

Cell pellets were prepared from cells expanded in culture and then harvested by gentle centrifugation @ 2500 RPM for 5min in a 50 ml conical tube. The supernatant was removed and the cells were resuspended in 10% neutral-buffered formalin (NBF) and fixed overnight. The fixed cells were again gently pelleted, then resuspended in warm (60°C) HistoGel (Thermo Scientific) in a 50cc tube. The HistoGel/cell suspension was cooled to 4°C and incubated for 30 min, then 10% NBF was gently added on top of the solidified cell pellet and fixed overnight at 4°C. The fixed cell pellets were processed through graded alcohols and xylene and embedded in paraffin using standard histological methods.

Immunohistochemistry and immunofluorescence staining protocols

CTLA-4 PC IHC (all reagents are Ventana kit reagents, unless noted)

1. Standard Ventana deparaffinization protocol: 69°C, 8 min, x3
2. Antigen Retrieval, CC1: 96C, 64 min
3. Discovery enzyme inhibitor (H_2O_2): 36°C, 12 min
4. Primary antibody (CTLA-4 Gt PC, AF-386-PB): diluted in Ventana Reaction Buffer, 36°C, 16 min
5. Anti-goat OmniMap-HRP: 37°C, 8 min
6. Discovery AMP TSA HQ: 37°C, 4 min
7. Discovery anti-HQ HRP AMP Multimer: 37°C, 4 min
8. Discovery Purple substrate: 37°C, 20 min
9. Hematoxylin: 37°C, 12 min
10. Bluing Reagent: 37°C, 12 min

CTLA-4 mAb IHC (all reagents are Ventana kit reagents, unless noted)

1. Standard Ventana deparaffinization protocol
2. Antigen Retrieval, CC1: 96C, 64 min
3. Discovery enzyme inhibitor (H_2O_2): 37°C, 12 min
4. Primary antibody (CTLA-4 mouse mAb, BSB-88): diluted in Dako diluent (S3022), 37°C, 32 min
5. Anti-Mouse HQ: 37°C, 12 min
6. Anti-HQ HRP: 37°C, 16 min
7. Discovery Purple substrate: 37°C, 24min
8. Hematoxylin: 37°C, 8 min

9. Bluing Reagent: 37°C, 4 min

CTLA-4 /FoxP3 Duplex IHC (all reagents are Ventana kit reagents, unless noted)

1. Standard Ventana deparaffinization protocol: 69°C, 8 min, x3
2. Antigen Retrieval, CC1: 96°C, 64 min
3. Discovery enzyme inhibitor (H_2O_2): 37°C, 12 min
4. Primary antibody 1 (CTLA-4 mouse mAb, BSB-88): diluted in Dako diluent (S3022), 37°C, 32 min
5. Anti-Mouse HQ: 37°C, 16 min
6. Anti-HQ HRP: 37°C, 16 min
7. Discovery Purple Substrate: 37°C, 24 min
8. CC1, 95°C, 20 min
9. Primary antibody 2 (FoxP3 rabbit mAb, SP97): diluted in Dako diluent (S3022), 37°C, 28 min
10. Anti-Rabbit NP: 37°C, 8 min
11. Anti-NP AP: 37°C, 12 min
12. Discovery Yellow substrate: 37°C, 28 min
13. Hematoxylin: 37°C, 8 min
14. Bluing Reagent: 37°C, 4 min

FoxP3 IHC (all reagents are Ventana kit reagents, unless noted)

1. Standard Deparaffinization protocol
2. Antigen Retrieval, CC1: 96°C, 30 min
3. Primary Ab (FoxP3 rabbit mAb, SP97): 37°C, 32 min
4. Anti-Rabbit OmniMap HRP: 37°C, 8 min
5. Hematoxylin: 37°C, 16 min
6. Bluing Reagent 37°C, 16 min

CTLA-4/CD3 Immunofluorescence (all reagents are Leica Bond (LB) or Ventana Discovery (VD) kit reagents, unless noted)

1. Load slides on Leica Bond instrument
2. DeWax Step (LB)
3. Antigen Retrieval, ER2 (LB): 100°C, 20 min
4. Peroxidase Block (LB): RT, 8 min
5. Blocking solution - 2% Fish Skin Gelatin (Sigma), 2% normal goat serum, 0.5% Tween 20, 1% casein in PBS: RT, 15 min
6. Primary antibody 1 (CTLA-4 mouse mAb, BSB-88): diluted in Dako diluent (S3022), RT, 25 min
7. Post Primary antibody, rabbit α-mouse IgG (LB): RT, 15 min

8. Refine Polymer goat α-rabbit-HRP (LB): RT, 8 min
9. Alexa 488, dilute 1/350 1X Amp diluent (Invitrogen): RT, 8 min
10. Remove Slides from Bond RX instrument
11. Rinse in Ventana Reaction Buffer (VD), 5 min
12. Load slides on Discovery Ultra instrument
13. Antibody denature step (CC2 (VD)): 96°C, 24 min
14. Discovery Inhibitor (VD): 37°C, 8 min
15. Primary antibody 2 (CD3 rabbit mAb, SP162, Spring Bioscience): diluted in Dako diluent (S3022), 37°C, 28 min
16. Refine Polymer-HRP (VD): 37°C, 8 min
17. Alexa 594, dilute 1/300 in 1X Amp diluent (Invitrogen): 37°C, 8 min
18. DAPI (Invitrogen), 16 µl of 20 mg/ml, diluted in 200 ml H20
19. Coverslip using Prolong Gold Mounting Media (Thermo Fisher)

CTLA-4/CD4 Immunofluorescence (all reagents are Leica Bond (LB) or Ventana Discovery (VD) kit reagents, unless noted)

1. Load slides on Leica Bond instrument
2. DeWax Step (LB)
3. Antigen Retrieval, ER2 (LB): 100°C, 20 min
4. Peroxidase Block (LB): RT, 8 min
5. Blocking solution - 2% Fish Skin Gelatin (Sigma), 2% normal goat serum, 0.5% Tween 20, 1% casein in PBS: RT, 15 min
6. Primary antibody 1 (CTLA-4 mouse mAb, BSB-88): diluted in Dako diluent (S3022), RT, 25 min
7. Post Primary antibody, rabbit α-mouse IgG (LB): RT, 15 min
8. Refine Polymer-HRP (LB): RT, 8 min
9. Alexa 488, dilute 1/350 1X Amp diluent (Invitrogen): RT, 8 min
10. Remove Slides from Bond RX instrument
11. Rinse in Ventana Reaction Buffer (VD), 5 min
12. Load slides on Discovery Ultra instrument
13. Antibody denature step, CC1 (VD): 95°C, 20 min
14. Primary antibody 2 (CD4, SP35), 37°C, 20 min
15. Anti-Rabbit OmniMap HRP (VD): 37°C, 8 min
16. Red 610 (VD), 37°C, 4 min
17. DAPI (Invitrogen), 16 µl of 20 mg/ml, diluted in 200 ml H20
18. Coverslip using Prolong Gold Mounting Media (Thermo Fisher)

CTLA-4/CD8 Immunofluorescence (all reagents are Leica Bond, unless noted)

1. Load slides on Leica Bond instrument
2. DeWax Step

3. Antigen Retrieval, ER2: 100°C, 20 min
4. Peroxidase Block: RT, 8 min
5. Blocking solution - 2% Fish Skin Gelatin (Sigma), 2% normal goat serum, 0.5% Tween 20, 1% casein in PBS: RT, 15 min
6. Primary antibody 1 (CTLA-4 mouse mAb, BSB-88): diluted in Dako diluent (S3022), RT, 25 min
7. Post Primary antibody, rabbit α-mouse IgG: RT, 15 min
8. Refine Polymer-HRP: RT, 8 min
9. Alexa 488, dilute 1/350 in 1X Amp diluent (Invitrogen): RT, 8 min
10. Antibody Denature Step: ER1, 95°C, 25 min
11. Peroxidase Block: RT, 5 min
12. Blocking solution: RT, 10 min
13. Primary antibody #2: (CD8, SP239): diluted in Dako diluent (S3022), RT, 25 min
14. Refine Polymer-HRP: RT, 8 min
15. Alexa 594: dilute 1/400 in 1x Amp diluent (Invitrogen): RT, 8 min
16. Remove slides in d-water
17. DAPI (Invitrogen), 16 µl of 20 mg/ml, diluted in 200 ml H20
18. Coverslip using Prolong Gold Mounting Media (Thermo Fisher)

In situ hybridization

In situ hybridization was performed using Ventana automated staining with a probe (cat. no. 554349; Advanced Cell Diagnostics, Newark CA) that recognizes human CTLA-4 and was designed for automated Ventana staining. The assay conditions were the same for the hu-CTLA-4 probe as well as the negative control probe, dapB (cat # 312039). All RNAscope reagents were supplied by Advanced Cell Diagnostics and loaded into Ventana fillable dispensers in our lab prior to use.

1. Samples are dewaxed with Ventana Wash Buffer 60°C
2. Target Retrieval Solution (ACD): 97°C, 24 min
3. Red Inhibitor Solution (ACD): 37°C, 16 min
4. mRNA Protease solution (ACD): 37°C, 16 min
5. Target Probe (CTLA-4 or dapB): 43°C, 2 hrs
6. Amplicon reagents Amp 1 through AMP 4 (ACD): all @ 37°C; duration fixed by RNAscope protocol
7. Reagent AMP 5: 37°C, 24 min
8. Reagents AMP 6 and AMP 7: 37°C; duration fixed by RNAscope protocol
9. Red Substrate (ACD): Application fixed by RNAscope protocol
10. Hematoxylin: RT, 8 min
11. Bluing solution: RT, 4 min
12. Rinse, air dry, coverslip with permanent mounting media