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Evaluating Loss of STING/cGAS Expression Using a Novel Multiplex Immunohistochemistry Detection Approach

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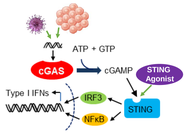
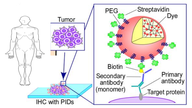
Introduction

The cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) pathway an important target in the expanding landscape of next-generation immunotherapies (1-3). STING agonists can induce systemic anti-tumor immunity and thus may be also synergistic with other immunotherapies (i.e. immune checkpoint blockade, cancer vaccines, and CAR-T cell therapy). Absence of cGAS-STING pathway regulates response to oncolytic virotherapy (2). Advanced stages of solid cancers (i.e. melanoma, colon adenocarcinoma) frequently show loss of STING/cGAS and would not be expected to respond to STING agonists (1, 3). There is an emerging need for predicting patient response which can accurately classify patients according to STING/cGAS expression.



STING signaling via cGAS or agonists activate innate immune response.



Schematic workflow of Quanticell™. The technique utilized regular IHC primary antibody, biotinylated secondary antibody, and detection using phosphor integrated fluorescent dots (PIDs). The assay offers a fully linear, quantitative assay that extended beyond detectable/saturated range of DAB. Adapted from (4).

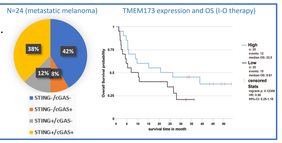
In this study, a novel immunohistochemistry (IHC)-based assay using fluorescent nanoparticles (Quanticell™) was tested for highly sensitive and quantitative analysis of STING and cGAS within the tumor contexture. The technique provides detection with high sensitivity (100-fold greater brightness than quantum dots enabling the detection of analytes not detected by conventional IHC methods), signal quantitation (300-fold greater linear dynamic range than IHC with no amplification steps to ensure robustness) with contextual visualization (pathologist validation at cellular and sub-cellular resolution) to allow for quantitative evaluation of gene products in clinical tissues.

Study Aims

- **Establish dual color Quanticell™ IHC assay by utilizing established melanoma cell lines, to measure STING/cGAS expression.**
- **Assess utility of Quanticell™ assay in biomarker evaluation of STING/cGAS in clinical melanoma samples.**
- **Compare Quanticell™ assay with chromogenic IHC to detect STING/cGAS expression in melanoma.**

Background

- In melanoma, STING pathway is deregulated at various nodes and also with disease progression (1, 2).
- High expression of TMEM173 predicts favorable prognosis of metastatic melanoma patients treated with monoclonal antibody against CTLA-4 (1).
- An accurate measurement of STING/cGAS in tumor cells is paramount for STING agonist therapy (cGAS$^+$STING$^+$, cGAS$^-$STING$^+$) and oncolytic virotherapy (cGAS$^+$STING$^-$).
- The requirement for non-DAB detection in melanoma (due to melanin) poses challenges to IHC.



Methods

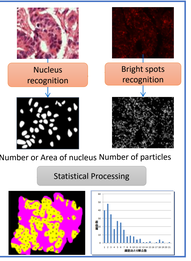
Clinical samples: Archived clinical melanoma blocks (FFPE, n=20) from primary tumor site (stage III or IV) were purchased (BioChain Institute Inc.). Sections (4 µm) were stained by chromogenic IHC and H&E (Invicro Boston) or Quanticell™ assay.

Chromogenic IHC: Dual-color IHC was performed sequentially using α-STING antibody (LSBio LS-C337801, 1:6,000 dilution) and α-cGAS (Abcam ab224144, 1:300 dilution) with Vina Green kit (Biocare BRR807AH, HRP) or Leica Red kit (DS9390, AP), respectively using antigen retrieval (pH 9, 10 min) and staining (Bond RX, Leica Biosystems).

IHC-PID: We utilized Quanticell™ (Ver 1.1) that offer 2-color PID staining and image analysis. Sections were stained with α-STING or α-cGAS antibody (control, no primary antibody), biotinylated secondary antibody, followed by pre-assembled PIDs (green or red, respectively). Slides were counter stained by hematoxylin. For each clinical sample, fluorescent images of both tumor region and internal "normal" skin were captured using DP80 camera (Olympus, 3-7 microscopic fields).

Image recognition algorithms: The number of PIDs on the digital image was calculated based on the light spot profile. The number of PID particles/ unit area (100 µm^2) was calculated for both fluorophores.

Data analysis: The IHC-PID data was visually compared to IHC-chromogenic data and Quanticell™ values provided.



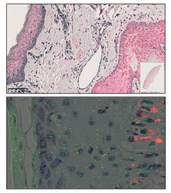
Results

Validation of dual-color Quanticell™ assay using established melanoma cell lines

	Quanticell Score (particles/cell ± SEM)			Immunoblotting*	
	STING	cGAS	Background	STING	cGAS
A375	39.0 ± 3.8	16.9 ± 0.5	**7.9 ± 0.4**	STING^{low}	cGAS^{-ve}
SK-MEL-28	127.9 ± 6.0	164.0 ± 8.1	**15.7 ± 0.3**	STING$^{-/+}$	cGAS^{low}

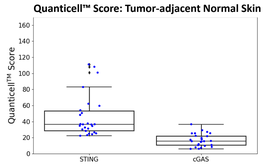
The Quanticell™ assay provides STING/cGAS scores for cell lines above the background signal, and performance was compared to cell lines with low protein expression of STING/cGAS as determined by Western blotting. *Adapted from (2)

STING/cGAS expression in control tissues for dual-color IHC or Quanticell™ assay

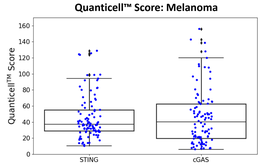


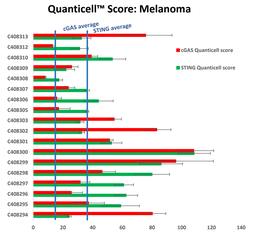
- A corresponding IHC assay was utilized to gain information on robustness of preanalytical or analytical phase testing (5). Dual color chromogenic IHC was developed using a normal skin control.
- IHC analysis of STING/cGAS in melanoma shows infiltration of immune and lympho-vascular cells, both cell types are cGAS$^+$STING$^+$ (1), as also shown by chromogenic IHC.
- In the Quanticell™ assay, staining performance and scores were calibrated against tumor adjacent skin, where cGAS and STING expression are present in discrete layers of the dermis.
- Quanticell™ showed STING expression in all 4 layers of the epidermis (stratum corneum, granulosum, spinosum and basale. However, cGAS expression is noted as localized to the stratum basale layer.

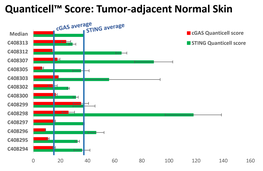
Clinical melanoma samples scored using the Quanticell™ assay for STING/cGAS expression

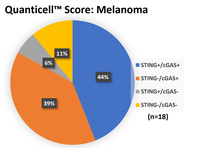


- The median Quanticell™ score for STING and cGAS in tumor-adjacent normal skin was 37.2 and 15.6, respectively.
- The median Quanticell™ score for STING and cGAS detection threshold for cell lines was 39.0 ± 3.8 and 16.9 ± 0.5, respectively (A375 cell line).
- 72% samples showed tumor adjacent skin that could also be scored for STING and cGAS expression.
- Based on the Quanticell™ score in internal control tissue, samples that showed altered expression for-
  - STING: n=5; C408312, C408307, C408303, C408298, C408296
  - cGAS: n=3; C408313, C408299, C408298





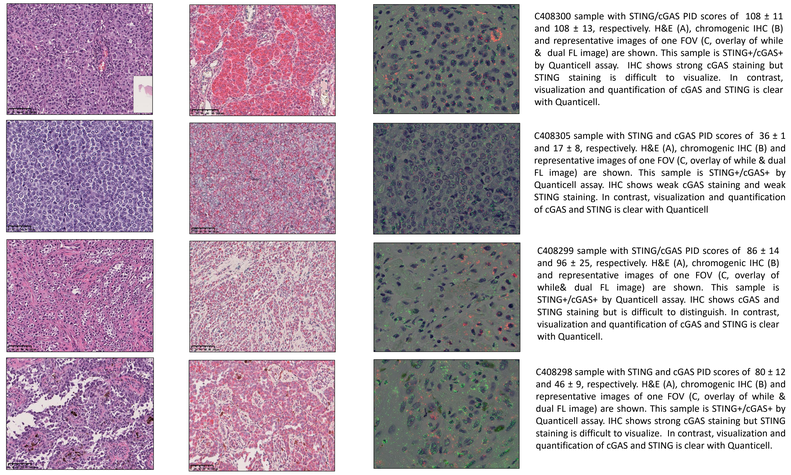




Quanticell™ assay results from tumor analysis show that:

- **50% of patients have below average STING expression and may not be candidates for STING agonists.**
- **17% patients have below average cGAS expression and may not be candidates for oncolytic virotherapy.**

Representative cGAS/STING Staining in Melanoma



C408300 sample with STING/cGAS PID scores of 108 ± 11 and 108 ± 13, respectively. H&E (A), chromogenic IHC (B) and representative images of one FOV (C, overlay of while & dual FL image) are shown. This sample is STING+/cGAS+ by Quanticell assay. IHC shows strong cGAS staining but STING staining is difficult to visualize. In contrast, visualization and quantification of cGAS and STING is clear with Quanticell.

C408305 sample with STING and cGAS PID scores of 36 ± 1 and 17 ± 8, respectively. H&E (A), chromogenic IHC (B) and representative images of one FOV (C, overlay of while & dual FL image) are shown. This sample is STING+/cGAS+ by Quanticell assay. IHC shows weak cGAS staining and weak STING staining. In contrast, visualization and quantification of cGAS and STING is clear with Quanticell

C408299 sample with STING/cGAS PID scores of 86 ± 14 and 96 ± 25, respectively. H&E (A), chromogenic IHC (B) and representative images of one FOV (C, overlay of while& dual FL image) are shown. This sample is STING+/cGAS+ by Quanticell assay. IHC shows cGAS and STING staining but is difficult to distinguish. In contrast, visualization and quantification of cGAS and STING is clear with Quanticell.

C408298 sample with STING and cGAS PID scores of 80 ± 12 and 46 ± 9, respectively. H&E (A), chromogenic IHC (B) and representative images of one FOV (C, overlay of while & dual FL image) are shown. This sample is STING+/cGAS+ by Quanticell assay. IHC shows strong cGAS staining but STING staining is difficult to visualize. In contrast, visualization and quantification of cGAS and STING is clear with Quanticell.

Conclusions

- STING/cGAS expression in the Grade III/IV melanoma samples analyzed varied widely. STING-IHC showed focal nuclear and cytoplasmic positivity in tumor-associated skin and endogenous immune cells as expected, while cGAS-IHC showed dense heterogeneous, cytoplasmic and nuclear staining in melanoma.
- The Quanticell™ assay for STING showed downregulation to below average in 50% of the samples; it is not clear if these patients will benefit from STING-agonist treatment compared to their counterparts.
- The Quanticell™ assay for cGAS showed downregulation to below average in 17% of the samples; it is not clear if these patients will benefit from oncolytic viral therapy compared to their counterparts.
- In contrast to IHC, the Quanticell™ assay enabled clear, simultaneous, and sensitive visualization and quantitative scoring of STING/cGAS expression, and may have value for predictive and prognostic application in STING agonist-based therapies and oncolytic viral therapy.

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