IHC and RT-PCR Assays for Detection of Cancer Antigen NY-ESO-1 in Human Tissues

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INTRODUCTION

NY-ESO-1 is a cancer testis antigen with documented expression in a broad range of tumors including lung, breast, ovarian, bladder, and liver cancers, melanoma, sarcoma, and myeloma. NY-ESO-1 prevalence reported in the literature varies and may be attributed to differences in methods or tissue quality between labs.

Celldex is developing an immunotherapy, CDX-1401, targeting NY-ESO-1 expressing cancers. CDX-1401 is a fusion protein composed of a fully human monoclonal antibody specific for a dendritic cell receptor (DEC-205) linked to full length NY-ESO-1 antigen. The direct targeting of NY-ESO-1 to dendritic cells has shown robust stimulation of NY-ESO-1-specific CD4 and CD8 responses in preclinical models and a Phase 1 clinical trial. To support further development of CDX-1401, we investigated diagnostic assays for determining NY-ESO-1 expression in tumor samples.

Immunohistochemistry (IHC) and quantitative RT-PCR (qRT-PCR) assays were developed to determine NY-ESO-1 expression in human tumors and normal adjacent tissues (NAT). A qRT-PCR assay to detect LAGE-1, a cancer testis antigen with significant DNA homology to NY-ESO-1, was also developed. An antibody to detect NY-ESO-1 by IHC was commercially available but no antibody was available that claimed to be LAGE-1 specific.

After development and validation, assays were used to survey mRNA message and antigen expression in tumor and NAT of different tissues types.

MATERIALS & METHODS

- FFPE tumor tissues and NAT were procured (Molecular MD).
- Two independent pathologists had discrepant views on tissue quality; Mosaic Labs offered additional tumor tissues to mitigate the foreseen low NY-ESO-1 positivity rate.
- Analysis of split samples for NY-ESO-1 by IHC (Mosaic), and for NY-ESO-1 and LAGE-1 by qRT-PCR (MolecularMD).
- Controls were 2 normal testis, 1-2 negative cell line(s) (SK-OV-3 +/- OV-CAR-3), and 1 positive cell line (HT-1080).

qRT-PCR Assays

- NY-ESO-1 and LAGE-1 kits (Life Technologies)
- Reference material was *in vitro* transcribed (IVT) RNA (IDT)
- mRNA housekeeping genes (HKG) were quantified with TaqMan kits (Life Technologies)

IHC Assays

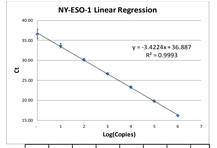
- NY-ESO-1 mouse mAb (Sigma); red chromogen (skin tissues); DAB chromogen (other tissue types).
- Assay performed on a DAKO Link Autostainer.

TOTAL NUMBER AND TISSUE TYPES

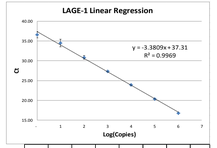
Sources (n=2)	Melanoma	Ovarian Carcinoma	Colorectal Cancer (CRC)	Head & Neck Squamous Cell Carcinoma (H&N SCC)	Non-Small Cell Lung Carcinoma (NSCLC)
Procured tumors	10	10	10	10	10
Tumors (Mosaic)	8	8	3	3	3
Total tumors (n=75)	18	18	13	13	13
Procured NAT (n=38)	8	5	10	7	8

qRT-PCR ASSAY DEVELOPMENT & VALIDATION

- Selection of HKG per tissue type:
  - Lung and ovary: MRPL19
  - Colon: RPL30
  - Skin, melanoma, and H&N SCC: IPO8, HMBS
- Assessment of the assay measurement range (AMR) with lower limit of quantitation (LOQ) and limit of detection (LOD):
  - Seven 10x dilutions (1-1x10^6 copies/reaction) for each IVT mRNA; analyzed in duplicates on 6 different runs
  - All Ct of ≥10 copies were <36.0
  - Assays were linear with LOQ = 100 copies/reaction and quantifiable transcripts in 100% of replicates; LOD = 1-10 copies/reaction and detectable transcripts in >50 - <100% of replicates



Replicates Detected	7/12	8/12	12/12	12/12	12/12	12/12	12/12



Replicates Detected	5/12	8/12	12/12	12/12	12/12	12/12	12/12

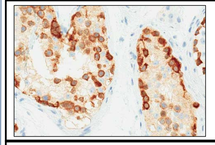
- Selectivity/specificity of NY-ESO-1 and LAGE-1 assays:
  - 1,000 copies NY-ESO-1 IVT RNA assayed in presence of 1x10^6 copies LAGE-1 IVT RNA in triplicate and vice-versa
  - Detection of NY-ESO-1 not affected by presence of high copies of LAGE-1 IVT RNA and vice-versa

Impact of LAGE-1 IVT RNA on NY-ESO-1 Assay			Impact of NY-ESO-1 IVT RNA on LAGE-1 Assay		
Replicate #	Without LAGE-1	With LAGE-1	Replicate #	Without NY-ESO-1	With NY-ESO-1
1	26.74	26.86	1	27.56	27.38
2	26.40	26.82	2	26.91	27.67
3	26.60	26.87	3	27.41	27.57
Average	26.58	26.85	Average	27.30	27.54
SD	0.17	0.02	SD	0.34	0.15
CV%	0.66	0.09	CV%	1.24	0.53

- Precision
  - Samples: 5 human tumors, 2 cell lines, 2 dilutions each NY-ESO-1 and LAGE-1 IVT RNA
  - 6 replicates on a single run; 6 independent RNA extractions on 6 different runs
  - In general, a high degree of agreement was observed: all Ct values <36.0; intra- and inter-assay SD values ≤0.50.

NY-ESO-1 IHC ASSAY DEVELOPMENT & VALIDATION

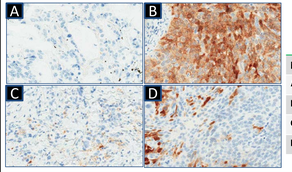
- Optimal antigen retrieval system and optimal antibody dilution
  - Selective binding verified with cell lines positive or negative by IHC or RT-PCR; normal testis tissue as positive control
- Intra-Run Precision: 3 replicates on a single run
  - DAB stain (4 samples)
    - Testis control, HT-1080 NSCLC cells, 2 positive NSCLC tissues
    - Single run: Average CV = 0% stained cells; H-score = 3.3%
  - Red stain (3 samples)
    - Testis control, HT-1080 NSCLC cells, 1 positive melanoma tissue
    - Average CV = 0% for stained cells; H-score = 1.9%
- Inter-Run Precision: 6 separate runs
  - Same samples as for intra-run precision
  - DAB stain: Average CV = 3.3% and H-Score = 17.4%
  - Red stain: Average CV = 8.9% and H-score = 17.8%



NORMAL TESTIS

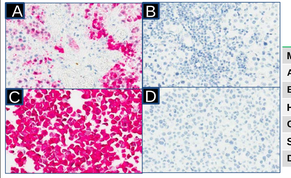
NY-ESO-1 IHC Stained Cells	NY-ESO-1 RT-PCR	LAGE-1 RT-PCR
85%	Positive	Positive

DAB IHC (20x) vs RT-PCR



	NY-ESO-1 Stained Cells	NY-ESO-1 RT-PCR	LAGE-1 RT-PCR
NSCLC			
A	0%	-	-
B	96%	+	+
C	1%	+	+
D	2%	+	-

Red Staining IHC (20x) vs RT-PCR



	NY-ESO-1 Stained Cells	NY-ESO-1 RT-PCR	LAGE-1 RT-PCR
Melanoma			
A	12%	+	+
B	0%	-	+
HT-1080 Cell Line			
C	100%	N/A	N/A
SK-OV-3 Cell Line			
D	0%	-	-

Cut-offs for Positivity:

- IHC staining was quite clean (no background noise); staining ≥1% epithelial cells considered positive
- Any RT-PCR signal Ct<36.0 considered positive

RESULTS

NY-ESO-1 and LAGE-1 Positivity

- 9/75 (12%) tumors but 0/38 (0%) NAT were positive for NY-ESO-1 (IHC). Cytoplasmic staining only.
- 10/75 (13.3%) tumors but 0/38 (0%) NAT were positive for NY-ESO-1 (RT-PCR).
- If PCR and/or IHC is considered, 11/75 (14.7%) tumor tissues would be considered positive for NY-ESO-1.
- Rate of concordance between NY-ESO-1 by IHC and by RT-PCR: 97.3%; only 1 sample positive by IHC and negative by PCR; 2 samples positive by PCR and negative by IHC.
- LAGE-1 by PCR: positive in 10/75 (13.3%) tumors and 1/38 (2.6%) NAT.
- NY-ESO-1 and LAGE-1: co-detected in 6/113 (5.3%) samples while the RNA were individually detected in 4/113 (3.5%) and 5/113 (4.4%) respectively.
- NY-ESO-1 by IHC: more prevalent in NSCLC (38%), ovarian cancer (11%) and melanoma (11%) vs. CRC and H&N.
- In general, Mosaic tumor tissues were better quality per pathologist, and significantly more positive than commercially procured samples.

POSITIVITY: TUMOR vs. NORMAL ADJACENT TISSUES

Tissue	NY-ESO-1 RT-PCR	LAGE-1 RT-PCR	NY-ESO-1 IHC	# SAMPLES
Tumor	+	+	+	6
	-	-	-	60
	+	-	-	2
	-	+	-	4
	-	-	+	1
	+	-	+	2
NAT	-	-	-	37
	-	+	-	1

POSITIVITY ACROSS TUMOR TYPES

	All	IHC	NY-ESO-1	LAGE-1
All	Procured samples (n=50)	2 (4%)	4 (8%)	5 (10%)
	Mosaic's (n=25)	7 (28%)	6 (24%)	6 (24%)
	Total	9 (12%)	10 (13.3%)	11 (14.7%)
NSCLC	Procured samples (n=10)	2	2	4
	Mosaic's (n=3)	3	2	2
	Total (n=13)	5 (38.5%)	4 (30.8%)	6 (46.2%)
Melanoma	Procured samples (n=10)	0	1	0
	Mosaic's (n=8)	2	2	3
	Total (n=18)	2 (11.1%)	3 (16.7%)	3 (16.7%)
Ovarian Carcinoma	Procured samples (n=10)	0	0	0
	Mosaic's (n=8)	2	2	1
	Total (n=18)	2 (11.1%)	2 (11.1%)	1 (5.6%)
CRC	Procured samples (n=10)	0	0	1
	Mosaic's (n=3)	0	0	0
	Total (n=13)	0 (0%)	0 (0%)	1 (7.7%)
H&N SCC	Procured samples (n=10)	0	1	0
	Mosaic's (n=3)	0	0	0
	Total (n=13)	0 (0%)	1 (7.7%)	0 (0.0%)

RATE OF CONCORDANCE

		NY-ESO-1 IHC +	NY-ESO-1 IHC -
NY-ESO-1 RT-PCR	+	8	2
	-	1	102

		LAGE-1 RT-PCR +	LAGE-1 RT-PCR -
NY-ESO-1 RT-PCR	+	6	5
	-	4	98

CONCLUSIONS

- Both IHC and RT-PCR assays are validated for clinical trials.
- RT-PCR assay designed to detect both NY-ESO-1 and LAGE-1.
- IHC assay specific for NY-ESO-1, does not cross-react with LAGE-1.
- High correlation between IHC and RT-PCR, few discrepant cases.
- This study provides evidence that poor (dry, under-fixed, difficult to section) or even unequivocal tissue quality impacts biomarker data and may lead to false decisions in clinical trials.
- Additional testing with good quality specimens may further verify the % positivity in the tumor types examined.
- Considering the pros and cons of each platform, implementing these assays, in particular IHC, in clinical trials may assist in prospectively identifying patients who could derive benefit from NY-ESO-1 vaccines, such as CDX-1401.

PROS AND CONS

	RT-PCR	IHC
Analysis	mRNA (may not represent protein levels)	Protein (target)
Robustness	In general, TaqMan-based PCR can be more objective than IHC	In this case, signal from IHC was more conclusive due to clean background
Cost	~2x > IHC; more if micro/macro dissection is needed	Direct staining of tissue section is less laborious and more cost effective
Minimum Specimen Requirements	5-8 slides with good amount of tissue and ~50% tumor content	2 slides with ≥100 cells
Turnaround Time	5-7 days	2-3 days