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Renewable IHC Control Tool CytoSections™ For Defining MAGEA3, MAGEA4, And MAGEA9 Antibody Specificity

Eden Zewdu[1], Rachel Gonzalez[1], Tianli Qu[1], Xiaomin Hu[2], Qi Ren[2], Zhaoying Guo[1],Yan Ma[1], Derek Ling[1], Ranran Zhang[2] , Xuan Liu[1], Wei Fu[1]

1) OriGene Technologies Inc.; 9620 Medical Center Drive, Suite 201, Rockville MD 20850

2) OriGene Wuxi Biotechnology Co., Ltd. No.168, Meiliang Road, Binhu District Wuxi, Jiangsu

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Abstract

Melanoma-associated antigen gene A (MAGEA) family proteins are expressed in a variety of tumors with each MAGEA protein having unique roles in cancer pathogenesis. The MAGEA members lack of expression in normal tissue and their role in cancer make them well suited for targeted cancer immunotherapy. Thus, knowing which MAGEA protein expression exists in a tumor is important. However, the MAGEA family shares sequence similarity that makes it difficult to find antibodies that are specific to just one family member. It is also challenging to source tissue generally with HIPAA regulation and even more so to get tissue representation of all 12 MAGEA family members. Here we present CytoSections as an alternative to patient control tissues. CytoSections are formalin fixed, paraffin embedded (FFPE) section of over-expression cell pellets. These cells were transfected with expression plasmids coding for genes of individual MAGES family. In this study, CytoSections are used to screen antibodies to all 12-member MAGEA family of proteins. The MAGEA family members were initially shown positive by targeted protein expression in western and immunohistochemistry using DDK tag antibody. Then multiple MAGEA3, MAGEA4, MAGEA9 antibodies were assessed by IHC to determine their specificity to their intended target. Using MAGEA family as a pilot project, we show that CytoSections is a verified, reproducible, and renewable alternative to human control tissues and serves as an ideal tool for antibody specificity assessment.

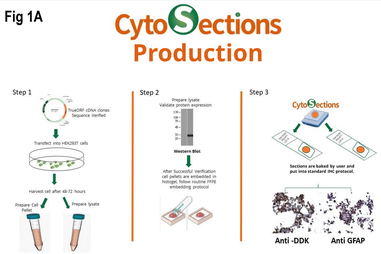
Introduction

Immunohistochemistry (IHC) assays are extensively used to localize specific epitopes in cells and tissues that enables cancer type classifications, prognostic predictions, or disease microenvironment characterizations. Appropriate positive and negative controls are critical for valid interpretation of these assays. The specificity of the antibody is influenced by the uniqueness of the target epitope, which is restricted to a five to six amino acid region of the protein. Determining specificity is challenging for genes in the same family, which have significant overlapping sequences. For example, the melanoma associated antigen (MAGE-A) family is composed of 12 plus members that encode proteins with 50 to 80% sequence similarity. MAGE-A family has a growing interest as a biomarker for cancer and a target of immunotherapy because of its extensive expression in cancer but limited expression in normal tissues. Not all MAGE-A family members are expressed in cancer thus knowing which MAGE-A protein is being expressed is important. It is crucial to know the specificity of the MAGE-A antibody to its target since there is so much sequence overlap with its family members.

Here we introduce how CytoSections function as a new control tissue source for IHC assay optimization. The steps for development of new targets of CytoSections are illustrated in the cartoon of Figure 1. Using CytoSections we can accelerate the screening of MAGE-A3, MAGE-A4, and MAGE-A9 antibodies against the MAGE-A family members. We incorporate negative control tissues which do not express the target or other similar family members to decipher between background and positive signal. Our study shows which antibodies are specific and which cross react to other proteins

Design & Methods

Fig 1A



Immunocytochemistry

Manual IHC staining of paraffin-embedded CytoSections using anti MAGEA3, 4, or 9 antibodies Table1 All antibodies required heat induced epitope retrieval HIER using OriGene-Citrate pH6.0 buffer for all MAGEA antibodies. OriGene's Polink-1 a one-step anti- mouse polymer HRP detection (Cat# D12-100) and DAB chromogen was used according to manufacturer's protocol. Scoring was based on the percentage of positive cells and not the intensity.

Table 1 MAGEA-3, 4, & 9 Antibodies

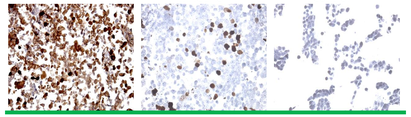
MAGEA3	MAGEA3	MAGEA4	MAGEA4	MAGEA9	MAGEA9
Ab Clone #	Ab SKU #	Ab Clone #	Ab SKU #	Ab Clone #	Ab SKU #
OTI1H1	TA800826	OTI1F9	TA505362	OTI1D8	TA800839
OTI1G9	TA800804	OTI2C1	TA505361	OTI1C6	TA800855
OTIF210	TA800802	OTI5E8	TA505423	OTI1A11	TA800909
OTI1A9	TA800828	OTI1F12	TA505396		

Western & Dot Blot

Overexpression cells were lysed with RIPA++ buffer, the concentration was measured using BCA assay and 5ug of each sample was loaded on 4-12% Bis- Tris gel. Anti-DDK mouse monoclonal antibody was used at 1:3000 concentration as primary.
https://www.origene.com/catalog/antibodies/tag-antibodies/ta50011-100/clone-oti4c5-anti-ddk-flag-monoclonal-antibody
HRP conjugated goat- anti mouse was used at 1:4000 concentration for secondary antibody.
https://www.origene.com/catalog/antibodies/secondary-antibodies/ta130001/goat-anti-mouse-igg-secondary-antibody
MAGEA3, MAGEA4, and MAGEA9 antibodies that tested using dot blot analysis to determine cross reactivity with the other MAGEA family members.

Results

Fig 2A DDK ON MAGEA Family Exhibit 2 Types of Staining



Figa2B IHC with DDK MAGEA 3, MAGEA4, AND MAGEA9 ANTIBODIES

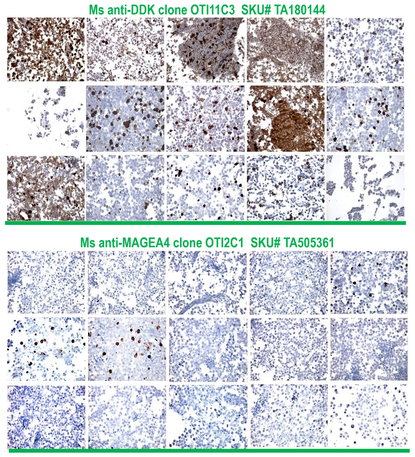


Table 3 MAGEA Family Member 1-12 CytoSections Images Map

MAGEA1-12 CytoSection Map				
MAGE-A1 TS402134	MAGE-A2 TS423561	MAGE-A3 TS403288	MAGE-A4v1 TS418952	MAGE-4v2 TS423938
MAGE-A4v3 TS404482	MAGE-4v4 TS423561	MAGE-A5 TS418575	MAGE-A6 TS423578	MAGE-A8 TS429878
MAGE-A9 TS401760	MAGE-A10 TS402501	MAGE-A11 TS402471	MAGE-A12 TS429868	HEK293T CONTROL

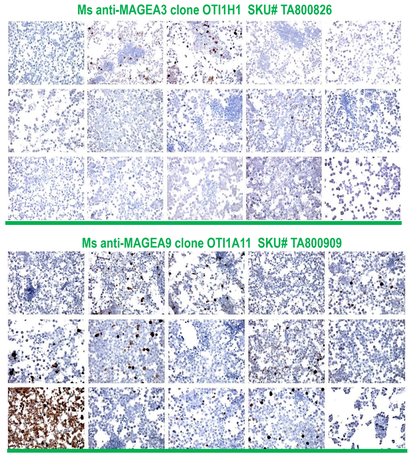
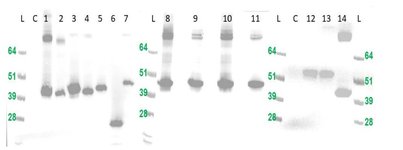


Table 4 MAGEA-3, 4, & 9 Antibodies Detection Pattern on MAGEA Family Member 1-12 CytoSections

Antibody sku #	OTI11C3	OTI1H1	OTI1G9	OTI2F10	OTI1A9	OTI1F9	OTI2C1	OTI5E8	OTI1F12	OTI1D8	OTI1C6	OTI1A11
AntibodyTarget	DDK -	MAGE-A3	MAGE-A3	MAGE-A3	MAGE-A3	MAGE-A4	MAGE-A4	MAGE-A4	MAGE-A4	MAGE-A9	MAGE-A9	MAGE-A9
CytoSection	Dilution = 1:600	Dilution = 1:10000	Dilution = 1:2000	Dilution = 1:2000	Dilution = 1:2000	Dilution = 1:1000	Dilution = 1:10000	Dilution = 1:1000	Dilution = 1:1000	Dilution = 1:10000	Dilution = 1:2000	Dilution = 1:5000
MAGE-A1	95	0	0	5	0	95	0	95	0	0	0	0
MAGE-A2	95	5	0	95	5	0	0	0	0	0	0	10
MAGE-A3	100	10	100	95	95	0	0	95	95	0	0.1	10
MAGE-A4 v1	100	0	0	95	0	95	0.1	95	95	10	0.1	10
MAGE-A4 v2	10	0	0	95	0	10	10	10	10	10	10	10
MAGE-A4 v3	10	0.01	0	na	0	10	10	10	10	10	10	10
MAGE-A4 v4	10	0	0	10	0	10	10	10	10	10	10	10
MAGE-A5	10	0	0	95	0	0	0	90	10	0.1	0.1	0
MAGE-A6	100	10	10	95	10	0	0	95	10	0	0	0
MAGE-A8	10	0	0	0	0	10	0	10	10	0.1	0	0
MAGE-A9	10	0	0.1	0	0	5	0	0	0	95	10	95
MAGE-A10	10	0	0	0	0	5	0	0	0	0	0.1	0.1
MAGE-A11	10	0.01	0	0.1	0	0	0	5	5	5	5	1
MAGE-A12	10	0	10	3	0	0.1	0	5	5	0	1	10
NEG CONTROL	0	0	0	0	0	0	0	0	0	0	0	0

Fig 3A Western Anti DDK 1:3000 on 10ug of MAGEA1-12 Lysate



Lane #	TARGET	RC CLONE	CytoSection #	MW	Lane #	TARGET	RC CLONE	CytoSection #	MW
1	MAGE-A1	RC202134	TS402134	34.3 kDa	9	MAGE-A4 V2	RC223938	TS423938	34.7 kDa
2	MAGE-A2	RC223561	TS423561	35.1 kDa	10	MAGE-A4 V3	RC204482	TS404482	34.9 kDa
3	MAGE-A3	RC203288	TS403288	34.9 kDa	11	MAGE-A4 V4	RC223991	TS423991	34.9 kDa
4	MAGE-A8	RC229878	TS429878	35.7 kDa	12	MAGE-A10	RC202501	TS402501	40.8 kDa
5	MAGE-A6	RC223578	TS423578	34.9 kDa	13	MAGE-A11	RC202471	TS402471	40.1 kDa
6	MAGE-A5	RC218575	TS418575	13 kDa	14	MAGE-12 V2	RC229867	TS429867	35.3 kDa
7	MAGE-A9	RC201760	TS401760	35.1 kDa	L	Ladder	NA	NA	NA
8	MAGE-A1 V1	RC218952	TS418952	34.7 kDa	C	HEK293T	CONTROL	CONTROL	NA

Fig 3B Dot Blot MAGEA Ab 1:2000 on MAGEA1-12 Lysate

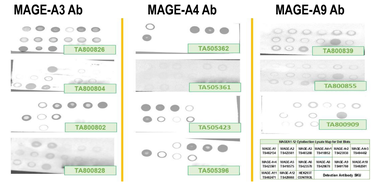


Fig 3C Dot Blot MAGEA Ab 1:10000 on MAGEA1-12 Lysate

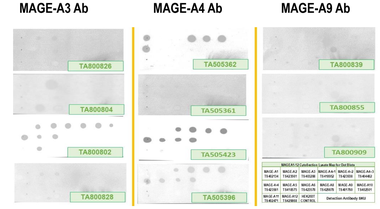


Fig 4 MAGEA1-12 Sequence Alignment

Conclusion

In this study, we show that screening multiple antibodies against all the member of the MAGEA family can be done expeditiously. We showed how easily we can incorporate negative control tissues which do not express the target or other similar family members to decipher between background and positive signal. MAGEA3, MAGEA4, and MAGEA9 antibodies that tested using dot blot analysis did not match the ICC/IHC results showing that with overlapping MAGEA family members CytoSections may be a better tool.

Trying to do this using traditional method of sourcing of accurate tissue for these studies is hindered by HIPPAA restrictions and tissue availability. Our study shows how CytoSections can reduce the time required to find the right tissue and mitigate the use of rare and less stable FFPE tissues.