MAGEA3 expression and secretion in MMR-MSH6 and MMR-PMS2 microsatellite-stable colorectal cancer

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Abstract

Despite the breakthrough of PD-1/PD-L1 blockade therapy in the recent years, mixed responses have been shown across multiple tumor types, including in colorectal cancer (CRC), the second leading cause of death worldwide. This immunotherapy has proven to be successful for a subtype of CRC that carries mismatch repair deficiency (dMMR) and high microsatellite instability (MSI-H), despite a lack of response by microsatellite-stable CRC. For these types of non-responsive cancers, new immunotherapy targets are currently being investigated for alternative therapy such as the MAGE-A cancer testis antigen family whose expression is restricted to germline cells in normal tissue but is overexpressed in many cancer cells. In this study, we examine the relationship between MAGEA3 and MMR proteins MSH6 and PMS2. Immunohistochemical staining of CRC tissues demonstrated co-expression between MAGEA3 with both MSH6 and PMS2. Immune cells were observed to express MAGEA3 in CRC as well. To investigate the possibility of secreted MAGEA3 from tumor cells, we performed western blot analysis of CRC cell lines, ELISA, and binding assay on cell lines and the positive results suggest that MAGEA3 protein is secreted. The results suggest that MAGE-A3 may be a promising target for microsatellite-stable colorectal cancer immunotherapy.

Introduction

Recent reports have shown that colorectal cancer with mismatch repair (MMR) mutations can benefit from PD-1/PD-L1 blockade therapy. The four main mismatch repair proteins (MSH2, MSH6, PMS2, MLH1) play an essential role in maintaining genome integrity during DNA replication by repairing mismatched DNA bases. Deficient MMR (dMMR) will lead to carcinogenetic pathway called microsatellite instability (MSI) due to DNA polymerase slippage and is defined by a lack of detectable MMR expression by immunohistochemistry. Despite favorable results with immunotherapy in patients with MSI/dMMR tumor, the 85% of colorectal cancer patients without dMMR did not have much success with PD-1/PD-L1 blockade therapy.

Here we look at Melanoma-associated antigen gene A (MAGEA3) as a potential target for MSH6 and PMS2 microsatellite-stable tumors. Here we use a highly specific MAGEA3 antibody, found using CytoSections (Figure 1) despite 60% sequence homology in the MAGEA family, to show the overlap of MAGEA3 and MSH6/PMS2 expression in 22 colon cancers. We then perform western blot, sandwich ELISA, and flow cytometry to find the source of MAGEA3.

Design & Methods

Immunocytochemistry

Manual IHC staining of paraffin-embedded colon cancer tissues using anti MAGEA3, PMS2, and MSH6 antibodies (Figure 2). All antibodies required heat induced epitope retrieval HIER using OriGene-Citrate buffer (**Cat# B05C**). Anti-MAGEA3 (**Cat# TA800804)** and anti-PMS2 (Abcam Cat# ab110638) was incubated overnight at 1:500 in 4C and anti-MSH6 (**Cat# UM800148)** was incubated for at 1:100 in 2h RT. OriGene Double Staining Kit for 2 mouse primary antibodies on human tissue (**Cat# DS203A**) and mouse and rabbit antibodies on human tissue (**Cat# DS201A**) was used according to manufacturer's protocol. Tissues were sourced from OriGene Technology's tissue collection.

Western Blot

MAGEA3 (**Cat# TA800804**; 1:50), MSH6 (**Cat# TA506683;** 1:25), PMS2 (**Cat# TA506683;** 1:25), and Actin (**Cat# TA811000;** 1:2000) specific antibodies were incubated overnight in 4C to detect signal from the lysates run on SDS-PAGE.

ELISA

MAGEA3 sandwich ELISA (**Cat# EA200011**) was used to measure MAGEA3 levels in cell culture supernatants and serum samples from 22 colon cancer patients and 30 non-cancer patient controls (BioVT). Data was analyzed using Student's t-Test.

Flow Cytometry

KM12 and SW620 was cultured in DMEM with 10% FBS. At 50% confluency, either 1:40 or 1:200 dilution of 7.14uM of proteins (MAGEA3 **Cat# TP303288**; B-actin **Cat# TP303643**) with DDK tag were added. After 24h, the cells were harvested through gentle scraping, and fixed with formalin for 2h. The cells were then stained with AF488 conjugated anti-DDK and ran on BD Accuri™ C6 Plus.

Conclusion

- MAGEA3 and MSH6 or PMS2 is co-expressed in many of the tumor epithelial cells, with MAGEA3 being expressed in the cytoplasm of the cell that is positive for MSH6 or PMS2 in the nucleus
- Often, the areas of the tumor that do not express MSH6 were also MAGEA3 negative
- Infiltrating immune cells near tumor were positive for MAGEA3, even though these are not tumor cells
- MAGEA3 is secreted, as shown by MAGEA3 positive cells adjacent to tumor and ELISA data with MAGEA3 overexpressed tissue culture supernatant and serum samples from colon cancer patients

Results

Fig 1 What are CytoSections and the benefits of using a reference slide

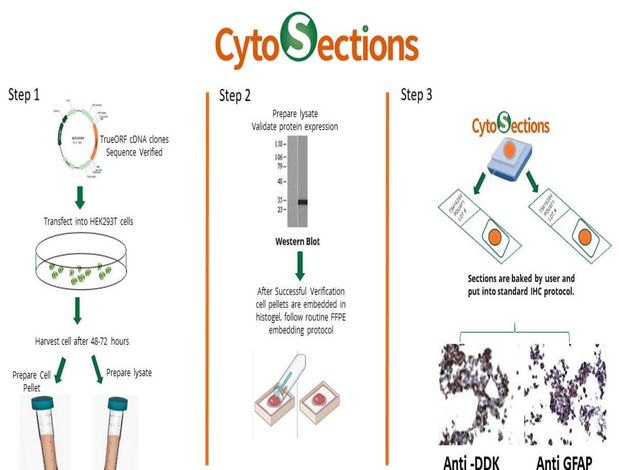


Fig 2 Colon cancer double staining with DS203A kit using two mouse primary antibodies and DS201A kit using a mouse and rabbit primary antibodies

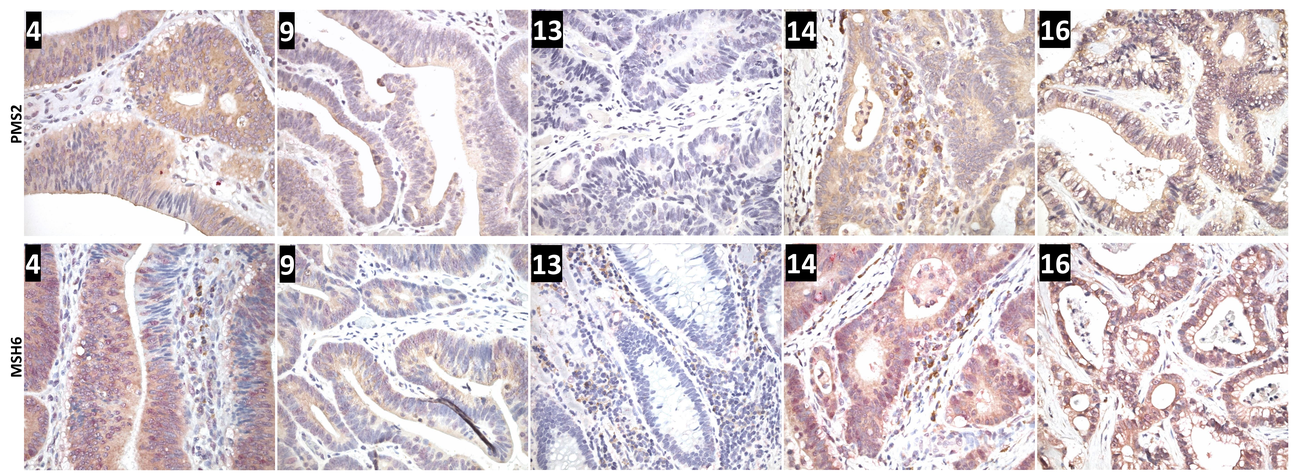


Table 1 IHC double stain of PMS2 or MSH6 with MAGEA3 Scoring shows correlation between MAGEA3 and MSH6 staining patterns

Colon Cancer Tissue	MAGEA3 (DAB)	MSH6 (AP) Loss	PMS2 (AP) Loss	MAGEA3 Immune Cells (DAB)
1	70% Positive	60% positive (40% loss)	90% positive (10% loss)	positive
2	35% positive	30% positive (70% loss)	60% positive (40% loss)	positive
3	70% positive	70% positive (30% loss)	50% positive (50% loss)	positive
4	80% positive	50% positive (50% loss)	80% positive (20% loss)	positive
5	70% positive	60% positive (40% loss)	90% positive (10% loss)	positive
6	90% positive	20% positive (80% loss)	90% positive (10% loss)	positive
7	70% positive	20% positive (80% loss)	50% positive (50% loss)	positive
8	80% positive	50% positive (50% loss)	50% positive (50% loss)	positive
9	80% positive	80% positive (20% loss)	80% positive (20% loss)	positive
10	90% positive	50% positive (50% loss)	5% positive (95% loss)	positive
11	20% positive	70% positive (30% loss)	80% positive (20% loss)	positive
12	negative	5% positive (95% loss)	30% positive (70% loss)	negative
13	negative	20% positive (80% loss)	0% positive (100% loss)	positive
14	90% positive	60% positive (40% loss)	0% positive (100% loss)	positive
15	90% positive	80% positive (20% loss)	60% positive (40% loss)	negative
16	90% positive	90% positive (10% loss)	70% positive (30% loss)	positive
17	30% positive	40% positive (60% loss)	30% positive (70% loss)	positive
18	100% positive	50% positive (50% loss)	60% positive (40% loss)	positive
19	100% positive	80% positive (20% loss)	90% positive (10% loss)	positive
20	5% positive	0% positive (100% loss)	5% positive (95% loss)	positive
21	20% positive	80% positive (20% loss)	80% positive (20% loss)	positive
22	80% positive	80% positive (20% loss)	95% positive (5% loss)	positive

Table 2. Increasing MAGEA3 levels in culture supernatant from cells overexpressing MAGEA3 protein suggests that MAGEA3 is being secreted

Sample	MAGE-A3 (pg/mL)
Culture Supernatant 12hrs	ND
Culture Supernatant 24hrs	25
Culture Supernatant 48hrs	570

Table 3. Serum MAGE-A3 levels is elevated in colon cancer patients compared against controls

Sample Type	Sample No.	Serum MAGEA3				Studen'ts t-test
		Range	Median	Mean	SD	p value
Colon Cancer	19	37-1030 pg/mL	210	298	234	<0.05 ‡
Control	30	23-308 pg/mL	127	140	66	

‡: p<0.05 compare to Control group

Fig 3 Western Blot of 3 colorectal cell lines and their expression of MMR proteins and MAGEA3

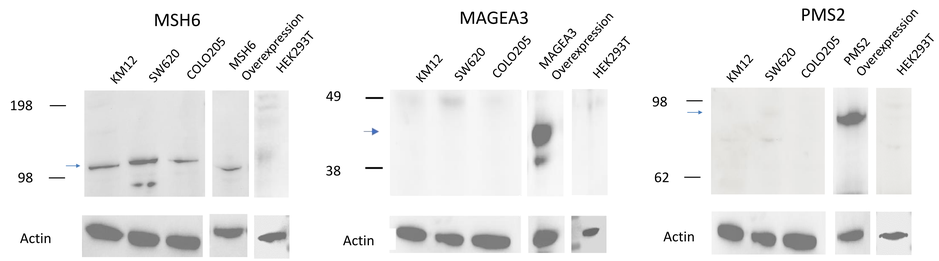


Fig 4 Gating Strategy for 24h incubation of colorectal cancer cells with MAGEA3 protein

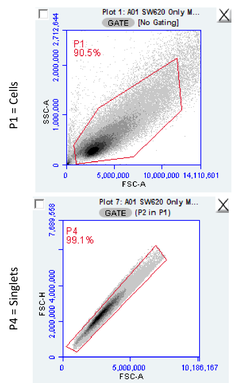
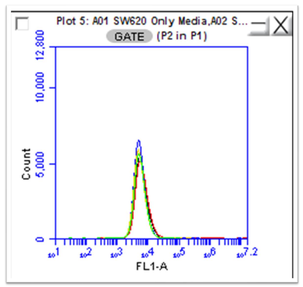


Fig 5 Flow cytometry results show that no significant binding activity has occurred between KM12 cells and MAGEA3 protein after 24h of incubation



Protocol:
Day 0: Seed cells
Day 1: 50% confluency. Add proteins
Day 2: Replace media with cold 1x PBS and gently scrape cells. Fix with 10% PFA for 2h

*KM12 cells clumped up and did not run properly. Need to repeat.

- A01 SW620 Only Media
- A02 SW620 MAGEA3 1:40
- A03 SW620 MAGEA3 1:200
- A04 SW620 Bactin 1:40
- A05 SW620 Bactin 1:200

Plot 5: Multiple Samples Gated on (P2 in P1)	Count	Volume (µL)	% of This Plot	% of All	Mean FL1-A	CV FL1-A	Median FL1-A
A01 SW620 Only Media : T...	66,613	139.4	100.00%	66.61%	6,547.52	65.64%	5,682.0
A02 SW620 MAGEA3 1:40 :...	66,710	54.4	100.00%	66.71%	8,225.85	1,142.75%	6,021.0
A03 SW620 MAGEA3 1:200...	74,931	50.2	100.00%	74.93%	5,961.67	605.28%	5,127.0
A04 SW620 Bactin 1:40 : T...	67,805	67.2	100.00%	67.80%	5,746.79	55.36%	5,070.0
A05 SW620 Bactin 1:200 : ...	64,605	58.7	100.00%	64.60%	5,958.40	48.72%	5,312.0