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Potential role of 5-aza-2'-deoxycytidine induced MAGE-A4 expression in immunotherapy for anaplastic thyroid cancer

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Abstract

Background—MAGEA4, a member of the cancer testis antigen (CTA) family has been reported in various cancers including melanoma, bladder, head and neck, oral, lung, and is a potential target for T cell receptor (TCR) based immunotherapy. Baseline expression levels of the MAGEA4 gene in thyroid cancer cell lines have not been previously thoroughly studied.

Method—Human thyroid cancer cell lines (8505c, HTh7, BCPAP and TPC-1) were treated with either 10 μ M 5'-azacytidine (Aza), 10 μ M 5-aza-2'-deoxycytidine (DAC) and evaluated for various MAGEA gene expression. Later melanoma cell line, A375 and 8505c were treated with PLX4720 in combination with DAC and evaluated for MAGEA4 expression.

Results—Only BCPAP cells expressed moderate levels of MAGEA3 and A6 at baseline. Treatment with DAC/ Aza induced the expression of MAGEA4 and A1 in 8505c cells. PLX4720 treatment did not affect MAGEA4 expression in 8505c cells but increased its expression in A375 cells. However, addition of PLX4720 to DAC treated 8505c cells decreased the previously induced MAGEA4 expression by DAC in these cells. Similar dampening of MAGEA4 expression by DAC was also seen in 8505cBRAF^{-/-} cells. While DAC treatment resulted in demethylation of the MAGEA4 promoter in two CpG sites, PLX addition to DAC did not affect the demethylation status.

Conclusion—Demethylating agents increased the MAGE's expression in thyroid cancer cells. The effect of BRAFV600E inhibitors on MAGEA4 expression suggest the role of downstream MEK/BRAF signaling in its expression apart from promoter demethylation being the sole requirement. Expression of MAGEA4 may make immunotherapeutic intervention possible in selected thyroid cancer patients.

Keywords

Cancer-testis antigens; MAGE; thyroid cancer; melanoma; demethylation agents; BRAF^{V600E} inhibitor

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Introduction

Thyroid cancer incidence is increasing dramatically in United States. While most do well, a subset of patients with anaplastic thyroid cancer (ATC), present with large invasive tumors and metastases that do not respond to treatment. Novel therapies are being intensely investigated, with a focus on targeting mutated pathways. (1). Both papillary thyroid cancers (PTCs) and ATCs show a high incidence of V600E mutation in the BRAF oncoprotein with elevated activity in the MAPKinase/ ERK pathway (2). Similarly BRAFV600E mutation is present in melanoma and colon cancer and leads to increased aggressiveness to some extent. Targeted therapies directed towards mutated BRAFV600E and MAPKinase pathway is dramatically effective in melanoma and is currently in phase II trials in metastatic thyroid cancer (3). Given limited success in using immune therapy for melanoma and the demonstrated role of mutated BRAF in modulating and masking immune surface antigens on melanoma cells (4-6), we wanted to see if antigens commonly used for cancer immune therapy could be demonstrated on the surface of thyroid cancer cells and whether their manipulation might lead to alternative novel immunotherapeutic interventions.

Cancer-testis antigens (CT antigens) are attractive targets for cancer immunotherapy. These antigens are expressed in various malignancies, but not in normal tissues with the exception of testis and placenta. Melanoma antigen gene family A (MAGE-A) is a CT antigen with 12 closely related genes (MAGE-A1 to MAGE-A12) located on chromosome-X (7). These antigens and their epitope peptides constitute important targets for antitumor immunotherapy with a number of clinical studies already completed or underway (8-10). Of the MAGE-A family, MAGE-A4 is expressed at differing levels at baseline in certain malignancies including melanoma, lung cancer, ovarian cancer, colorectal cancer, head and neck cancer, sarcoma, esophageal cancer, and urothelial carcinoma (11). Since recent studies have identified novel HLA-A*0201-restricted CTL epitopes derived from either MAGE-A4 or native peptides that can elicit cytotoxic CTL response against MAGE-A4 expressing tumor cells (12-14), we were interested in whether thyroid cancer cells express these potentially useful antigens at baseline. Furthermore, low expression of the MAGE genes has been primarily attributed to hypermethylation of the promoter regions (15), and treatment with demethylating agents induced expression of these genes in tumor cell lines. (16, 17). Enhanced expression of these antigens on the surface of malignant cells might lead to a more effective “kill” of the tumor cells by cytotoxic T cell receptor based immunotherapeutics.

So far expression of CTAs in thyroid malignancy has only been verified by immunohistochemical analysis in thyroid cancer tissue samples. Earlier studies showed MAGE-A3 expression in 65% (18) and MAGE-A1 in 30% of thyroid tumors (19), both of them predominantly in PTC. NYESO-1, also a member of CT antigens, was expressed in 7.5% of medullary thyroid cancer tissues (20). In a similar study by Melo et al, 2011, none of the thyroid tumor samples analyzed showed any expression of MAGE-A4 or MAGE-C1 antigens (21). However, neither baseline expression levels =nor whether use of demethylating agents can enhance expression of CTAs has yet been addressed in thyroid cancer cell lines. Therefore, in this study we analyzed the expression profile of various CTAs in multiple thyroid cancer cell lines to evaluate whether these antigens might someday prove useful for generating specific antitumor vaccines for the treatment of thyroid cancer patients. Furthermore, since there is more knowledge of the effects of demethylating agents and BRAF inhibitors on these antigens in melanoma, our study aims to study both these agents in our thyroid cancer cell lines and compare them to melanoma.

Materials and methods

Chemicals, antibodies and Cell culture

5'-Azacytidine (Aza) and 5'-Aza-3'-deoxycytidine (DAC) were purchased from Sigma and PLX4720 from Plexxikon. Antibodies- β -actin, phospho-ERK1/2 (p44/p42), total ERK1/2(p44/p42) was purchased from Cell signaling technology. Thyroid cells 8505c, BCPAP, TPC-1 and HTh-7 were grown in RPMI1640, melanoma cell lines A375 in DMEM, supplemented with 10% FBS and penicillin/ streptomycin antibiotics (Gibco). Cells were plated at 30% confluency and either treated for 72 hours with 10 μ M DAC or Aza or 1-10 μ M of PLX4720 replacing the medium every 24 hours. At the end of the treatment, the cells were harvested for RNA isolation or protein lysates. IC50 for all three drugs was calculated using the MTS cell viability assay (Promega) after treatment with a range of drug concentration from 0.1 μ M to 500 μ M for 72 hours and analyzed with Graphpad Prism statistical software.

RNA isolation and real time PCR

RNA isolation was performed using Trizol (Invitrogen). Gene validation was performed by relative quantification using real-time RT-PCR with technical triplicates. Initially, cDNA was synthesized from 1000 ng of total RNA using Superscript VILO cDNA synthesis kit (Invitrogen). The cDNA was used as the template for subsequent real-time PCR with primers designed specifically for MAGE-A genes. (TaqMan Gene Expression Assays; Applied Biosystems,). Controls included samples with or without Taq Polymerase, and RNA alone without reverse transcription. MAGE gene expression was normalized to β -actin gene. Genes showing more than 2.0-fold difference in relative expression and p values <0.05 in unpaired two-tailed Student's t test were considered to be differentially expressed and statistically significant. To allow standard comparisons among all cell lines and various MAGE expression profiles, expression of MAGE genes in each of the treatment conditions were compared to the corresponding MAGE gene expression in melanoma cell line A375 (a positive control) at baseline.

Western blot

All treated cells were washed twice with PBS and rapidly lysed in RIPA lysis buffer containing protease and phosphatase inhibitor cocktails (Invitrogen) by incubating on ice for 10 minutes. The total lysates were then centrifuged at 15000 $\times$ g for 15 minutes at 4°C and the supernatants was collected and quantified using Barford protein assay kit (Pierce). Western blot analysis was performed using standard procedure and the proteins were identified using specific antibodies.

Bisulfite treatment

Genomic DNA was isolated using Purelink genomic DNA mini kit (Invitrogen) and the bisulfite reaction was carried out using a Methylcode bisulfite conversion Kit (Invitrogen). The methylation of the promoter region flanking the MAGE-A4 transcription start site, was amplified using the bisulfite PCR primers - FGGTTATGTTAGGAATTAAGGATTGAG and RCAACTCTCTCTTAAATCCTAACTTTC. The amplified PCR product was ligated into the pCR2.1 vector (Invitrogen) and transformed into One shot INV α F' chemically competent bacteria (Invitrogen). The ligated PCR products were then sequenced at the MGH DNA core facility using the T7 sequencing primer.

Suppression of BRAFV600E using Lentiviral transfection

Two lentiviral constructs, sh#1 anti-BRAFV600E and a control shRNA GL3B (anti-firefly luciferase sh) cloned into the GFP reporter plasmid HIV-U6-GL3B-GFP (kindly provided

by Dr. Yutaka Kawakami Keio University School of Medicine) were used to stably suppress the BRAFV600E gene expression in 8505c cells, as previously described (3). BRAFV600E knockdown was confirmed by western blot.

Results

Expression of MAGE genes in thyroid cancer cell lines treated with Aza and DAC

Thyroid cell lines with different mutational status – 8505c (BRAF^{V600E/-}), BCPAP (BRAF^{V600E/WT}), TPC-1 (RET/PTC) and HTh7 (NRAS^{Q61R}); were tested for the baseline expression MAGE-A1, 3, 4, 6 genes using real time PCR. To allow standard comparison among all the cell lines and the various MAGE expression profiles, expression of MAGE genes in each of the treatment conditions were compared to the corresponding MAGE gene expression in melanoma cell line A375 at baseline. The melanoma A375 cell line has been extensively studied for baseline MAGE expression and was used here as a positive control and for creating comparable ratios. None of these thyroid cell lines expressed MAGE genes at base line (listed as control in Fig. 1a-1d) except BCPAP which expressed MAGE-A3 (0.35 fold, ie 35% of the MAGEA3 expression level in A375 cells in comparable conditions) and MAGE-A6 (0.38 fold) as compared to the melanoma cell line A375 (Fig.1b and Fig.1c). The IC₅₀ for DAC in TPC-1, HTh-7, 8505c and BCPAP were ~ 50, 31, 74 and 46 μ M respectively while the IC₅₀ for Aza in these cell lines were ~10.5, 6.0, 15.5 and 36.5 μ M as determined by the MTS assay. Treatment with DAC (10 μ M) or Aza (10 μ M) resulted in the expression of some of the MAGE genes in all thyroid cell lines studied. DAC was more effective in increasing expression of MAGE genes than Aza. None of the thyroid cell lines tested showed MAGE-A1 expression at base line. However, treatment with DAC for 72 hours resulted in the expression of MAGE-A1 mRNA in all the four thyroid cell lines - TPC-1 (~0.75 fold), BCPAP (~0.25 fold), 8505c (~0.7 fold) and HTh-7 (~0.32 fold), as compared to the base line expression of this gene in A375 cells (Fig.1a). In BCPAP cells, both DAC and Aza treatments resulted in the expression of MAGE-A3 (Fig.1b) and MAGE-A6 (Fig 1d) mRNAs to an order equal to the expression of these genes in A375. In 8505c cells, DAC treatment induced the mRNA expression of MAGE-A4 (~0.2 fold) (Fig.1c) gene as compared to A375 melanoma cells. None of the thyroid cell lines expressed the melanoma differentiation antigens, gp100 and Mart-1 that have been previously extensively studied for immunotherapy in melanoma, either at base line or in the treatment with Dnmt inhibitors (data not shown).

Relative mRNA expression profiles showed that the 8505c cells treated with DAC had the highest absolute mRNA expression levels of the MAGE-A4 gene with maximal expression even at 5 μ M of DAC (range of treatment 0.1–10 μ M) at 72 hour (data not shown). Given this data and the recent published data on using MAGE-A4 for immunotherapy in humans (14) and the fact that certain melanoma surface antigens levels (4, 6) appear to increase when melanoma cells are treated with BRAF inhibitors, further experiments in this study were limited to MAGE-A4.

Inhibition of MAPK pathway with BRAFV600E inhibitor PLX4720 in DAC treated thyroid and melanoma cancer cell lines

Targeted therapies aimed at the BRAFV600E oncoprotein activation of the MAPK/ERK pathway are under clinical trials for the treatment of both melanoma and thyroid cancer. It is clear from preclinical studies in both thyroid and melanoma that therapy with BRAFV600E inhibitor PLX4720 alone does not result in apoptosis of the malignant cells and relapses occur in fairly short order (3, 22). Combining cytotoxic immunotherapy against MAGE in combination with PLX4720 may result in better long term results, especially if cell surface

expression of MAGE-A4 could be enhanced either as a result of BRAF inhibition (4-6) or as a result of treatment with demethylating agents (as implied by our results above).

While A375 expressed MAGE-A4 mRNA at base line, 8505 cells did not (Figs.2a and 2c). The IC50 for PLX in A375 and 8505 cells were determined to be 52 nM and 23 μ M. In 8505c cells, DAC treatment for 72 hours showed an increase of MAGE-A4 expression while treatment with PLX4720 (1 and 10 μ M) alone did not cause the expression of MAGE-A4 (Fig.2a). However, combining PLX4720 with DAC treatment in these cells caused a decrease by five fold in the previously induced MAGE-A4 mRNA expression by DAC alone (Fig.2a). The effect of PLX4720 in DAC treated 8505c cells was also verified by knocking down the BRAFV600E oncoprotein in 8505c cells using shRNA. Western blot showed a decrease of pERK44/42 in 8505cBRAF^{-/-} as compared to the 8505c cells. A comparison between both these cell lines treated with DAC for 72 hours resulted in a decreased induction of MAGE-A4 mRNA in 8505cBRAF^{-/-} to 8505c cells by at least 2.4 folds (Fig. 2b).

In contrast to 8505c cell line, DAC treatment in A375 did not cause any change in the MAGE-A4 mRNA expression. However, treatment with PLX4720 alone or in combination with DAC increased the baseline expression of MAGE-A4 in A375 cells by ~1.6 folds (Fig. 2c). Western blot showed the decrease of pERK44/42 protein (Fig.2c).

Methylation profile of MAGE-A4 promoter in the DAC treated thyroid cancer cells

As shown earlier, the MAGE genes were not expressed at baseline in the thyroid cancer cell lines; however the treatment with DAC and Aza induced expression of these genes, highlighting the importance of promoter methylation in the expression of these genes. Previous studies have shown that MAGE-A4 gene promoter contains CpG islands which are methylated in normal cells (15). We used the previously reported bisulfite sequencing primers (BSP) by Qui G et al. 2005 (23), to verify the methylation status of the MAGE-A4 promoter spanning the region of 276bp around the transcription start site. Two cell lines 8505c and BCPAP in untreated and DAC treated conditions were analyzed for methylation patterns using these primers. The sequenced PCR product amplified by the BSP primers on the bisulfite treated promoter regions of the MAGE-A4 showed that- (a) In untreated 8505c and BCPAP cells CpG sites -150, -77, -71, -35, -13, +10, +20, +45 were methylated and CpG site -5 was in a demethylated condition (Fig.3a and Fig.3b). (b) DAC treatment of 8505c and BCPAP cells lead to the demethylation of CpG sites -77 and -71 that had been methylated in the untreated cells (Fig.3 and Fig.3b). (c) 8505c cells treated with a combination of DAC and PLX4720 showed the same pattern of methylation as that of 8505c cells treated only with DAC although the MAGE-A4 mRNA expression got decreased by the addition of PLX4720 to DAC treated 8505c cells (Fig.3a).

Discussion

MAGE family antigens have been mined as potential targets for immunotherapy in recent years due to their unique expression on the surface of tumors cells and not normal cells. Previous studies in clinical thyroid cancer samples were limited and our study is the first to comprehensively evaluate a thyroid cancer cell line panel for MAGE genes at baseline and with treatment using demethylating agents and BRAF inhibitors. Only one PTC cell line (BCPAP) showed any baseline expression of MAGE antigens. However, treatment with demethylating agents resulted in enhanced expression of a variety of MAGE genes. Generally speaking DAC was more effective in increasing the expression of MAGE genes than Aza. Not all the thyroid cancer cell lines behaved the same, and the highest alteration in mRNA expression was seen in MAGE-A4, when 8505c cells were treated with DAC. 8505c cells are derived from an aggressive anaplastic thyroid cancer cell line, which has been

shown to be exquisitely sensitive to the BRAF inhibitor PLX4720 in both *in vivo* and *in vitro* (3). While PLX4720 causes G0/G1 cell cycle arrest and reduces the tumor volume in an 8505c orthotopic model chiefly by necrosis, long term remission in humans seems unlikely due to development of resistance and lack of apoptosis (24).

Earlier study in melanoma cells showed that targeting BRAF oncoprotein by PLX4720 resulted in increased cell surface expression of melanoma differentiation antigens and also an enhanced T-cell recognition of melanoma tumors (4-6). Based on these facts, we aimed to study the influence of BRAF inhibition on MAGE-A4 expression in the 8505c thyroid cancer cells and compared it to a well studied melanoma cell line A375. While BRAF inhibition by PLX4720 increased the MAGE-A4 expression in the melanoma cell line it did not show any effect on the expression of MAGE-A4 in the thyroid cancer cell line 8505c. In contrast, the MAGE-A4 expression induced in 8505c cells by the treatment with DAC was decreased by the BRAF inhibition by PLX4720. Additionally, we showed that DAC treatment demethylated the promoter region in 8505c cells around the transcriptional start site influencing the expression of the MAGE-A4 gene. Combining PLX4720 with DAC allowed the MAGE-A4 promoter to become similarly demethylated but the mRNA expression was significantly decreased. These facts taken together shows that the demethylation of the MAGE-A4 promoter is important for its expression in thyroid cancer cell lines (15), however, it is influenced by the downstream transcription proteins of the pERK pathway. Previous studies showed that the MAGE-A4 promoter is regulated by the transcriptional factors ETS-1 and SP1 (25), ETS-1 being the downstream transcription factor regulated by BRAF/ ERK pathway. The contrasting MAGE-A4 mRNA expression patterns exhibited in the melanoma and thyroid cancer cell line studied here under the influence of BRAF inhibition implies regulation of MAGE-A4 beyond the MAPKinase pathway. Understanding the intricacies of these in the complex networks and the relation to the surface antigens will be important if we are to identify the patients in which anti-BRAF therapies can be combined with effective MAGE-A4 immunotherapy.

The results in this study demonstrate members of the cancer testis gene antigens are present at very low quantities on the surface of some thyroid cancer cells and that the expression of certain specific MAGE genes can be increased through the use of demethylating agents such as DAC. Studying these important cell surface immune antigens might lead to rational treatment of anaplastic thyroid cancer patients with aggressive and recurrent tumors using demethylating agents such as DAC to increase thyroid cancer cell immune antigens while administering the corresponding immunotherapy vaccines towards the specific MAGE genes.

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Figure 1.

Aza and DAC induced the expression of MAGE genes in thyroid cancer cell lines. Four thyroid cancer cell lines 8505c, TPC-1, BCPAP and HTh-7 were treated with 10µM Aza or DAC for 72 hours and analyzed for the induction of MAGE genes using real time PCR. Melanoma cell line A375 was used as positive control. (a) None of the thyroid cancer cell lines expressed MAGE-A1 at base line. However, treatment with Aza or DAC induced

MAGE-A1 expression in all the four thyroid cell lines. (b) Only BCPAP cells showed significant expression of MAGE-A3 at baseline and treatment with DAC and Aza increased its expression by ~2 folds. 8505c cells also showed MAGE-A3 mRNA expression in the DAC and Aza treatment but to a lesser order than BCPAP cells. (c) None of the thyroid cell lines expressed MAGE-A4 at base line. DAC induced a significant expression of this gene in 8505c cells when compared to melanoma cell line A375. (d) BCPAP cells showed significant expression of MAGE-A6 at baseline and treatment with DAC and Aza increased its expression by ~2 folds. 8505c cells also showed MAGE-A6 mRNA expression in the DAC and Aza treatments but also to a lesser order than BCPAP cells.

Figure 2.

Combination of PLX4720 and DAC showed varied effect on the MAGE-A4 gene expression in thyroid cancer cells 8505c and melanoma cell line A375. (a) In the thyroid cancer cell line 8505c cells, combination of PLX4720 to the DAC treated cells decreased the MAGE-A4 gene expression by at least 5 folds when compared to the 8505c cells treated with DAC alone. Western blot showed the PLX4720 ability to decrease the pERK42/44 protein by inhibiting BRAFV600E oncoprotein in 8505c cells. (b) DAC treatment of 8505cBRAF^{-/-} cells (shRNA knock down of BRAFV600E in 8505c cells) showed ~2.4 fold decreased expression of MAGE-A4 gene when compared to 8505cBRAF^{V600E/-} cells. Western blot showed the decreased expression of pERK in 8505cBRAF^{-/-} compared to the

8505cBRAF^{V600E/-} cells (c) Melanoma cell lines A375 showed MAGE-A4 expression at base line. Treatment with PLX4720 alone or in combination with DAC increased the MAGE-A4 gene expression in A375 cells by at least 1.6 fold. Western blot showed the decrease of pERK by PLX4720 treatment

Figure 3.

Methylation analysis of the CpG sites of the MAGE-A4 using bisulfite sequencing method. The CpG positions are numbered comparing their location with the transcription start site. The methylation status of CpG residue is represented by the shaded squares, black (methylated), and white (unmethylated). MAGE-A4 promoter was demethylated in the treated cells with DAC (alone or in the combination of PLX4720) at the CpG sites -77 and -71 in 8505c cells (a) and BCPAP cells (b).