Complement Regulatory Protein CD46 Manifests a Unique Role in Promoting the Migration of Bladder Cancer Cells

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CD46 is a membrane-bound complement regulatory protein (mCRP) possessing a regulatory role with the complement system. CD46 protects the host cells from damage by complement. Expression of CD46 is also highly maintained in many cancers, including bladder cancers, and thus functions as a receptor for many cancer therapeutic viruses. In this study we report a unique role of CD46 as a progression factor of cancer cells in bladder cancers. Resulting data from a DNA microarray using CD46-altered HT1376 bladder cancers demonstrated a pool of target genes, including complement C3 α chain (C3α), matrix Gla protein (MGP), AFAP-AS1, follicular dendritic cell secreted protein (FDCSP), MAM domain containing 2 (MAMDC2), gamma-aminobutyric acid A receptor pi (GABRP), transforming growth factor, beta-induced (TGFBI), a family of cytochrome P450 (CYP24A1), sialic acid binding Ig-like lectin 6 (SIGLEC6), metallothionein 1E (MT1E), and several members of cytokeratins. Subsequent studies using quantitative RT-PCR and Western blot analyses confirmed CD46-mediated regulation of C3α, MGP, and keratin 13 (KRT13). MGP and KRT13 are known to be involved in cell migration and cancer cell metastasis. A cell migration assay demonstrated that CD46 enhanced migratory potential of bladder cancer cells. Taken all together, this report demonstrated that CD46 is generally overexpressed in bladder cancers and plays a unique role in the promotion of cancer cell migration. Further detailed studies are needed to be performed to clarify the action mechanism of CD46 and its application to cancer therapeutics.

Key Words: *CD46; mCRP; Urinary Bladder Neoplasms*

INTRODUCTION

There have been an estimated 82,290 diagnoses of bladder cancer in the United States in 2023.[1] Men are 4 times more likely than women to be diagnosed with the disease. Among men, bladder cancer is the fourth most common cancer. It is the eighth most common cause of cancer death among men with an estimated 16,710 deaths from this disease expected in the United States in 2023. In 2020, an estimated 212,536 people worldwide died from bladder cancer. Bladder cancers are mostly urothelial carcinoma, also called transitional cell carcinoma that originates from the urothelial cells of the bladder, the urethra, ureters and the outer track of the kidneys.[2] Cancers can be treated with various approaches including surgery, intravesicular therapy using BCG, chemotherapy, radiotherapy, targeted therapy, and immunotherapy.[3] Despite of recent interest in various immunotherapy which utilizes the body's immune system to recognize and attack cancer cells and offers a more targeted and potentially effective approach compared to traditional treatments, not all patients respond to immunotherapy, and ongoing research is aimed at understanding the factors that determine treatment response and resistance.

CD46, also known as membrane cofactor protein (MCP), is a membrane-bound complement regulatory protein (mCRP) which is found on the surface of human cells. It plays a role in regulating the complement system, which is a part of the immune system that helps in identifying and eliminating

foreign invaders such as bacteria and viruses.[4] CD46 is not typically associated with cancer cells. However, alterations or dysregulation of CD46 expression or function have been observed in certain types of cancers including the stomach, ovary, breast, bladder, hematologic malignancies.[5-10] This upregulation of CD46 in leukemic cells may help protect them from complement-mediated lysis and contribute to leukemic disease. However, the precise role of CD46 in these cancers is not clearly understood. Previously we have demonstrated that almost half of bladder cancers over-express CD46 with its overexpression shown to confer a better prognosis to bladder cancer patients.[5] CD46 also protects cancer cells from antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[11] Since many cancer cells overexpress CD46, we questioned whether CD46 has a unique role in cancer development and progression other than cancer cell protection mediated through immune cells. The results of the current study provide a hint of evidence that CD46 may have direct role in the cancer cell progression in terms of motility and migration.

MATERIALS AND METHODS

1. Cell lines and cell culture

Human bladder cancer cell lines (HT1376 and 5637) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in the Roswell Park Memorial Institute-1640 medium (RPMI, Welgene, Korea). The complete media were supplemented with 5% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY, USA) and 1% pen-icillin/streptomycin (Gibco) before use. All cultures were maintained at 37℃ and 5% CO_2, and the medium was re-newed every 3-4 days. Overexpressing CD46 cell lines were generated by using the lentiviral vector pBlasti-eGFP-CD46 as described previously.[12] Lentiviral particles with CD46 shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfected into cells to construct CD46-suppressed cells as previously described.[5] For constructing control cells, pBlsti vectors were used. Cells were cultured in media with blasticidin (10 μg/mL) for selection over three weeks and positive clones were confirmed by Western blotting. CD46 overexpression clones were grown in a medium supplemented with 10 μg/mL blasticidin (Sigma-Aldrich, St. Louis, MO, USA).

2. DNA microarray

Either CD46 overexpressing or suppressing HT1376 cells were grown to purify total RNA using the Qiagen RNeasy system. The pBlsti-tranfected HT1376 cells were used as a control. Quality control of RNA was made by both spectrophotometry and agarose gel separation of ribosomal RNA. Gene chip analysis was done on contractual basis with Macrogen Inc (Seoul, Korea). Two sets of the GeneChip human gene 2.0 ST array (Affymetrix, Santa Clara, USA) were used and hybridization were performed in duplicate

for each group. Arrays were then scanned using a scanner, controlled by Affymetrix GCOS software. Images were examined for defects. The Affymetrix® Microarray Suite version 5.1 algorithm analyzed the hybridization intensity data from GeneChip® expression probe arrays and calculated a set of metrics that described probe set performance. The average intensity on each array was normalized by global scaling to a target intensity of 1,000. Target genes that were altered ~2 fold in both CD46-overexpressed and CD46-suppressed HT1376 cells were analyzed and sorted by ToppGene Suite (https://toppgene.cchmc.org).

3. Western blotting

Cells were lysed in the RIPA buffer supplemented with cocktails of protease/phosphatase inhibitors (Cell Signaling Technology, Beverly, MA, USA). Proteins (20 μg) were separated using a 10% SDS-polyacrylamide gel and the Bio-Rad electroporation system and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). CD46 antibodies were obtained from OriGene Technologies, Inc. (Rockville, MD, USA); antibodies against MGP, AFAP1-AS1, and TGFB1 were from Proteintech (Rosemont, IL, USA); MT1G from Lifespan Biosciences (Shirley, MA, USA); KRT13, GABRP, and C3-α chain from Abcam (Seoul, Korea); SIGLEC6 from R&D systems (Minneapolis, MN, USA); and CYP24A1 and β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). The bands were visualized and analyzed using the Immobilon Western detection system (Millipore, Billerica, MA, USA) and ChemiDOC™ MP Gel Imaging System (Bio-Rad, Hercules, CA, USA).

4. Quantitative RT-PCR

Total RNA extraction from selected cultured cells was performed as previously described.[11] RNA from wild type HT1376 cells was used for standardizing the expression levels of each of the genes. Fifty nanograms (ng) of total RNA from each sample were used to detect real-time RT-PCR products with Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. PCR cycling conditions for all of the samples were as follows: 10 min at 95℃ for enzyme activation; 40 cycles for the melting (95℃, 15 s): and annealing/extension (30 s) steps. Using the standard curve method generated by amplification of 0.08-50 ng of HT1376 cDNA, the resulting Ct values were converted to picogram quantities. Then, the quantity of each gene was normalised by β-actin and subtracted from no reverse transcriptase controls. This value was then averaged for each duplicate. The experiment was repeated once to increase the power analysis. The sequences of each of the primers are listed in Table 1.

5. Scratch wound healing assay

For the measurement of cell migration during wound healing, cells were seeded onto 6-well plates and allowed to grow to confluence. The confluent cell monolayer was wounded by pressing a sterile 100 μL pipette tip down onto

TABLE 1. List of primers used in quantitative RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
MGP	CTG CTG AGG GGA TAT GAA GG	CTG CTG AGG GGA TAT GAA GG
MT1G	ACT CCG CCT TCC ACG TGC AC	ACT TGG CAC AGC CCA CAG GG
KRT13	ATG AGC CTC CGC CTG CAG AG	AGC TCA CGC CGC CTC CAT AG
C3a	GCT GCT CAC TCC TCC CCA TC	CAG TGC AGG GTC AGA GGG AC
SIGLEC6	CAT GCA GGG AGC CCA GGA AG	TGC AGG GTA CGA GGA CGC AC
CYP24A1	AGC TCC CCC ATC AGC AAG AG	TCT CGC CAC CAG CTG TCA GC
AFAP1-AS1	CTG CTG CCA CGT AAG AAG TG	CGT CTT CTC CAC TTG GTC ATT C
FDCSP	TTC TCC TCC TGA TCA CAG CC	GGA AAT GGA ATT GGT GGA AG
TGFBI	CCA AGT CGC CCT ACC AGC TG	TGG ACA GCC TTC TCC CCC AG
GABRP	CCT CCA CTT GGC CTT CGT GTG	CCA CCA AAA TTG GGC CTG AG
MDMDC2	CGC GAC CAT GCT GTT AAG GG	TCT CCC CCT GCT TGC CAA AG
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC

TABLE 2. List of selected CD46 target genes in CD46-overexpressed HT1376 cells

Symbol	Accession	Gene description	Fold change	p-value
CD46	NM_002389	CD46 molecule, complement regulatory protein	3.350998	0.013
C3	NM_000064	Complement component 3	1.997857	0.002
MGP	NM_000900	Matrix Gla protein	5.0386	0.019
AFAP1-AS1	NR_026892	AFAP1 antisense RNA 1	5.573683	0
FDCSP	NM_152997	Follicular dendritic cell secreted protein	5.142967	0
MAMDC2	NM_153267	MAM domain containing 2	4.752284	0
SIGLEC6	NM_001177547	Sialic acid binding Ig-like lectin 6	−1.573844	0.031
GABRP	NM_001291985	Gamma-aminobutyric acid (GABA) A receptor, pi	2.020296	0.023
TGFBI	NM_000358	Transforming growth factor, beta-induced, 68kDa	1.940037	0.06
MT1E	NM_175617	Metallothionein 1E	−1.540313	0.134
CYP24A1	NM_000782	Cytochrome P450, family 24, subfamily A, polypeptide 1	1.990046	0.014
KRT13	NM_002274	Keratin 13, type I	1.78627	0.024
KRT15	NM_002275	Keratin 15, type I	1.787982	0.057
KRT34	NM_021013	Keratin 34, type I	2.301502	0.011

the plate to cut the cell sheet and mark the plate with a sharp and visible demarcation at the wound edge. The medium and debris were aspirated away and replaced by a fresh serum-free medium, and cells were incubated for 24 h at 37℃. For the evaluation of the 'wound closure', five randomly selected points along each wound were marked and the horizontal distance of migrating cells from the initial wound was measured. This assay was imaged using a microscope (Leica Microsystems, Wetzlar, Germany). All presented data are from at least three independent experiments performed in duplicate.

6. Statistical analyses

Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). Bar charts were generated using means and the standard deviation. Student's t-test was used for the comparison between two groups and p-values of < 0.05 were considered statistically significant.

RESULTS

1. CD46 target gene expression is profiled using a DNA microarray in HT1376 bladder cancer cells

Total RNA counts from CD46-overexpressed and CD46-suppressed HT1376 bladder cancer cells were analyzed by DNA microarray. Vehicle transfected cells were used as a control. Two sets of the Affymetrix GeneChip human gene 2.0 ST array were used for each group. Target genes altered ~2 fold in both CD46-overexpressed and CD46-suppressed HT1376 cells were analyzed. There were 150 genes sorted in CD46-overexpressed cells and 75 genes in suppressed cells. Genes are selected based on mutual regulation in both overexpressed cells and suppressed cells, known genes, or significantly altered genes (Tables 2 and 3). In CD46-overexpressed cells (veh-CD46), CD46 stimulates the expression of matrix Gla protein (MGP), C3-α chain, AFAP-AS1, follicular dendritic cell secreted protein (FDCSP), MAM domain containing 2 (MAMDC2), gamma-aminobutyric acid (GABA) A receptor pi (GABRP), transforming growth factor, beta-induced (TGFBI), a family of cytochrome P450 (CYP24A1), and several keratins including

TABLE 3. List of selected CD46 target genes in CD46-suppressed HT1376 cells

Symbol	Accession	Gene description	Fold change	p-value
CD46	NM_002389	CD46 molecule, complement regulatory protein	−2.251482	0.013
C3	NM_000064	Complement component 3	−1.948803	0.002
MT1G	NM_001301267	Metallothionein 1G	2.502524	0
KRT13	NM_002274	Keratin 13, type I	−4.563162	0.024
KRT4	NM_002272	Keratin 4, type II	−3.800207	0.026
KRT1	NM_006121	Keratin 1, type II	−2.704641	0.004
CYP24A1	NM_000782	Cytochrome P450, family 24, subfamily A, polypeptide 1	−1.819037	0.014
GABRP	NM_001291985	Gamma-aminobutyric acid (GABA) A receptor, pi	−1.676769	0.023
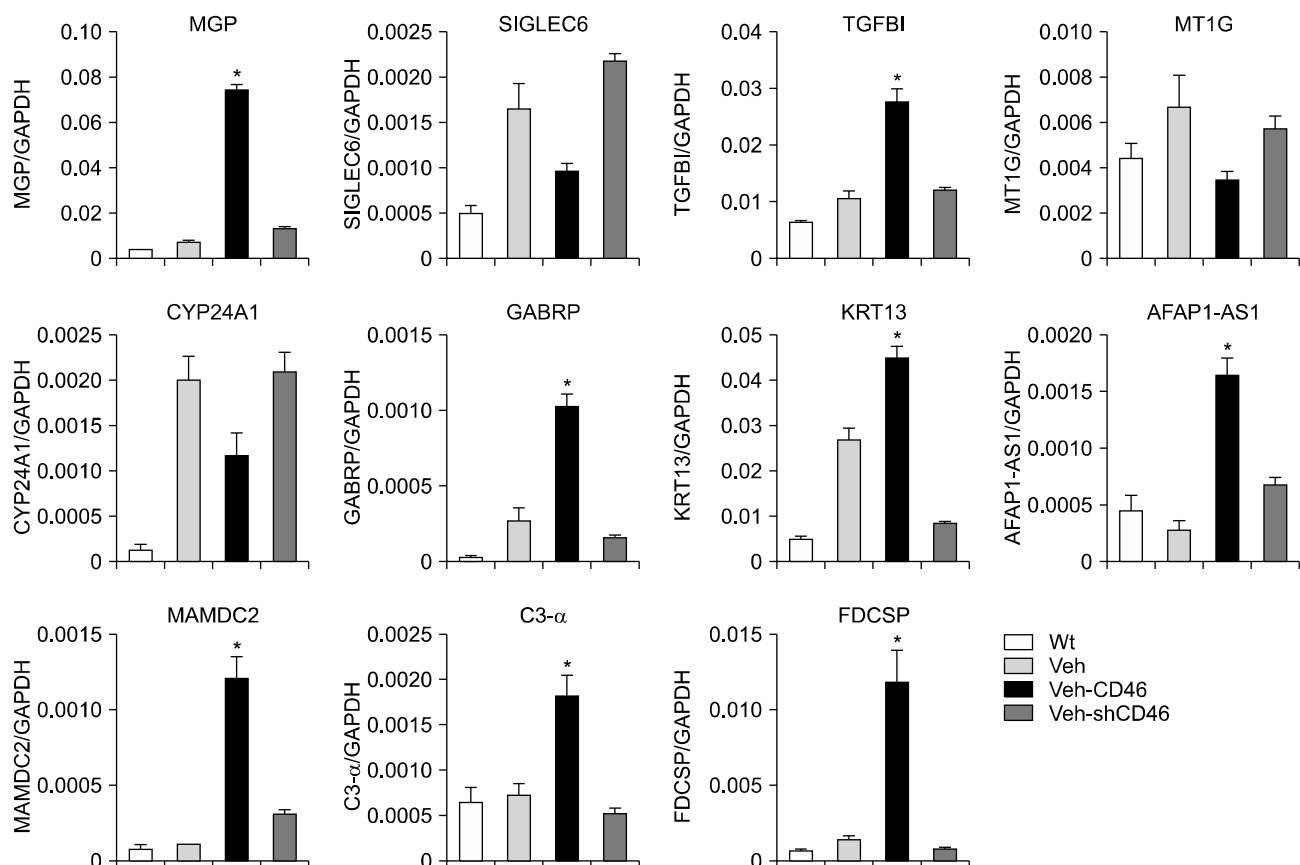
FIG. 1. Expression of selected target genes in CD46-altered HT1376 cells. SYBR green-based quantitative RT-PCR analysis was performed with mRNA from HT1376 cells. Expression of each genes were finally normalized by GAPDH and shown in bar graph. *Denotes that difference is significant (p < 0.05) compared to both wild type (wt) and vehicle-trasfected (veh).

KRT13, KRT15 and KRT34. CD46 also suppressed expression of sialic acid binding Ig-like lectin 6 (SIGLEC6) and metallothionein 1E (MT1E). In CD46-suppressed cells (veh-shCD46), suppression of CD46 inhibited the expression of keratins (KRT1, KRT4, KRT13), CYP24A1, C3-α chain, and GABRP but stimulated the expression of MT1G.

2. CD46 significantly regulates expression of MGP, KRT13, and C3-α chain

To verify CD46 regulated genes, quantitative RT-PCR was performed using the total RNAs from CD46 regulated HT1376 cells. Both CD46-overexpressed (veh-CD46) and

suppressed (veh-shCD46) HT1376 cells were used as well as wild type (wt) and control vector transfected (veh) cells. Out of the selected genes from Tables 1 and 2, overexpression of CD46 promoted expression of MGP, TGFBI, GABRP, KRT13, AFAP1-AS1, MAMDC2, C3-α chain, and FDCSP (Fig. 1). However, CD46-suppressed cells did not consistently show altered expression of target genes altered in CD46-overexpressed cells. In suppressed cells, the expression of KRT13, GABRP, and C3-α chain was altered compared to wt and/or veh cells. Several CD46-regulated genes were further analyzed for their protein expression by Western blot analysis. In HT1376 cells, CD46 overexpression

upregulated expression of MGP, KRT13, and C3-α chain, but not TGFBI, GABRP, AFAP1-AS1, MT1G, SIGLEC6, and CYP24A1 (Fig. 2A). Again, CD46-suppressed cells did not consistently regulate expression of selected proteins, maybe due to the poor efficiency of gene suppression. In 5637 bladder cancer cells, the expression of MGP, KRT13, and TGFBI was observed (Fig. 2B). These results suggest that CD46 consistently stimulates the expression of both mRNA and protein of MGP and KRT13 in bladder cancer cells.

3. CD46 promotes migratory potential of bladder cancer cells

Forced expression of CD46 consistently upregulates the expression of MGP and KRT13 which are tightly involved in cell migration and invasion, we sought to examine a new role of CD46 as a cancer cell migration. Using various bladder cancer cells, we performed a scratch assay. Overexpression of CD46 into HT1376 and 5637 bladder cancer cells that we tested ha previously been reported not to affect pro-

liferation of cancer cells.[11] As demonstrated by the scratch assay in Fig. 3, CD46 significantly enhanced motility and migration of HT1376 and 5637 cells. CD46-HT1376 and CD46-5637 cells filled the scratch area much faster than control cells. Cell migration was represented as a number of cells filling the central gap area after making the scratch. These results suggest that CD46 plays a unique role in promotion of cell motility and migration to bladder cancer cells.

DISCUSSION

We have previously demonstrated that many bladder cancer cells overexpress CD46 and its overexpression predicts better survivability of bladder cancer patients.[5] CD46 is also overexpressed in some human cancers, including lymphomas, breast cancers, ovarian cancers, and hepatocellular carcinomas,[13-16] thus protecting cancer cells from the complement system. We also observed that the EGFR inhibitor enhanced the therapeutic effect in bladder
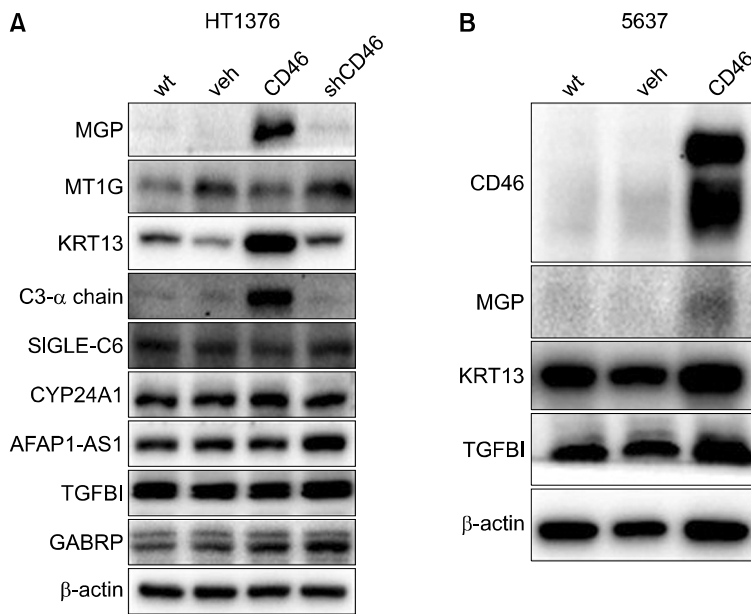
FIG. 2. Expression of selected target proteins in CD46-altered HT1376 and 5637 cells. Western blot analysis was performed with cellular lysates from HT1376 (A) and 5637 (B) cells. Total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods.
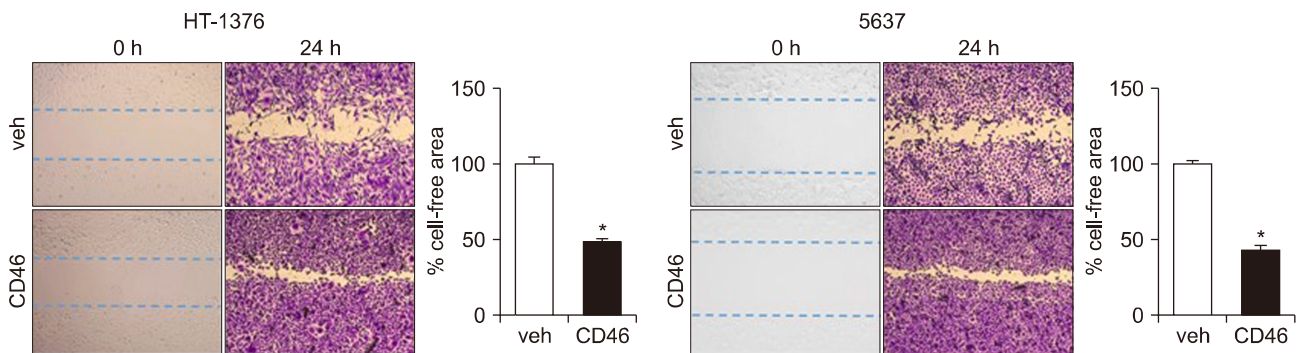
FIG. 3. Forced expression of CD46 promotes migration potential of HT1376 and 5637 bladder cancer cells. Cells were plated onto 60 mm dishes and scratched with pipet tips and photographed at 24 and 48 hours by phase contrast microscope. *Student t-test, p < 0.05.

cancer cells by retaining EGFR signals via both antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), suggesting that CD46 expression might be a beneficial action mechanism in monoclonal antibody-mediated immunotherapy for cancers.[11] As we mentioned, CD46 is a type I membrane protein that plays an inhibitory role in complement-dependent cytotoxicity (CDC).[4] Throughout these studies, however, we have frequently observed unexpected biological behavior of CD46-altered bladder cancer cells in terms of cell motility without the direct involvement of immune cells. Subsequent DNA microarray analysis demonstrated that a pool of CD46-regulated genes. Quantitative RT-PCR and Western blot analyses confirmed that CD46 positively regulated the expression of complement factor C3 α chain, matrix Gla protein (MGP), and cytokeratin 13 (KRT13).

CD46 regulates activity of complement factor C3 to regulate complement activation cascades of the innate immune system. The complement modulates the antitumor activity of various monoclonal antibodies (mAbs) which activates the complement component C1. Activated C1 cleaves C4 and C2 to form the C3 convertase, which further cleaves C3 into C3a and C3b.[17] C3b and C5b are ultimately involved in the lysis of the cells. While CD46 protects the cells from complement-mediated cell lysis and its activity is mediated through the activation of C3, in this study, we have observed that the expression of the α chain of C3 was consistently upregulated by CD46 overexpression, although by which mechanism is not clearly understood. Matrix Gla protein (MGP) is a 12 kD secreted protein that originally isolated from bone tissue. MGP is a secreted protein that could inhibit calcification by binding to calcium ions and is involved in the inhibition of calcification of kidney, heart, cartilage, and vascular smooth muscle cells.[18,19] Aberrant expression of MGP was shown in various types of cancers and functions as either oncogene or tumor suppressor by regulating several target genes.[20] MGP was overexpressed in glioma, testicular cancer, ovarian cancer, and cervical lesions.[18,21-23] Expression of MGP also correlates with poor prognosis and tumor aggressiveness.[23-26] KRT13 is a type I keratin that often pairs with type II KRT4.[27,28] KRT13 is expressed in well-differentiated urothelial carcinoma, squamous cell carcinomas, and Brenner's tumors.[27] Keratins may play role in cancer progression through cell signaling and skeletal organization. KRT19 may contribute high metastatic properties of breast cancer cells.[29] KRT8 is highly expressed in malignant breast cancer cells and enhances cell adhesion to the extracellular matrix.[30] Cells expressing de novo KRT13 results in rapid development of bone metastases in immunocompromised mice.[31] In our study, the gene expression profile of CD46-suppressed cells did not consistently match compared to CD46-overexpressed cells, which was probably due to fluctuating activity from the U6 promoter used in the lentiviral shRNA expression vector. At the same time, the gene expression profile obtained by RT-PCR did not always match with protein expression obtained by western blot analysis. Both RNA and protein expression level of MGP, GABRP, KRT13, C3 were consistently altered by CD46. Levels of SIGLEC6, MT1G, and CYP24A1 were not confirmed by RT-PCR. Protein levels of TGFBI and AFAP1-AS1 were not matched with RNA level. Protein levels of MAMDC2 and FDCSP could not be confirmed due to the lack of antibody availability. Considering the amplification nature of QRT-PCR, it is not unusual that false positive genes are picked up through the selection processes.

In summary, the main function of CD46 is to protect cells from complement-mediated cell lysis. CD46 also provide a cancer-protective effect against both direct (by involvement of PBMC or complement) and indirect cytotoxic activity by mAb in bladder cancer cells. CD46 is generally overexpressed in solid cancers including bladder cancer and colon cancer, in which the function of CD46 protects normal and cancerous cells from complement-mediated cytotoxicity and/or antibody-mediated cell cytotoxicity.[5,11,32] For this reason, maintenance of CD46 is important for cells escaping from unwanted cell killing mechanisms in the milieu of the tumor microenvironment setting including various immune cells, implying that the overexpression of CD46 indicates better survival of bladder cancer patients.[5] In this report, we showed a glimpse of the evidence that CD46 manifests unique role of cancer cell migration and invasion without the involvement of immune cells, namely KRT13 and MGP, which may not be the only targets possessing cell migratory roles regulated by CD46. Further studies are in need to fully investigate the migration and invasion targets of CD46 and to clarify the action mechanism by which CD46 mediated cancer cell migration. The results presented in this report suggest that CD46 may have unique role in cancer cell migration alone without direct involvement of the immune cells in bladder cancers.

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CONFLICT OF INTEREST STATEMENT

None declared.

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