MARCKS phosphorylation is modulated by a peptide mimetic of MARCKS effector domain leading to increased radiation sensitivity in lung cancer cell lines

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Abstract. Lung cancer is the leading cause of cancer-associated mortality in the United States. Kinase hyperactivation is a known mechanism of tumorigenesis. The phosphorylation status of the plasma membrane-associated protein myristoylated alanine rich C-kinase substrate (MARCKS) effector domain (ED) was previously established as being important in the sensitivity of lung cancer to radiation. Specifically, when MARCKS ED was in a non-phosphorylated state, lung cancer cells were more susceptible to ionizing radiation and experienced prolonged double-strand DNA breaks. Additional studies demonstrated that the phosphorylation status of MARCKS ED is important for gene expression and in vivo tumor growth. The present study used a peptide mimetic of MARCKS ED as a therapeutic intervention to modulate MARCKS phosphorylation. Culturing A549, H1792 and H1975 lung cancer cell lines with the MARCKS ED peptide led to reduced levels of phosphorylated MARCKS and phosphorylated Akt serine/threonine kinase 1. Further investigation demonstrated that the peptide therapy was able to reduce lung cancer cell proliferation and increase radiation sensitivity. In addition, the MARCKS peptide therapy was able to prolong double-strand DNA breaks following ionizing radiation exposure. The results of the present study demonstrate that a peptide mimetic of MARCKS ED is able to modulate MARCKS phosphorylation, leading to an increase in sensitivity to radiation.

Introduction

Lung cancer is the leading cause of cancer-associated mortality in the United States (1). Common molecular properties of lung cancer include hyperactivation of receptor tyrosine kinases (RTKs) (2). Phosphatidylinositol 3-kinase (PI3K) is a potent downstream RTK signaling target, which is able to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3) (3). PIP3 recruits and activates phosphoinositide-dependent kinase 1 and Akt serine/threonine kinase 1 (Akt) leading to a pro-growth, pro-survival phenotype (4). The plasma membrane associated protein myristoylated alanine rich C-kinase substrate (MARCKS) is able to bind to the plasma membrane via electrostatic interactions and sequester PIP2 (5-11). MARCKS does not possess endogenous enzymatic activity and therefore a 25 amino acid peptide mimetic of MARCKS ED was engineered in order to modulate MARCKS phosphorylation.
phosphorylation (14). In the present study, the ability of MARCKS targeted therapy to influence MARCKS phosphorylation levels and increase the sensitivity of lung cancer cells to radiation was investigated.

Materials and methods

Cell culture. Human lung cancer cell lines A549, H1792 and H1975 (American Type Culture Collection, Manassas, VA, USA) were cultured in filtered (22 µm vacuum filtration; cat no. 431097; Corning, Inc., Corning, MA, USA) RPMI-1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 1% penicillin-streptomycin and 1% GlutaMAX™ (Thermo Fisher Scientific, Inc.). All cells were maintained at 37°C in 5% CO₂.

MARCKS plasmid production. A549 lung cancer cell lines were engineered to overexpress MARCKS in a tetracycline-dependent manner as described previously (14). WT-MARCKS and NP-MARCKS sequences (GenScript USA, Inc., Piscataway, NJ, USA) were cloned into a pLenti6.3/V5 (ViraPower HiPerform T-Rex Gateway Expression System; cat no. A11141; Invitrogen; Thermo Fisher Scientific, Inc.) lentiviral plasmid as described previously (5). The NP-MARCKS was engineered by substituting the four serine residues in the ED domain on the C-terminus of WT- and NP-MARCKS.

Lentiviral particle production. Lentiviral particles were produced as described previously (14). A total of 4 µg lentiviral packaging plasmid psPAX2 (plasmid no. 12260), lentiviral envelope plasmid PCMV-VSV-G (plasmid no. 8454; both Addgene, Cambridge, Inc., MA, USA) and lentiviral vector plasmid, were mixed with Lipofectamine® 2000 (cat no. 11668) and Opti-MEM™ media (cat no. 11058; both Invitrogen; Thermo Fisher Scientific, Inc.) lentiviral plasmid as described previously (5). The NP-MARCKS was engineered by substituting the four serine residues in the ED domain on the C-terminus of WT- and NP-MARCKS.

Stable cell line selection. A549 cells were transduced with equal amounts (~30 ng/ml) of p24 quantified tetracycline-repressor (Tet-R) packaged lentiviral particles along with 8 µg/ml polybrene (EMD Millipore, Billerica, MA, USA). A total of 500 µg/ml Geneticin (cat no. G418; Thermo Fisher Scientific, Inc.) was used to select for Tet-R positive cells. Tet-R cells were subsequently transduced with equal amounts (~30 ng/ml) of p24 quantified WT- and NP-MARCKS lentiviral particles. A total of 1 µg/ml Blasticidin (Thermo Fisher Scientific, Inc.) was used to select for WT- and NP-MARCKS-expressing cells. MARCKS expression was induced by culturing with 2 µg/ml of the tetracycline homologue doxycycline overnight at 37°C (14).

MARCKS peptide. MARCKS peptide was used as described previously (14). MARCKS-ED tyrosine aminotransferase (TAT) peptide was engineered by conjugating the cell permeable human immunodeficiency virus (HIV) TAT peptide via cysteine bonds to the 25 amino acid ED sequence (KKKKRFSFKKSFKLGSFSFKKNKK) (15). The control peptide sequence was designed using the ExPasy random protein sequence generator (www.expasy.org) using the average amino acid composition computed from Swiss-Prot (www.uniprot.org) (CEIEEHWATVEMFSFFPTQLYNDA) to control for peptide size. To eliminate the effect of the positive charges, lysine and arginine residues were changed to glutamates. Peptide was added to cultures 1 h prior to performance of in vivo assays, including immunoblotting, RNA expression analysis, cellular survival and DNA double-strand damage quantification. A dose establishment study identified 6.25 µM as the lowest effective dose (tested, 1-25 µM), which was selected for subsequent studies.

Immunoblotting. Immunoblotting was performed as previously described by Jarboe et al (5). Mammalian protein extraction reagent lysis buffer supplemented with protease (cat no. P8340; Sigma-Aldrich; Merck Millipore) and phosphatase inhibitors (cat nos., P0044 and P5726; Sigma-Aldrich; Merck Millipore) was used to lyse cells. Protein concentration was determined by using Pierce Bicinchoninic Protein Assay kit (Thermo Fisher Scientific, Inc.) and ~10 µg of protein were separated via electrophoresis on an 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Immolon; Merck Millipore). Blots were blocked in 5% bovine serum albumin (BSA) (Sigma-Aldrich; Merck Millipore) at room temperature for 1 h and probed with the following primary antibodies: Phosphorylated-MARCKS (cat no., ab81295), MARCKS (cat no., ab52616; both Addcam, Cambridge, UK), phosphorylated-Akt (Ser473) (cat no., D9E, 4060), Phospho-Akt (Thr308) (cat no., C31E5E, 2965), Akt (cat no., C67E7, 4691; all Cell Signaling Technology, Inc., Danvers, MA, USA). Antibodies were used at a dilution of 1:1,000 with overnight 4°C incubation. Additionally, actin antibody (cat no., sc-1616; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used at a concentration of 1:5,000 and incubated overnight at 4°C. Blots were washed 3 times for 5 min in TBST. Peroxidase-conjugated Affini Pure Goat Anti-Mouse IgG (cat no., 115-035-166) and Affini-Pure Donkey Anti-Rabbit IgG (cat no. 711-035-152) secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) was added at a dilution of 1:5,000 and incubated overnight at 4°C. RNA expression analysis. RNA expression analysis was performed at the UAB Nanostring Laboratory (Birmingham, AL, USA) (www.uab.edu/medicine/radonc/en/nanostring). The nCounter® GX Human Cancer Reference Kit (NanoString Technologies, Inc., Seattle, WA, USA) was used to analyze RNA expression of 230 human cancer-associated genes. MARCKS expression in A549 NP-MARCKS and A549 WT-MARCKS cells was induced by overnight incubation at 37°C with doxycycline, and total RNA was collected the following day. A total of 100 ng RNA was prepared in a 30 µl
reaction volume and run on the automated nCounter system. Quality control and normalization were performed on the raw data using positive and negative control spots on the included GX Human Cancer Reference Panel chip and five housekeeping reference genes (16,17).

Cell viability and survival assay. Cells were plated in black 96-well plates (cat no. 3603; Costar; Thermo Fisher Scientific, Inc.) and allowed to adhere overnight. The following morning the control or ED peptides were added. A total of 1 h following peptide treatment, cells in the irradiation group were subjected to 0, 5 or 8 Gy irradiation using a 320 kV X-ray irradiator (Kimtron Inc., Woodbury, CT, USA). A total of 4 days following peptide addition or irradiation, ATP levels were measured via the ATPlite™ Luminescence Assay System (PerkinElmer, Inc., Waltham, MA, USA) using a Synergy H1 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, USA).

In vivo studies. The animal protocol was provided by the University of Alabama at Birmingham’s Institutional Animal Care and Use Committee. Mice were housed at ~24°C and given free access to food and water. A 12 h light/dark schedule was provided for the mice. A total of 1 million WT- or NP-MARCKS-expressing A549 lung cancer cells, were injected into the flanks of ~7 week old female athymic nude mice (~17 g) (Charles River Laboratories, Madison, WI, USA). Cells were mixed in a 1:1 mixture of PBS:Matrigel® (BD Biosciences, San Jose, CA, USA) to a final volume of 50 µl and loaded into a 1 ml syringe with a 25 g/8” needle (Monoject™ Tuberculin Syringe; cat no. #8881501640; Covidiens, Dublin, Ireland) (18). When tumors were palpable, mouse food was subsequently supplemented with doxycycline (cat no. TD.05125; Harlan Laboratories, Madison, WI, USA) (19). WT- and NP-MARCKS expression was induced with doxycycline-supplemented mouse food for 7 days prior to tumor volume calculation. Tumor volume was measured using Vernier calipers using the formula: (Length x width^2)/2 (20,21). Values are presented as the mean fold change in tumor volume ± standard error of the mean (SEM). Mice were euthanized with CO2 inhalation as set forth by institutional guidelines.

Double-strand DNA damage quantification. Following overnight induction of MARCKS expression, lung cancer cells were treated with the control or ED peptides 1 h prior to 8 Gy irradiation. As described previously, cells were fixed and stained with an anti-phosphorylated-γH2AX-S139 antibody and counterstained with DAPI (14). γH2AX staining foci marking DNA double-strand breaks were analyzed using an EVOS FL digital inverted fluorescence microscope (Thermo Fisher Scientific, Inc.). Positive events were defined as ≥10 foci per cell, and positive and negative controls were included in all experiments (14,22-24).

Statistical analysis. Statistics calculations and data graphing was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Analysis of variance was performed using the Bonferroni correction post hoc test was used to quantify DNA damage, and a Student’s t-test was performed to analyze survival assays. All statistics were two-sided and P<0.05 was considered to indicate a statistically significant difference. Values are presented as the mean ± SEM.

Results

Phosphorylation status of MARCKS ED influences lung cancer biology. MARCKS ED is known to have multiple biological functions; however, limited information is available regarding the involvement of MARCKS in lung cancer biology (25). Reversible phosphorylation of MARCKS ED is important in regulating the function and subcellular localization of MARCKS (10,26). In the present study, A549 lung cancer cells were engineered to express WT- or NP-MARCKS under the regulation of a tetracycline inducible promoter. Western blotting (Fig. 1A) confirmed that A549 cells were able to overexpress WT- and NP-MARCKS in a tetracycline/doxycycline-inducible manner. Due to the ability of MARCKS to regulate multiple signaling cascades (25), the effect of MARCKS phosphorylation on gene expression was analyzed. Using the nCounter® GX Human Cancer Reference kit, differences in gene expression between A549 cells overexpressing WT- and NP-MARCKS were investigated. A total of 19 genes exhibited a ≥1.5 fold increase in expression in the WT-MARCKS cell line compared to the NP-MARCKS cell line, including siah E3 ubiquitin ligase 1, androgen receptor, matrix metalloproteinase (MMP) 1, platelet derived growth factor subunit A, protein tyrosine kinase 7, notch 1, MYB proto-oncogene, transcription factor, caspase 10, Wnt family member 10B, MMP9, retinoic acid receptor alpha, WD repeat domain 87, v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog, B-cell CLL/lymphoma 6, leukemia inhibitory factor, fibroblast growth factor receptor 4, S100 calcium binding protein A4, cytochrome P450 family 1 subfamily A member 1 and Fox proto-oncogene, AP-1 transcription factor subunit (Fig. 1B). Several of these genes are associated with tumorigenesis and cancer progression (27-31). Cell viability assays were performed to investigate if the ED phosphorylation status was important in lung cancer proliferation. The ATPlite assay indicated significantly increased levels of ATP in the WT-MARCKS A549 cells compared with the NP-MARCKS cell line (Fig. 1C; P<0.002), suggesting that non-phosphorylated MARCKS impedes cell viability. In addition, in vivo mouse studies were used to assess the ability of MARCKS ED phosphorylation status to influence lung cancer growth. WT- and NP-MARCKS A549 cells were injected into the flanks of athymic nude mice. Mouse food was supplemented with doxycycline to induce the expression of WT- and NP-MARCKS (19). The change in tumor volume was compared between the WT- and NP-MARCKS A549 cells over a period of 40 days. WT-MARCKS transfected mice exhibited a greater increase in tumor volume compared with NP-MARCKS transfected mice (Fig. 1D). By modeling the data to a nonlinear regression fit for exponential growth, a significant difference between the WT- and NP-MARCKS tumors (P<0.0001) was observed. The results of the present study suggest that the phosphorylation status of MARCKS ED influences gene expression, viability and tumor growth. MARCKS ED provides a potential therapeutic intervention to alter lung cancer biology. Previously, a 25-amino
acid peptide mimic of MARCKS ED was used to alter lung cancer biology (14). The 25-amino acid ED peptide was conjugated to the cell permeable TAT-peptide, which allowed the ED mimic to enter the cell. Swiss-Prot software calculated a control peptide comprised of 25 random amino acids to control for peptide length. The previous investigation observed changes in cell physiology due to the ED peptide; however, the ability of the peptide to influence MARCKS phosphorylation or proliferation was not investigated (14). Dose titration determined that 6.25 µM was the ideal concentration (data not shown). A549, H1792 and H1975 lung cancer cell lines were cultured without peptide, with 6.25 µM control peptide or with 6.25 µM ED mimetic peptide. Western blot analysis revealed that the ED peptide mimetic was able to decrease the level of phosphorylated MARCKS in the three lung cancer cell lines (Fig. 2A). Decreasing the level of phosphorylated MARCKS may lead to increased plasma membrane-associated MARCKS and decreased Akt activation. Treatment of A549, and to a lesser extent, H1975, cells with the ED-peptide was able to decrease levels of Akt phosphorylated at the serine 473 and threonine 308 residues (Fig. 2A). In addition, the ED-peptide was able to reduce the total level of Akt, which was most pronounced in the H1792 cells (Fig. 2A). Notably, minimal phosphorylated T308 Akt was observed in the H1792 cells in all conditions, though phosphorylated serine 473 Akt was detectable and was mildly suppressed with the ED-peptide. The effect of MARCKS ED peptide on lung tumor growth was investigated. ATP levels in A549, H1972 and H1975 lung cancer cells treated with the ED peptide mimetic were normalized to ATP levels in lung cancer cells with no peptide intervention. A significant decrease in ATP levels in the A549 (P=0.045), H1792 (P=0.000002) and H1975 (P=0.000003) lung cancer cells following 4 days of treatment with the ED peptide was observed compared with the control cells (Fig. 2B).

**MARCKS targeted peptide increases the sensitivity of lung cancer cells to radiation and increases γH2AX foci staining.** Previously, lentiviral manipulation was used to overexpress NP-MARCKS in A549 cells and their sensitivity to radiation increased (14). In the present study, the effect of treatment with the MARCKS ED peptide mimic on the radiation sensitivity of lung cancer cells was analyzed. A549, H1792 and H1975 cells were cultured with either the control or ED peptides 1 h prior to treatment with ionizing radiation. The three cell lines exhibited significantly decreased survival...
following ED peptide treatment compared with the control group at irradiation levels 0, 5 and 8 Gy (Fig. 3A; P<0.05). DNA double-strand breaks in A549 and H1975 cells were examined by measuring γH2AX foci (22). γH2AX foci increased in all groups 1 h following irradiation; however, 24 h following irradiation A549 and H1975 cells treated with the control peptide exhibited basal level γH2AX foci staining. Conversely, A549 and H1975 cells treated with the ED peptide demonstrated persistent γH2AX foci staining (Fig. 3B; P<0.01 compared with the control). The results of the present study suggest that altering the phosphorylation status of MARCKS ED increases lung cell sensitivity to radiation and prolongs DNA damage following irradiation.

Discussion

MARCKS appears to have tumor suppressive properties in glioblastoma and colon cancer (5,32), however, may function as a tumor promoter in breast and cholangiocarcinoma cancers (33,34). The exact role MARCKS serves in cancer biology remains unclear. The subcellular localization and phosphorylation status of MARCKS ED are important in the normal biological and oncogenic function and regulation of MARCKS (25,35), particularly in lung cancer (36).

Hanada et al. (37) reported that squamous cell carcinoma with increased MARCKS expression indicated poor prognosis, however subcellular localization and phosphorylation status were not investigated. Chen et al. (38) demonstrated that MARCKS phosphorylation is involved in invasiveness and that lung tumors with elevated and phosphorylated MARCKS exhibited increased invasion in vitro and in patients. Recently, Chen et al. (36) demonstrated that increased levels of phosphorylated MARCKS are associated with decreased patient survival and increased tumor growth. A similar 25-amino acid peptide was used to modulate MARCKS phosphorylation and led to reduced migration (36). Previously, the present authors demonstrated that the phosphorylation status of MARCKS is important in subcellular localization, drug sensitivity and the DNA damage response (14). Specifically, lentivirally manipulated A549 lung cancer cell lines were used and showed that NP-MARCKS-expressing cells exhibited increased levels of MARCKS fluorescent staining around the plasma membrane compared with the WT-MARCKS-expressing cells, suggesting ED phosphorylation status may impact MARCKS location (14). In the present study, A549 NP-MARCKS-expressing cells exhibited increased sensitivity to radiation and prolonged double-strand DNA breaks, when assessed using γH2AX.
foci staining. A549 lung cancer cells are typically inherently resistant to radiation (39). The present results demonstrated that the phosphorylation status of MARCKS is essential to radiation sensitivity. As MARCKS lacks endogenous enzymatic activity, a peptide mimetic was designed to modulate MARCKS phosphorylation. The 25-amino acid sequence of MARCKS ED was conjugated to the HIV TAT peptide, which increased the ability of the peptide to enter the cell.

In the present study, the importance of MARCKS ED phosphorylation status in lung cancer biology was further characterized and the therapeutic benefit of ED peptide treatment in lung cancer was investigated. RNA expression was observed to be altered dependent on the phosphorylation status of MARCKS in lentivirally-engineered A549 cells. Furthermore, the phosphorylation status of MARCKS ED in vivo was observed to impact on lung tumor growth. The efficacy of the ED peptide was tested. Parental A549, H1792 and H1975 lung cancer cells treated with the ED peptide exhibited a decrease in MARCKS phosphorylation. In addition, a decrease in T308 and S473 phosphorylation and total Akt levels was observed. While it is unclear why T308 phosphorylation was so low in the H1792 cells, the ED peptide was able to decrease A549, H1792 and H1975 proliferation, and increased radiation sensitivity, though it caused prolonged double-strand DNA breaks only in A549 and H1975 cells following irradiation.

Lung cancer is the leading cause of cancer-associated mortality in the United States, and despite targeted therapy and early detection, drug resistance frequently develops (40). Additionally, resistance to ionizing radiation has been observed (41). Targeting MARCKS has the potential to provide a novel mechanism for targeting the RTK-PI3K-Akt signaling cascade. The ED peptide may be able to modulate radiation sensitivity and serve as a future method of avoiding drug resistance (42).

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