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Interferon inducible antiviral MxA is inversely associated with prostate cancer and regulates cell cycle, invasion and Docetaxel induced apoptosis

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Abstract

Background—The interferon inducible Myxovirus (influenza virus) resistance A (MxA) is considered as a key mediator of the interferon -induced antiviral response. Mx proteins contain the typical GTP-binding motif and show significant homology to dynamin family of GTPases. Strong interaction of MxA with tubulin suggests that Mx proteins could be involved in mitosis. Studies have shown that MxA inhibit tumor motility/metastasis and virus induced apoptosis. However the clear association between MxA expression and cancer remains unknown. Meta-analysis suggested that MxA expression was inversely correlated with prostate cancer (PCa). In this study we demonstrate the expression MxA in PCa and its functional significance on the cancer phenotype.

Methods—The expression of MxA protein in prostate cancer was examined by immuno-histochemistry. MxA was knocked down (shMxA) or over-expressed (pMxA) in DU145 or LNCaP PCa cell lines respectively. These cell lines were used to study proliferation, apoptosis, invasion, migration and anchorage independent growth. Co-localization of MxA with tubulin was performed by immuno-cytochemistry following Docetaxel treatment.

Results—The expression of MxA protein was significantly decreased in PCa as compared to the normal tissues. DU145 cells lacking MxA (DU145+shMxA) showed significant increase in proliferation, associated with decreased expression of CDKN1A and B. Increased migration, anchorage independent growth in DU145+shMxA cells was associated with increased MMP13 expression. Tubulin organization was also dependent on MxA expression. Tubulin polymerizing agents such as Docetaxel was less effective in promoting apoptosis in cells lacking MxA due to altered tubulin organization. Gain of MxA expression in LNCaP cells (LNCaP+pMxA) resulted in cell cycle arrest that was associated with increased expression of CDKN1A. MxA expression was also down-regulated by dihydrotestosterone in LNCaP cells.

Conclusions—MxA expression is inversely correlated with prostate cancer. Down-regulation of MxA in LNCaP cells by DHT suggests that MxA could play a significant role in disease

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progression. Loss of MxA expression results in increased metastasis and decreased sensitivity to Docetaxel suggesting that MxA expression could determine the outcome of chemo-therapeutic treatment. Additional studies will be required to fully establish the cross-talk between androgen receptor-IFN pathway in regulating MxA expression in the normal prostate and prostate cancer.

Keywords

MxA; Prostate Cancer; Docetaxel

INTRODUCTION

MxA (Mx1 in mice and MxA in humans) is a 76kDa cytoplasmic protein is strongly induced by IFN and its expression is a preferred marker for IFN biological activity in vivo (1,2). Mx proteins are also considered as key mediators of the interferon -induced antiviral response in vertebrates and hence of great biological and clinical importance (2). Mx proteins contain the typical GTP-binding motif and show significant homology to dynamin family of GTPases (3). The mouse Mx1 protein was originally found in influenza virus-resistant mice and was shown to have intrinsic antiviral activity.

The critical role of mouse and human Mx proteins in mediating the antiviral activity of IFNs against specific viruses became evident early on but other roles for these proteins have not been extensively studied. Results show that ovine Mx is up-regulated during early pregnancy in the uterus (4). A strong interaction with tubulin suggests that Mx proteins could be involved in mitosis (5) and transporting proteins or vesicles throughout the cell (vesicle trafficking) (6). These studies indicated alternative roles of Mx proteins outside of known antiviral activity.

Differential display revealed that MxA is specifically expressed in prostate cancer cell line PC-3 but not in its highly metastatic derivative PC3-M (7). Consequently, ectopic MxA expression decreased motility, invasiveness and metastasis of PC-3M cells (7). Moreover, the MxA gene located on chromosome 21 is frequently deleted as a consequence of TMPRSS2-ERG fusion in aggressive and invasive prostate cancer (8). MxA also mimics the inhibitory effect of IFN effect on motility, suggesting that it might be a critical downstream mediator in the IFN pathway (7). Down-regulation of a number of IFN target genes has been reported in several studies of global gene expression in prostate cancer. Studies have also shown that a significant number of the genes whose down-regulation was associated with prostate cancer tumorigenesis or tumor progression were IFN-inducible genes, including MxA.(9–11). Thus meta-analysis and limited functional studies have suggested that MxA could act as a potent tumor suppressor. However, a systematic analysis of MxA expression on prostate cancer tissue and the underlying molecular mechanism remains to be investigated. Here we report for the first time that MxA expression is progressively decreased in advanced prostate cancer. We also demonstrate through gain and knockdown approaches that MxA expression promotes apoptosis and sensitivity to tubulin polymerizing chemo-therapeutic agents such as Docetaxel.

MATERIALS AND METHODS

Cell culture and reagents

Cell lines and Transfections—Human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were cultured in RPMI-10% Fetal Bovine Serum (FBS) with antibiotics and DU145 and PC3 cells were maintained in Hams F-12 media supplemented with 10% Calf Serum with antibiotics. Cells were cultured at 37°C in a fully humidified atmosphere containing 5% CO₂. Universal Type I Interferon was obtained from R&D Systems and used at a concentration of 300 Units/mL. Jak Inhibitor 1 (In Solution JAK Inhibitor 1, Calbiochem) was used at concentrations of 15 nM and 30 nM for 24 hours. Docetaxel (Enzo) was used at a concentration of 1 µM/mL for 2, 4, and 24 hours. Controls were treated with respective vehicles (media or DMSO).

The expression of MxA in response to androgens was measured in LNCaP cells. The cells in culture were treated with RPMI media supplemented with 10% FBS, 10% charcoal stripped FBS or 10nM 5- α Dihydrotestosterone (DHT). RPMI with 10% Charcoal stripped FBS was used as androgen deprived media (Testosterone concentration 15.6–19pM), RPMI with 10% FBS as media containing castration levels of testosterone (51–97.5pM) and cFBS supplemented with 10nMDHT as media with physiological levels of the androgen (12).

MxA was stably silenced in DU145 cells using gene specific shRNA retroviral vectors (Open Biosystems) in pSM2c vector (DU145+shMxA). The cells transfected with non-silencing shRNA (RHS1707) was used as control (DU145+shNS). Full length human MxA was transiently overexpressed in LNCaP cells using A0257-MX1 (pReceiver-M10 plasmid, GeneCopypia) mammalian expression plasmid (LNCaP+pMxA). Transfections and selection of transfectants (Neomycin) were performed as suggested by the manufacturer. Successful gene silencing/overexpression were confirmed by qRT-PCR and Western blot analysis.

Reverse transcription (RT) and quantitative (q) PCR

Total RNA was isolated from all cells using a Total RNA Extraction Kit (Omega BioTek). cDNA was generated from 4 µg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was performed using GoTaq qPCR reagent (Promega) and transcript levels of MxA, IFN α , IFN β , p21, p27, Cyclin D1, GAPDH, GADD45, MMP13 were measured on a Bio-Rad System. All qPCR data was normalized to GAPDH expression used as a loading control. Sequences of all primers are shown in Appendix Table 1.

Western blotting analysis

Total cellular protein was prepared from cultured prostate cancer cell lines using M-PER (Thermo Scientific). Protein samples were quantitated using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. A standard curve was determined and sample absorbance read at 750 nm. 10–30 µg of total protein was used in a 1:1 (v/v) ratio with 2X Laemmli sample Buffer for electrophoretic separation on 4–20% SDS-polyacrylamide gel (BioRad) and subsequently blotted onto a nitrocellulose membrane (BioRad). The blotted nitrocellulose membrane was subjected to Western blot analysis using protein specific MxA

antibody (Proteintech Lab Group). After washing with 1x PBS with 0.5% Tween 20, the membranes were incubated with a secondary antibody against rabbit/mouse IgG and the signal was visualized using the Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and blots visualized using the Fuji Film LAS-3000 Imager.

Immunohistochemistry

Prostate cancer tissue microarrays (TMA) were used to investigate Mx1 expression in 41 prostate cancers (Stage I-III), 6 BPH and 8 normal prostate core biopsies (1.5mm) in duplicate (BC19014, BC19111 and T192, BioMax, Inc.,). The Cancer stage, grade and histological type information were available from the manufacturer for each of the sections. The IHC was performed using Mx1 specific antibody according to the protocol described earlier (13). Non immune IgG was used as a negative control.

MTT proliferation assay

Cell proliferation was quantitated using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). Cells were seeded (5×10^3 cells/well) in 96-well plates (in triplicate) in recommended growth media. After allowing cells to attach overnight, media was replaced and 15 μ l Dye Solution added and incubated at 37 °C in the dark for 4 h. Following 4 h incubation, solubilization/stop solution was added and incubated for 1 h. Absorbance was read at 570 nm using a 96-well plate reader. Three independent experiments were carried out with similar results.

Flow Cytometric analysis of cell cycle and apoptosis

The cells were cultured in 24 well plates to a sub- confluent density (~70%). Cells were detached from the plates by adding trypsin, collected and washed with phosphate buffered saline. The cells were then fixed with 70% ethanol and stored at -20°C overnight. The fixed cells were washed twice with ice cold phosphate buffered saline PBS/FBS (10%) followed by a final wash in 1x PBS. The cells were then finally resuspended 1 mL of PBS (1x) containing 50 μ g/ml RNase A, 0.1% TritonX-100 and 1 mM EDTA and incubated at 37°C for 30 minutes. Finally, 20 μ g/mL of propidium iodide was added. Data acquisition and analysis were performed on an Accuri CFlow flow cytometer. The cell cycle profiles were then analyzed using FLOWJO (for cell cycle analysis) and CFlow for apoptosis. At least 50,000 cells in each sample were analyzed to obtain a measurable signal.

Migration assay

In vitro cell migration assay was performed using 24-well trans-well inserts (8 μ m) as described previously (14). Briefly, cells were washed once with F-12 and harvested from cell culture dishes by EDTA-trypsin into 50 ml conical tubes. The cells were centrifuged at $500 \times g$ for 10 minutes at room temperature; pellets were re-suspended into media supplemented with 0.2% bovine serum albumin at a cell density of 3×10^5 cells/ml. The outside of the trans-well insert membrane was coated with 50 μ l of rat tail collagen (50 μ g/ml) overnight at 4°C. The next day, aliquots of rat tail collagen (50 μ l) were added into the trans-well inserts to coat the inside of the membranes. The inserts were left to stand for 1.5 h at room temperature before being washed thoroughly with 3 mL of Ham's F-12.

Aliquots of 100 μ L cell suspension were loaded into trans-well inserts that were subsequently placed into the 24-well plate. The trans-well insert-loaded plate was placed in a cell culture incubator for 5 h. At the end of the incubation, trans-well inserts were removed from the plate individually; the cells inside trans-well inserts were removed by cotton swabs. The cleaned inserts were fixed in 300 μ L of 4% paraformaldehyde (pH 7.5) for 20 minutes at room temperature. Cells were stained using HEMA 3 staining kit (Fisher Scientific, Inc.). Stained cells were counted in four non-overlapping fields under the microscope. The average number of cells reflected the cell migration status in each trans-well insert. To avoid experimental bias, a systematic random sampling technique was applied in the selection of representative fields, in which sample preparation and handling was executed by different persons. Each experiment was repeated at least three times using a different cell preparation.

Colony formation in soft agar

Cells were incubated in the presence of 0.5 μ g/mL Neomycin before reseeding in soft agar at low density to ensure the selection of transfected cells. After trypsinization and counting, the cells were resuspended in medium containing 0.3% agarose and 0.5 ml containing 500 cells was added to each well of 6-well plates. The cells were incubated at 37°C in a humidified incubator with 5% CO₂ in for 14–20 days and colonies were counted in an inverted phase contrast microscope.

Statistical Analysis

Quantitative RT-PCR data was analyzed using the Ct method. Within group Student's t-test was used for evaluating the statistical differences between groups.

RESULTS

MxA expression is decreased in prostate cancer

Meta-analysis was first used to investigate the expression of MxA in prostate cancer. Decreased MxA expression in prostate adenocarcinoma as compared to adjacent normal prostate (Fig. 1A) was observed in The Cancer Genome Atlas (TCGA) prostate cancer adenocarcinoma (PRAD) gene expression (IlluminaHiseq) database. MxA expression was also found to be down-regulated (mRNA expression, all complete tumors, Z-score threshold +2.0) in the MSKCC Prostate Adenocarcinoma with a reduction in disease free survival as compared to cases expressing MxA (Fig. 1B).

MxA immuno-histochemistry was performed on normal/benign prostate and prostate cancer tissue microarrays to determine their association with prostate cancer. MxA expression was low to undetectable in majority of prostate adenocarcinoma (Fig. 1C, stage I and III) whereas 100% of the normal and benign prostate tissue (Fig. 1C 100x and 400x) showed strong MxA expression. The cellular localization of MxA was mostly cytoplasmic with some perinuclear staining. Occasionally MxA expression was observed in stage I (Fig. 1C) but rarely observed in stage III prostate cancers (Fig. 1C).

Expression of MxA in prostate cancer cells

The expression of MxA was high in the immortalized normal prostate epithelial cell line RWPE1 (Fig. 2A). These results are consistent with MxA expression in the normal prostate cells (Fig. 1). MxA expression was also observed in prostate cancer cell lines DU145 and PC3 which was lower than that observed in RWPE1 cells. MxA expression in PC3 cell lines was also reported earlier (7). Surprisingly, MxA was undetectable in LNCaP cells when analyzed by Western blot (Fig. 2A). The expression of MxA transcript measured by quantitative real time RT-PCR (qRT-PCR) was also highest in RWPE1 as compared to prostate cancer cell lines DU145 and PC3 (Fig. 2B). Significantly lower MxA mRNA was observed in LNCaP cells as compared to RWPE1 (Fig. 2B) but the corresponding protein was undetectable (Fig. 2A)

Regulation of MxA

Inclusion of a specific JAK inhibitor in the culture media led to undetectable levels of MxA expression in DU145 cells (Fig. 2C). These results suggested that MxA expression is regulated in a well-established JAK1/Stat dependent manner (15) in DU145 cells also. We speculate that lack the functional JAK/STAT1 pathway in LNCaP cells could result in low to undetectable MxA expression (16). In order to investigate alternate pathways relevant to prostate cancer initiation and progression such as androgen receptor, we investigated whether androgens could regulate MxA expression in LNCaP cells. As opposed to DU145, PC3 and RWPE1 cells which lack androgen receptor expression, LNCaP cells are both androgen receptor positive and respond to androgens (17,18). Since we were unable to detect MxA by western blot analysis, we therefore used qRT-PCR to investigate MxA expression in LNCaP cell. MxA expression was measured in LNCaP cells cultured in the presence of 10nM DHT in media containing charcoal stripped FBS which lacks any measurable amounts of androgens as opposed FBS (Fig. 2D) which contains approximately 55–97pM of testosterone (12). Surprisingly, low MxA expression was observed in LNCaP cells cultured in media containing 10% charcoal stripped FBS (cFBS) which. Treatment with DHT led to a significant decrease in MxA expression. In order to ensure that the response was specifically DHT-Androgen receptor mediated, we treated the cells with Casodex, a competitive inhibitor of androgen receptor. Treatment with Casodex led to the recovery of MxA expression which was comparable to untreated cells cultured in charcoal stripped media alone (Fig. 2D). We next performed a western blot analysis using 30ug of total protein as opposed to 10ug of protein used to detect MxA levels in LNCaP cells shown in Fig. 1A. Using this approach, we were able to detect MxA expression albeit at significantly lower levels in cells cultured in cFBS and FBS (Fig. 2E). The results indicated that DHT indeed down-regulated MxA expression in LNCaP as inferred from the qRT-PCR experiment shown in Fig. 2D. These results confirmed that MxA is down-regulated by androgens in LNCaP cells.

MxA overexpression leads to cell cycle arrest

The focus of this study was to investigate the functional significance of MxA in prostate cancer cells through either MxA knockdown or over-expression. Based on the MxA expression profile, we selected LNCaP and DU145 cells as models to over-express or

silence MxA respectively. We attempted stable transfection of MxA in LNCaP cells which proved lethal since we always ended with massive cell death. Previous studies have also shown that constitutive high-levels of MxA is detrimental (19) to cell survival. As an alternate approach, MxA was transiently transfected in LNCaP cells. Transient over-expression of MxA in LNCaP cells (Fig. 3A) showed a significant G1 cell cycle arrest as compared to cells transfected with an empty vector ($P<0.001$) (Fig. 3B). The G1 checkpoint is tightly regulated by cyclin dependent kinase inhibitors such as CDKN1A (p21). As expected, a significant increase in the expression of CDKN1A was observed in LNCaP cells transiently over-expressing MxA (Fig. 3C). On the contrary, the expression of cyclin D1, a cyclin that associates with and functions as a regulatory subunit of CDK4/6 decreased in LNCaP cells in which MxA was transiently over-expressed (Fig. 3D). These results led us to conclude that MxA dependent cycle cell arrest is in part mediated by increased expression of CDKN1A and decreased expression of cyclin D1.

Silencing of MxA increased proliferation of DU145 cells

At least 50% reduction in the expression of MxA was observed in DU145 cells in which shMxA RNA was stably expressed (DU145+shMxA) (Fig. 4A). These cells were then used to investigate the effect of loss of MxA expression on various cancer phenotypes such as cell cycle, proliferation, apoptosis, transwell migration/invasion, colony formation in soft agar (anchorage independent growth) and sensitivity to chemo-therapeutic agents such as Docetaxel.

Results demonstrated a modest decrease in G1 phase of the cell cycle in DU145+shMxA cells as compared DU145 cells stably transfected with non-silencing shRNA (DU145+shNS) (Fig. 4B). A corresponding increase in S-phase of the cell cycle suggested an increased DNA synthesis in DU145+shMxA as compared to DU145+shNS (Fig. 4B). Consistent with these results, an increase in cell proliferation as measured by MTT assay was also observed over 24 and 72 hrs ($P<0.05$) (Fig. 4C). The decrease in G1 phase and increased cell proliferation was also associated with decreased expression of cyclin dependent kinase inhibitors CDKN1A (p21, Fig. 4D) and CDKN1B (p27, Fig. 4E).

Silencing of MxA promotes migration/invasion and anchorage independent growth

We next investigated the effect of MxA silencing on migration/invasion and anchorage independent growth of DU145 cells in Matrigel and soft agar assay respectively. As compared to non-silencing controls (DU145+shNS, Fig. 5A), a larger fraction of DU145+shMxA cells crossed the Matrigel barrier (Fig. 5B). Quantitation of cells revealed at least 3-fold higher ($P<0.001$) invasion by DU145+shMxA cells through Matrigel as compared to DU145+shNS (Fig. 5C). The anchorage independent growth of DU145+shMxA cells in soft agar also demonstrated approximately 2.5 fold increase ($P<0.001$) in number of colonies by DU145+shMxA cells compared to the DU145+shNS used as controls (Fig. 5D). Increased invasion of DU145+shMxA was associated with 2.7 fold higher expression of matrix degrading enzyme matrix metalloproteinase 13 (MMP13) (Fig. 5E, $p<0.001$) that is generally associated with aggressive metastatic PCa (20). These results suggested that loss of MxA promotes cell migration, invasion and anchorage independent growth, the hallmarks of an aggressive cancer.

MxA knockdown blocks apoptosis and promotes sensitivity to Docetaxel

DU145+shMxA cells were significantly less apoptotic than their control DU145+shNS counterparts as quantitated by Annexin V staining followed by flow cytometry, suggesting that loss of MxA promotes cell survival (Fig. 6A). Previous studies have shown that MxA physically associates with tubulin (7). Tubulin re-organization resulting in microtubule stability plays a major role in promoting apoptosis (21). Based on these observations, we hypothesized that interaction of MxA with tubulin could be required for cell-cycle arrest and apoptosis. Immuno-cytochemistry based co-localization was performed to study MxA-Tubulin interaction in DU145 cells in the presence or absence of Docetaxel (DTX), a tubulin binding chemotherapeutic drug (22). DTX stabilizes microtubules (23) leading to mitotic catastrophe and eventually apoptosis (24). In control DU145-shNS cells, a clear co-localization of MxA was observed with tubulin (Fig 6B1). In the presence of DTX, the association between MxA and Tubulin was significantly decreased (Fig. 6B2). Interestingly, MxA re-organized into a punctate pattern in DU45+shNS cells following DTX treatment (Fig. 6B2, white arrow in the inset). A larger fraction of DU145+shNS cells treated DTX exhibited microtubules as seen by intense tubulin staining (Fig. 6B2.2, white asterisk) as compared to untreated DU145+shNS cells (Fig. 6B1.2), suggesting mitotic catastrophe. These results are consistent with decreased cell cycle (Fig. 4) and increased apoptosis (Fig. 6A), hallmarks of DTX treatment. A similar set of experiments were then performed in DU145 cells lacking MxA.

Treatment of DU145+shNS cells with the chemotherapeutic drug Docetaxel (DTX) for a period of 24 hours demonstrated significantly increased apoptosis as compared to untreated cells (Fig. 6A). Surprisingly, the DU145+shMxA cells were less sensitive to DTX induced apoptosis as compared to DU145+shNS cells treated with DTX (Fig. 6A). These results suggested that MxA expression promotes whereas lack of MxA results in resistance to DTX induced apoptosis.

At the cellular level, no MxA expression was observed in DU145+shMxA (Fig. 6B3 and B4) confirming successful silencing of MxA (see Fig. 4A). Few DU145+shMxA cells demonstrated the presence of microtubules (Fig. 6B3.2) as compared to non-silencing controls (Fig. 6B1.2). A larger fraction of cells with microtubules were observed when DU145+shNS cells were treated with DTX (Fig. 6B2.2) as compared to DU145+shMxA cells treated with DTX (Fig. 6B4.2)

We also observed a correlation between MxA expression and tubulin. In non-silencing controls, the tubulin organization was dependent on the level of MxA expression. Cells with lower MxA expression demonstrated diffused tubulin expression and appeared flattened (indicated by white arrows in Fig. 6B1.2 and B1.3), as opposed to round cells with high MxA and tubulin expression. Consequently, in cells lacking MxA, a significantly larger fraction of cells appeared to be flattened with diffused tubulin expression (Fig. 6B3.2 and 3.3). In the presence of DTX, the cell morphology was similar to cells with MxA (See round cells in Fig. 6B1.2, B2.2 and B4.2). These results suggested that MxA could mimic the effects of DTX and promote apoptosis.

DISCUSSION

We show for the first time that MxA expression is inversely correlated with prostate cancer. This is supported by the meta-analysis (TCGA) followed by immuno-histochemical analysis of MxA expression on prostate cancer tissue microarray. The mechanistic studies based on loss and gain of MxA expression in prostate cancer cell lines DU145 and LNCaP cells respectively also support the tumor suppressive effect of MxA that is consistent with its expression profile in prostate cancer.

AR acts as a tumor suppressor in the normal prostate but transitions to an oncogene in prostate cancer. Thus many of the AR up-regulated genes and pathways are down-regulated in prostate cancer and vice versa. MxA could represent one such gene that is up-regulated in normal prostate but down-regulated by AR in prostate cancer further supporting its role as a tumor suppressor.

Apart from AR, the major mechanism involved in down-regulation of MxA in prostate could be due to a general decrease in IFN expression and signaling in prostate cancer (9). Independent studies have also demonstrated the suppression of innate immune response and MxA during prostate cancer progression (11). Alternatively, the overall low expression of MxA in prostate cancer could possibly be due to the polymorphism in the proximal promoter region of MxA. Our previous study established an association of MxA with prostate cancer risk, specifically the genotype with low promoter activity (25) which results in reduced expression of MxA. Alternatively, MxA gene could be deleted in prostate cancer as a consequence of TMPRSS2-ERG fusion in aggressive and invasive prostate cancer (8).

The prostate cancer cell lines demonstrated variable expression of MxA but overall expression was low as compared to immortalized normal RWPE1 prostate epithelial cell line. Low MxA expression that is further down-regulated by androgens in androgen sensitive LNCaP cells is particularly interesting. Previous studies have shown that activation of AR is associated with down-regulation of IFN signaling which in turn promotes cell survival and suppress apoptosis (26). MxA being a classical IFN response gene its down-regulation by androgens is thus expected. However, the synergy between androgen-interferon pathway in LNCaP cells is likely not at the level of Jak/Stat1 since this pathway is non-functional in LNCaP cells (16). We speculate that in LNCaP cells the androgen – interferon negative cross-talk could be independent of STAT1 and involve yet unknown alternate mechanisms (27). Whether these alternate pathways are involved in other androgen sensitive prostate cancer cell lines or prostate cancer in general remains to be investigated.

In normal prostate epithelial cells Androgen receptor levels are increased by IFN type I (28) suggesting a cross-talk between IFNs and AR is likely to be involved in high MxA expression in normal prostate epithelial cells. However, a clear correlation between these two pathways could not be ascertained in this study due to the lack of a relevant model system. The cell lines that express AR and respond to androgens such as LNCaP lack the functional JAK/STAT pathway (16) whereas the cells with a functional JAK/STAT pathway such as DU145 and PC3 lack AR. The lack of AR expression in normal immortalized prostate epithelial cell line RWPE1 also lacks AR expression. Thus, inclusion of additional

cell lines that express both AR and respond to IFNs could be used to study AR-IFN interaction in regulation MxA expression in the future.

Cells with constitutive expression of MxA such as DU145 are sensitive to the classical Jak/Stat1 dependent interferon signaling as shown in this study. It is therefore not surprising that the anti-proliferative effects (G1 arrest) of interferons alpha and gamma on DU145 cells is mediated by increased expression of CDKN1A (p21) (29) and increased STAT1 phosphorylation (30,31). Decreased G1 arrest and CDKN1A expression observed after MxA silencing DU145 cells could therefore be the primary mechanism involved in interferon dependent cell cycle arrest. The role of MxA in cell cycle regulation is further supported by the observation that ectopic MxA expression in LNCaP cells results in CDKN1A dependent cell cycle arrest. This was supported by a reduction in Cyclin D1 which is sufficient to cause a G1 cell cycle arrest (32,33). CDKN1A can also down-regulate Cyclin D1 in a p53 dependent manner (34).

Massive apoptosis observed in LNCaP cells following stable transfection of MxA but not in DU145 and PC3 or even RWPE1 cells that express MxA is puzzling. Previous studies have shown that MxA dependent apoptosis is in part mediated through a p53 dependent mechanism (35–37). All three cell lines i.e. RWPE1, DU145 and PC3 contain mutant, deleted or inactivated p53 respectively (38) whereas LNCaP cells express wild type p53. The apoptosis seen in LNCaP cells either stably or transiently transfected with MxA could therefore be in part through p53 dependent activation of the apoptotic cascade. The increase in CDKN1A expression, a well characterized p53 response gene (39) further supports the activation of p53 pathway by MxA in LNCaP cells, however alternate pathways cannot be ruled out.

Silencing of MxA clearly prevented apoptosis in a p53 independent manner in DU145 cells, suggesting alternate mechanisms. Previous studies including this study have demonstrated that MxA interacts with tubulin (7) and other subcellular components (3). The interaction of MxA with tubulin is particularly interesting due to the role of tubulin in regulating multiple cellular processes including cell cycle, cell migration and apoptosis (40). Microtubules, which are essentially polymers of tubulin monomers comprise an essential part of the cytoskeleton. The currently used anti-cancer acts by suppressing of microtubule dynamics by either by de-polymerizing (e.g. vinblastine) or by excessive polymerization (e.g. paclitaxel and Docetaxel), thus driving the cell into apoptosis and/or cell cycle arrest (41). Increased apoptosis in part due to tubulin polymerization and re-distribution of MxA following DTX treatment clearly suggested that MxA expression could be involved in regulating tubulin function in DU145 cells. Protection against apoptosis in response to DTX in cells lacking MxA (DU145(-)MxA (+DTX)) clearly supports the role of MxA in tubulin polymerization. These results suggest that interaction of MxA with tubulin could determine the sensitivity of cancer cells to chemotherapeutic drugs that promote tubulin polymerization. Whether such a mechanism is also involved in determining sensitivity to tubulin depolymerizing ant-cancer drugs will be an attractive next step in understanding the role of MxA in tubulin dynamics.

MxA inhibits motility and colony formation which is comparable to that observed by IFN (7) (42) suggesting that MxA may be a critical molecular mediator of the IFN effect in prostate cancer cells (30). We show that at the molecular level, increased invasion and anchorage independent growth could be mediated by increase in MMP13 in cells lacking MxA. Increased MMP13 expression is known to promote migration and motility in cancer cells (43).

Clinically, up-regulating the expression of MxA in prostate cancer would therefore be advantageous. Strategies promoting MxA expression such as through small molecules identified recently are promising (7). Alternatively, treatment of prostate cancer patients with IFN-alpha that is known to induce MxA expression is a viable approach (2,3). Phase I and II trials using IFN-alpha as a therapeutic produced a response rate 5% and a >50% reduction in 23% of patient but was associated with high toxicity (44). In a recent study, low dose IFN-alpha improved the clinical outcome of Docetaxel in patients with castration resistant prostate cancer (45). Our studies therefore support a model where the IFN inducible MxA could potentiate DTX induced apoptosis.

Conclusions

MxA appears to regulate multiple tumor suppressor pathways. The strong overlap between the effects of MxA and IFN on cell cycle, apoptosis and cell migration/invasion suggests that MxA could be a major down-stream effector of IFN in cells. One of the major roles of MxA uncovered in this study is its role in promoting sensitivity (apoptosis) of cancer cells to chemotherapeutic tubulin polymerizing agents such as DTX, which seems to be independent of p53. In fact mutant p53, plays a major role on protecting cancer cells against chemotherapeutic agents. The lack of MxA in cells with mutant p53 would further enhance this resistance directly at the level the target molecule i.e. tubulin by preventing its polymerization. The down-regulation of MxA due to androgens, promoter polymorphism, down-regulation in IFN signaling and deletion due to TMPRSS-ERG2 fusion could contribute to various prostate cancer disease modalities.

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Figure 1.

Expression of MxA correlates inversely with prostate cancer. **A:** MxA expression in prostate adenocarcinoma (PCa, blue) as compared to adjacent normal prostate (ANP, Pink) in The Cancer Genome Atlas (TCGA) prostate cancer adenocarcinoma (PRAD) gene expression (IlluminaHiSeq) database. **B:** MxA expression is also associated with Disease free survival. MxA is down-regulated (Red, mRNA expression, all complete tumors, Z-score threshold ± 2.0) in the MSKCC Prostate Adenocarcinoma with a reduction in disease free survival as compared to cases expressing MxA (blue). The data is obtained from the MSKCC Prostate Adenocarcinoma dataset (cBio cancer genomics portal, mRNA expression, all complete tumors, Z-score threshold ± 2.0). **C:** Representative MxA immuno-histochemistry on normal

prostate and prostate cancer tissue microarray. MxA expression is indicated by brown staining. The left panel is 100x and the right panel is 400x.

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Figure 2.

Expression and regulation of MxA in prostate cancer cell lines: **A:** Representative Western blot analysis illustrating MxA expression in prostate cancer cell lines LNCaP, DU145 and PC3 and in normal immortalized prostate epithelial cell line RWPE1. **B:** MxA expression as determined by Quantitative RT-PCR in prostate cell lines (Mean±SEM). The data is normalized to RWPE1. **C:** MxA expression is regulated by interferon through JAK/STAT dependent pathway in DU145 cells. Cells were incubated with either JAK/STAT inhibitor, IFNα alone or in combination. MxA expression was determined by Western blot analysis. Representative results from experiments performed in triplicate are shown. **D:** Regulation of MxA by Androgens in LNCaP cells cultured in media containing 10% charcoal stripped media (cFBS). Quantitative RT-PCR analysis of MxA expression after 24hr DHT(10nM) in the presence or absence of the anti-androgen Casodex (CSD). The data is expressed (mean

$\pm$ SEM) as fold change compared to LNCaP cells cultured in cFBS (***: $P < 0.001$). **E:** MxA protein expression by western blot analysis in LNCaP cells cultured in cFBS, FBS or DHT. Representative results from experiments performed in triplicate are shown.

Figure 3.

Over-expression of MxA attenuates cell cycle in LNCaP cells. **A:** MxA was transiently over-expressed in LNCaP cells (LNCaP+pMxA). The extracted RNA and protein was subjected to western blot analysis (top panel) and qRT-PCR (bottom panel) for detection of MxA using protein specific antibody and gene specific primers respectively. GAPDH was used as a loading control for both analyses. The qRT-PCR data is expressed as fold change relative to LNCaP cells transfected with control plasmid (LNCaP+pcDNA) (mean±SEM, ***: $p < 0.001$). **B:** Cell cycle analysis by flow cytometry analysis in LNCaP+pMxA and LNCaP+pcDNA. The data from 10,000 events is expressed as % cells (mean±SEM, ***: $p < 0.001$) in each phase of the cell cycle (G1, S and G2). **C and D:** Expression of p21 (CDKN1A, C) and Cyclin D1 (D) in LNCaP+pMxA and LNCaP+pcDNA. The data expressed as (mean±SEM, ***: $p < 0.001$, *: $P < 0.05$) is relative to the expression of respective genes in LNCaP+pcDNA cells.

Figure 4.

Knockdown of MxA promotes cell cycle and proliferation in DU145 cells. **A:** MxA was stably silenced in DU145 cells (DU145+shNS). The extracted RNA and protein was subjected to western blot analysis (top panel) and qRT-PCR (bottom panel) for detection of MxA using protein specific antibody and gene specific primers respectively. GAPDH was used as a loading control for both analyses. The qRT-PCR data is expressed as fold change relative to DU145 cells transfected with control plasmid (DU145+shNS) (mean±SEM, ***: $p < 0.001$). **B:** Cell cycle analysis by flow cytometry analysis in DU145+shMxA and DU145+shNS. The data from 10,000 events is expressed as % cells (mean±SEM, ***: $p < 0.001$) in each phase of the cell cycle (G1, S and G2). **C:** Rate of proliferation between DU145+shMxA and DU145+shNS cells as measured by MTT assay over 24, 48 and 72 hours. The data is expressed as mean±SEM (*: $p < 0.01$) from three experiments performed in triplicates. **D and E:** Expression of p21 (CDKN1A, D) and p27 (CDKN1B, E) in DU145+shMxA and DU145+shNS. The data expressed as (mean±SEM, ***: $p < 0.001$, *: $P < 0.05$) is relative to the expression of respective genes in DU145+shNS cells.

Figure 5.

Knockdown of MxA promotes invasion and anchorage independent growth in DU145 cells.

A, B and C: transwell migration assay was performed on DU145+shNS (A) and DU145+shMxA (B). Each panel shows representative images from four different experiments. The cells were counted in 10 random field from each well are expressed as average cell/well (mean±SEM, ***: $p<0.001$) in panel C. **D:** Number of individual colonies formed by DU145+shNS and DU145+shMxA cells were counted in a soft agar assay and are expressed as colony forming units (CFU) (mean±SEM, ***: $p<0.001$). **E:** Expression of MMP13 in DU145+shMxA and DU145+shNS. The data expressed as (mean±SEM, ***: $p<0.001$, *: $P<0.05$) is relative to the expression of MMP13 in DU145+shNS cells.

Figure 6.

Knockdown of MxA promotes apoptosis. **A:** Percent apoptotic cells expressed as fold change relative to DU145+shNS cells. The cells were either cultured in regular growth media or treated with Docetaxel (DTX) for 24 hrs before measuring apoptosis by Annexin V staining followed by flow cytometry. The data is expressed as mean±SEM (***: $p < 0.001$) from three experiments in duplicate. **B:** Representative images demonstrating co-localization of MxA (Red) with tubulin (green). The merged images show co-localization as seen by yellow color. Asterisks represent microtubules. Arrows in B2 represent punctate MXa expression and a flattened cell with diffused tubulin and MxA expression (Legend: (+): cells treated with DTX, (-) control cells or cell lacking MxA).