RESEARCH ARTICLE

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Evaluating the Oncogenic and Tumor Suppressor Role of XPO5 in Different Tissue Tumor Types

Munazza Khan[1], Zafaruddin Khan[2], Yaser Uddin[3], Sadaf Mustafa[4], Irfan Shaukat[4], Jianbo Pan[5], Naseruddin Höti[5]*

Abstract

The miRNAs nuclear export protein XPO5 has been previously studied in several individual malignancies. In our recent study we have demonstrated that excess levels of XPO5 enhanced the proliferation of prostate cancer cells. Similarly, there are studies to support the inhibitory role of XPO5 in cancers. In order to evaluate discrepancies in the expression levels of XPO5 in differential tumor types, we quantified the expression of XPO5 using gene expression RNA-seq data for several tumor types which were independently confirmed by immunohistochemistry in multiple organs cancer tissue microarray (TMAs) experiment. We found that while some tumors (Breast, Bladder, Lymph-node, Lung, Esophagus and Ovary) showed higher differences between normal and malignant tumors in XPO5 expression, there were tissues (Kidney and Brain) that have a significantly lower XPO5 expression in malignant tumors. We further studies these observations of overexpression and down-regulation of XPO5 in breast and kidney cancer cell lines and found that XPO5 might have a dual role in promoting or inhibiting tumor growth in different cancer tissue types.

Keywords: miRNA- TMA- XPO5- Tumors- IHC

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Introduction

The intact microRNA processing machinery is required for the proper processing and maturation of miRNA (Slezak-Prochazka et al., 2010). Recent studies have shown that dysregulated expression of the miRNA processing machinery might be responsible in the progression of cancers (Li et al., 2015). miRNAs are small non-coding RNA molecules (19 to 22nt) that has been shown to regulate gene expression by binding to the 3' UTR of the transcriptome (Li et al., 2015; Kumar et al., 2016). Due to the appearance of large number of non-coding miRNAs within the human genome, it was predicted that around 30%–80% of human genes are under the influence of miRNA regulation control (Li et al., 2015). The nuclear export of hairpin looped miRNAs into the cytoplasm is uniquely performed by the Exportin-5 (XPO5) protein in the RNA-GTP dependent manner (Yi et al., 2003; Leisegang et al., 2012). In the cytoplasm the hairpin looped miRNAs are intercepted by the RNase III enzyme called Dicer that perform a cleavage at the hairpin loop resulting in a double stranded RNA molecule. Once the Dicer cleaves the hairpin loops the RNA inducing silencing complex associates with the RNA duplex, retaining one RNA stand, the mature miRNA leading to mRNA degradation or translation silencing (Lund and Dahlberg, 2006).

While the function of XPO5 remains the rate limiting step in the production of mature miRNAs (Sun et al., 2016), very little is known about the association of XPO5 in different cancers. Several studies including ours have shown dysregulated expression of XPO5 in cancers. In the colorectal cancer models, XPO5 has been shown to harbor genomic mutants which renders its function by trapping XPO5 inside the nucleus (Melo et al., 2010). Similarly, in hepatocellular carcinoma overexpression of XPO5 was shown to support the tumor suppressive role of the miRNA passenger XPO5 protein (Li et al., 2016). On the other hand, there are studies which support the oncogenic roles of XPO5 and targeting of which could lead to significant therapeutic responses in cancer models (Shigeyasu et al., 2017). In our recent study we have found higher levels of XPO5 in prostate cancers (Hoti et al., 2017). Using several cell lines models we demonstrated that excess levels of XPO5 in prostate cancer negatively impact the miRNA regulation control supporting cancer cells proliferation (Hoti et al., 2017). In order to understand whether XPO5 might be having tumor suppressive or oncogenic role in different tissue types we evaluated the TCGA genomic RNA seq data and deep proteomics data for multiple tumor

*[1]Department of Physiology, Institute of Medical Sciences, Kohat, [2]Department of Urology and Transplantation, Shaikh Zayed Hospital, [3]Department of Neurosurgery unit II, Lahore General Hospital, Lahore, Pakistan, [4]Department of Internal Medicine, MedStar Good Samaritan Hospital, [5]Department of Ophthalmology, Johns Hopkins School of Medicine, Baltimore, USA. *For Correspondence: nhoti1@jhmi.edu*

types. We further confirmed these results by using multi organ tumor microarray experiment and in different cell line models. Our results suggest that XPO5 expression might have differential (inhibitory or cancer promoting) roles in different cancer tissue types.

Results

Differential cancer tissue type specific expression of XPO5

To evaluate cancer tissue specific expression of XPO5, we first examined the TCGA RNA seq data sets for the relative expression of XPO5 mRNA. As shown in the Figure 1A we discovered a large variability in the expression profiles of XPO5 among different cancer types. Interestingly, while the expression levels of XPO5 remained high in majority of the tumors (breast, bladder, colon, head and neck, lungs, prostate) compared to normal tissues, we found renal and thyroid cancers to have the lower mRNA expression in the TCGA data sets. Similarly, we also evaluated the number of mutations (missense, nonsense, in-frame or frameshift mutations) associated with these tumors (Figure 1B). While we found no significant gene alterations in testicular, ovarian, uterine colorectal, glioma and RCC cancers, a number of missense mutations were identified in breast, melanomas, and bladder cancers suggesting a cancer tissue specific type functional expression of XPO5 protein. We also evaluated the mRNA expression levels for the other two miRNA biogenesis genes, the Dicer and Drosha (Figure 1 C, D) and found that the mRNA levels for Drosha was upregulated in many cancer tumor types. Similarly, the Dicer mRNA expression levels were statistically different between tumor and normal samples except KICH (p=0.08), LUSC (0.93) and PRAD (0.32) (Figure 1C).

In order to confirm these observations we further evaluated the expression profiles of XPO5 in individual tumor types using multiple organ tissue (Figure 1 E & F) as described in our material and method section. Briefly, multiple organ TMAs that contain duplicate or triplicate malignant tissue and normal adjacent tissues were stained with XPO5. A higher expression of XPO5 staining was detected in ovarian, breast, esophagus, bladder and lymph node cancers compared to the normal adjacent tissue. While significant lower expression of XPO5 was observed in Kidney and adrenal gland cancers compared to the normal cells supporting the RNA-seq data. Interestingly, there were tissues (Liver, Colon, Rectum and Brain) that didn't show significant differences among the XPO5 staining in the TMA while in the TCGA RNA-seq data the same tissues showed higher expression of XPO5 mRNA (data not shown). Further studies using large sample size will be needed to clarify these results.

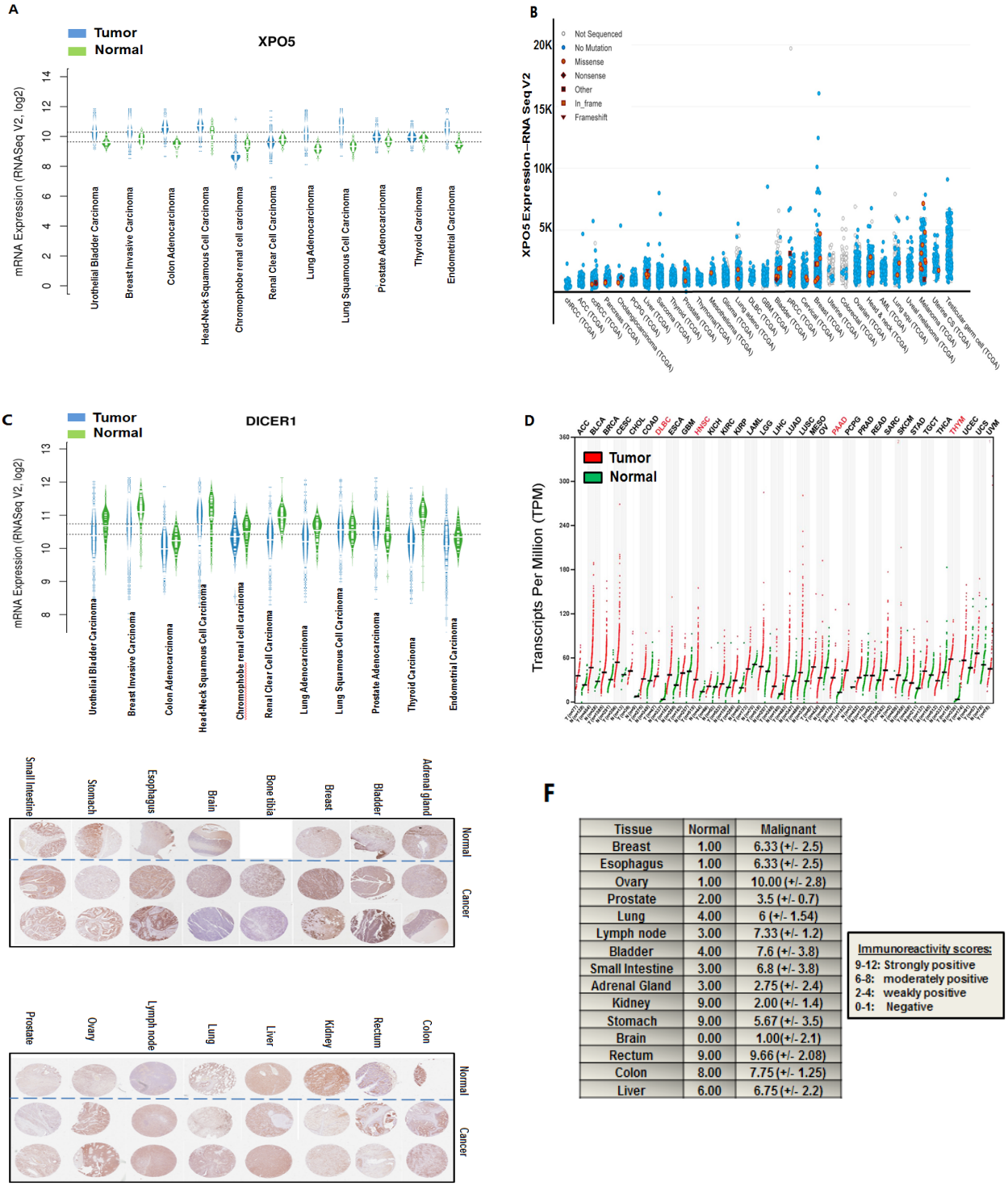
Overexpression of XPO5 in breast cancer supports tumor cell proliferation

In order to evaluate and confirm the tumor and cell proliferation function of XPO5 in cancer cells we select the breast cancer model that have shown significant higher expression for XPO5 in both the RNA-seq TCGA data sets and in the cancer TMA experiment (Figure 1A and C). Using one normal (MCF-10A) and four different breast cell lines (MCF-7, MDM MB231, BT-20 and T4-7D) we studied the relative mRNA expression of XPO5 between normal and breast cancer cell lines using qRT-PCR. As shown in the Figure 2A there was a significant higher expression of XPO5 in all of the breast cancer cell lines compared to the normal MCF-10A suggest a tumor promoting role of XPO5 in breast cancer. These observation were further independently confirmed in breast cancer and normal tissues by IHC experiment (Figure 2B). XPO5 is a nuclear and cytoplasmic shuttling protein, there were reports of genomic point mutations in XPO5 in colorectal cancer cells which renders its function by trapping it inside the nucleus. Similarly within the RNA-seq data sets we found several missense and in frame mutations for XPO5 in breast cancer tumors suggesting to explore whether there were differences in XPO5 localization among normal and breast cancer cell lines. In order to evaluate the XPO5 localizion we performed the immunofluorescent assay (IF). Briefly, cells grown on coverslip slides were stained with XPO5 antibody followed by nuclear DAPI staining. As shown in the Figure 2C, GFP staining for XPO5 was detected in cytoplasm and nucleus for all of the cell lines suggesting no differences in XPO5 function between normal and breast cancer cells. To explore whether overexpression of XPO5 support the proliferation of breast cancer cells and knocking down XPO5 might suppress the proliferation of MCF-7 cells we performed the cell proliferation assay. Briefly MCF-7, transfected for 48h with XPO5 (pcDNA-3.1 XPO5) or knockdown XPO5 using shRNA (n=8) were subjected to MTS assay. Cells that were knockdown for XPO5 expression showed significant lower proliferation compared to the XPO5 transfected MCF-7 cells suggesting tumor promoting role of XPO5 in breast cancer cells (Figure 2D).

We next sought to determine whether XPO5 gene copy number alteration (GCN increase) might be affecting the overall survival of breast and kidney cancer patients. Using the TCGA RNA-seq data that only contains patient tumor samples, we first evaluate the breast invasive carcinoma data set that contained a total of 1098 patient samples. We measured the copy number alteration (CNA) for XPO5 gene which were around 2.8% with the gene amplification of around 1.91%. Evaluating the overall survival for patients with XPO5 copy number alteration suggested a negatively association between the two variables, although the log rank p- value didn't meet statistical significance p=0.165 (Figure 2E) which might be because of the sample size and the analysis were done between the breast cancer patients

Anti-proliferative role of XPO5 in Kidney Cancers

Based on the staining pattern of XPO5 in multiple cancers TMA and from the TCGA, RNA- Seq data sets we observed a significant lower XPO5 expression in kidney cancers. In order to confirm these data in cell line models, we evaluated the expression pattern of XPO5 in two commercially available renal cancer cell lines (786-0 and A498) and a normal transformed human



F

Tissue	Normal	Malignant
Breast	1.00	6.33 (+/- 2.5)
Esophagus	1.00	6.33 (+/- 2.5)
Ovary	1.00	10.00 (+/- 2.8)
Prostate	2.00	3.5 (+/- 0.7)
Lung	4.00	6 (+/- 1.54)
Lymph node	3.00	7.33 (+/- 1.2)
Bladder	4.00	7.6 (+/- 3.8)
Small Intestine	3.00	6.8 (+/- 3.8)
Adrenal Gland	3.00	2.75 (+/- 2.4)
Kidney	9.00	2.00 (+/- 1.4)
Stomach	9.00	5.67 (+/- 3.5)
Brain	0.00	1.00(+/- 2.1)
Rectum	9.00	9.66 (+/- 2.08)
Colon	8.00	7.75 (+/- 1.25)
Liver	6.00	6.75 (+/- 2.2)

Immunoreactivity scores:
9-12: Strongly positive
6-8: moderately positive
2-4: weakly positive
0-1: Negative

Figure 1. The mRNA Expression of XPO5 in the TCGA RNA-seq Data Sets for Pan Cancer and Normal Tissues. P-values for average XPO5 expression difference between tumor and normal samples in pan-cancer were BLCA: 3.86 e-12, BRCA: 1.24 e-50, COAD: 8.45 e-35, HNSC: 3.55 e-08, KICH: 2.44 e-06, KIRC: 4.53e-05, LUAD: 9.82 e-54, LUSC: 5.20 e-47, PRAD: 4.69 e-10, THCA: 1.98 e-06, UCEC: 1.37 e-20 (A). Copy number alterations (CNA) in multiple cancer identified from the RNA-seq TCGA data sets (B). Dicer mRNA expression for Pan Cancer and normal tissue. The precomputed p-values for average Dicer expression difference between tumor and normal samples were, BLCA: 0.017, BRCA: 4.29 e-15, COAD: 2.06e-05, HNSC: 0.0341, KICH: 0.080, KIRC: 2.221e-32, LUAD: 4.02 e-09, LUSC: 0.93, PRAD: 0.32, THCA: 1.740 e-17 and UCEC: 0.002 (C). Drosha mRNA expression in multiple normal and pan cancer tissues (D) Multiple organ tissue microarray (TMA) for cancer and normal tissues (E), along with the German immunoreactive score indicating low staining in Kidney cancer compared to the breast and ovarian carcinomas (F).

embryonic kidney 293HEK cells line. The RECA-7 cell line that was developed in the Dr. William Isaacs lab (Johns Hopkins School of Medicine) from a renal cancer patient was also evaluated for XPO5 expression. Using qRT-PCR

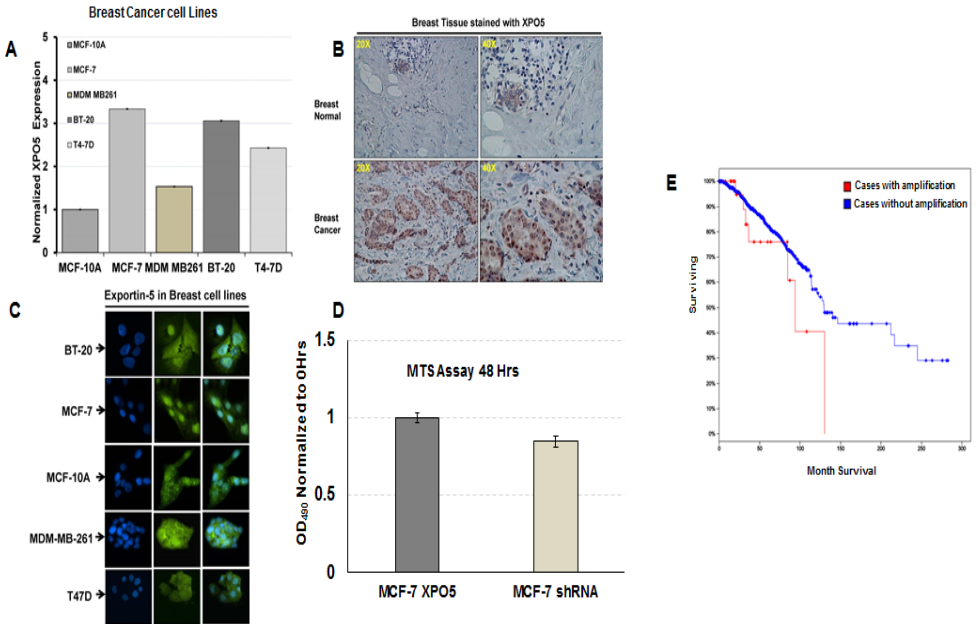


Figure 2. Multiple Breast Cancer Cell Lines Expressing Higher Levels of XPO5 mRNA Compared to the Normal MCF-10A (A). IHC staining for XPO5 in breast cancer and normal adjacent tissue (B). Immunofluorescent microscopy for XPO5 expression in multiple breast cancer cells and normal MCF-10A cell line (C). MTS assay for MCF-7 cells that were transfected to overexpressed XPO5 or knockdown using shRNA against XPO5. * indicated p≤ 0.05 (D). Survival data in breast cancer patients with copy number alteration (CAN) having 1.91% amplification for XPO5 (red) and without CNA in XPO5 gene (blue), log rank p- value p=0.165 (E).

analysis we evaluated the relative mRNA expression of XPO5 among these cells lines. A prostate cancer cell line (C4-2) was included as reference for positive control. As shown in the Figure 3A there was a significant lower expression of XPO5 in three renal cancer cell lines compared to the normal 293HEK cells or C4-2 prostate cancer cells. These data were independently validated by the IHC experiment (Figure 3B). We also studies the expression pattern of XPO5 in these cells (293 HEK, 786-0 and A498) using immunofluorescent microscopy. Interestingly, we found while there was no significant differences between the nuclear and cytoplasmic intensity among 293HEK and 786-0 RCC cell lines, a significant lower cytoplasmic staining of XPO5 was observed in A498 cell line suggesting the dysregulated function of XPO5 in A498 cell lines (Figure 3B). To further

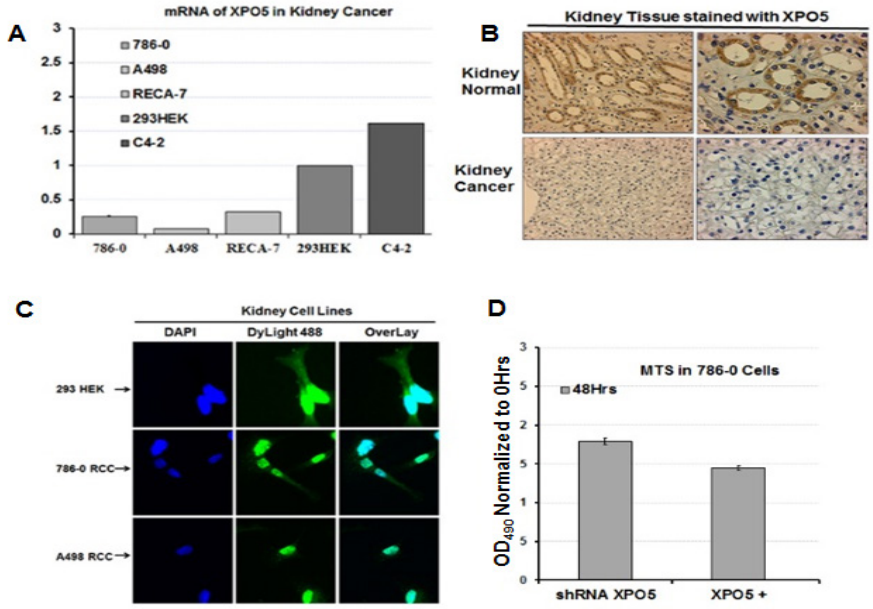


Figure 3. Kidney Cancer Cell Lines and Normal Transformed 293HEK Cell Line Showing Differential mRNA Expression of XPO5 Gene. C4-2, a prostate cancer cell line was included as a reference for positive control (A). IHC staining for XPO5 in kidney cancer and normal adjacent kidney tissue (B). Immunofluorescent microscopy for XPO5 expression in multiple Kidney cell lines (C). MTS assay in 786-0 kidney cancer cell line that were either knockdown with shRNA or transiently overexpressed using plasmid (pcDNA 3.1- XPO5), * indicated p≤ 0.05 (D).

demonstrate the tumor promoting or inhibitory role of XPO5 in kidney cancer cells, we selected the 786-0 RCC cell line that showed both nuclear and cytoplasmic staining for XPO5 and overexpressed (pcDNA 3.1- XPO5) or knockdown XPO5 using shRNA against XPO5 in 786-0 kidney cancer cells. MTS assay was performed to evaluate the proliferation differences between the XPO5 overexpressed or knockdown 786-0 kidney cancer cells. As shown in the Figure 3C overexpression of XPO5 in 786-0 cell significantly suppress the proliferation of 786-0 cells. On the other hand, knocking down XPO5 support the proliferation of these cells. Taken together, these data suggest an inhibitory role of XPO5 in renal cancer cells. Further studies are needed to fully understand the molecular mechanism responsible for the tumor suppressive role of XPO5 in kidney cancers.

Discussion

While significant advances has been made in the diagnosis of cancers, the prognosis for advanced cancers still remain quite disappointing (Stone and Lund, 2007; Tavares et al., 2017). Each year, millions of people that are diagnosed with advanced cancers will eventually scramble to the disease. Therefore a detailed molecular understanding of cancers and cancer related pathways are needed to design specific therapies to target cancers. The non-coding miRNAs has been shown to play important regulator roles in the progression of many disease including cancers (Martinez and Peplow, 2017; Qian et al., 2017; Gao et al., 2017). MicroRNAs are 19-22 nt small RNA moieties that are initially transcribed as long, 5'-capped, polyadenylated (Xie et al., 2013), known as primary miRNAs (pri-miRNAs). Within these transcripts, the mature miRNA sequences are locked in ~60-80 nucleotide hairpin structures (Wahid et al., 2010). The canonical processing by the RNase III enzyme Drosha, and its co-factor DGCR8, removes the precursor hairpin (pre-miRNA) from the pri-miRNA transcript (Yeom et al., 2006). The resulting pre-miRNA is then exported by a uniquely involved XPO5 protein out of the nucleus to the cytoplasm where the RNase III enzyme Dicer performs a second cleavage to generate a double-stranded 21-23 nucleotide RNA molecule. The RNA-induced silencing complex (RISC), then associates with the RNA duplex (Pratt and MacRae, 2009), retaining one RNA strand, the mature miRNA which then sequence-specifically targets complementary messenger RNAs, leading to mRNA cleavage or translational silencing. It is currently believed that the degree of complementarity between the miRNA and its target mRNA determines the method of silencing (Li and Rana, 2012) i.e. perfect identity leads to cleavage and imperfect matching to translational repression.

Several studies have demonstrated a global down regulation of mature miRNAs and the shortening of 3'UTR in proliferation cells leading to dysregulated suppression of the mRNA transcripts in cancers (Hoffman et al., 2016). Very recently, we and other have evaluated the miRNA regulatory machinery in cancer progression and found that overexpression of XPO5 and DICER protein might be responsible for the dysregulated functions of miRNA regulation control (Hoti et al., 2017; Bennasser et al., 2011; Iwasaki et al., 2013). While our initial studies were confined to only prostate cells and prostate cancer tissues, in this study we evaluated a number of cancer tissues to determine whether XPO5 might have a differential expression in tumor types. Using cancer TMAs and the TCGA RNA seq-data sets we found overexpression of XPO5 in several tumors (Breast, Ovary, Prostate, and Bladder) compared to the normal adjacent while in some tumors (Kidney and adrenal gland) we found a significant lower expression of XPO5 suggesting a tissue specific differentially regulation for miRNA (Figure 1A and C). While the expression of XPO5 has been previously shown to hamper by genomic mutation (Melo et al., 2010; Melo and Esteller, 2014), we also evaluated the copy number alteration in different tumor types. Using the TCGA RNA-seq data sets we found some missense and frameshift mutation in several tumors, however, the frequencies of which were almost negligible. Mutation within the XPO5 gene has been shown to trap the protein inside the nucleus with a profound impact on the levels of mature miRNAs. Using florescent microscopy we evaluated the nuclear and cytoplasmic expression pattern of XPO5 in several breast and normal cell lines however, we did not observed any staining differences among the nuclear and cytoplasmic compartment between the breast cancer and normal cells. Interestingly, in one of the kidney cancer cell line (A498) we observed some staining differences that need to be validated by genomic analysis. We further validated the expression patterns of XPO5 in breast and kidney cancer cell line models. Using a quantitative qRT-PCR we found a significant higher expression of XPO5 in breast cancer cells compared to the normal MCF-10A cells, while on the other hand a significant lower XPO5 expression was detected in kidney cancer cells when compared to the normal transformed 293 HEK cells (Figure 2A and 3A).

Based on the functions of XPO5 in the maturation biology of miRNAs, it was characterized as a tumor suppressor protein (Melo and Esteller, 2011). However, we and other have shown the overexpression of XPO5 in cancers was oncogenic with tumor promoting properties (Hoti et al., 2017; Shigeyasu et al., 2017). In order to understand whether overexpression of XPO5 in cancer cells were inhibitory or if excess levels of XPO5 were supporting tumor growth we overexpressed or knockdown XPO5 in breast and kidney cancer cell lines. Interestingly we found that excess levels of XPO5 in breast cancer were promoting cellular proliferation while in kidney cancer cell line it was suppressing cellular proliferation, suggesting a dual, oncogenic and tumor suppressive role for XPO5 in different tissue types. While these findings are preliminary in nature, they open up a series of questions as to whether the levels of mature miRNAs and the processing between the two tissues might be different. Similarly, the tumor suppressive or oncogenic miRNAs that regulate the mRNA targets needed to be validated across different tissue types. In conclusion, we have shown that XPO5 might be playing a dual role in promoting cancer in some tissues while in others it might have a tumor suppressive role.

Cell Culture and reagents

Human kidney cancer cell lines 786-0, A498, RECA-7 and 293HEK cells were obtained from American Tissue Type Culture Collection (Manassas, VA). Human breast cancer cell lines MCF-10A were provided by Dr. Dipali Sharma (Johns Hopkins University) while the C4-2 prostate cancer cells were obtained from Dr. William. B. Isaacs lab (Brady Urology Institute, The Johns Hopkins University). The breast cancer cell lines MCF-7, MDM MB261, BT-20 and T4-7D were a kind gift from Dr. Zafar Nawaz (University of Miami, Miller school of Medicine). All cells were grown as in RPMI-1640 or DMEM media (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) with 5 µg/mL ciprofloxacin hydrochloride (U.S. Biological, Swampscott, MA), and 50 µg/mL gentamicin (Quality Biological, Inc., Gaithersburg, MD) as recommended by the supplier. Cells were passaged twice a week using 0.05% trypsin/0.53 mmol/L EDTA (Cellgro) at the confluency of around 70 to 80%. All cells were maintained at 37 °C in an atmosphere containing 5% CO2. The anti-XPO5 polyclonal Ab (HPA018402) for the TMA staining (dilution 1:200) and Anti-mouse IgG HRP-conjugated (1:1000) were obtained from Sigma Aldrich, St Louis, MO). Majority of all other chemical reagents and compounds were ordered from Sigma, unless otherwise specified.

Tissue Microarray and Staining

Multi-tumor tissue microarray TMA (ab178234) was obtained from abcam (San Diego, CA) containing 95 cases in formalin fixed paraffin embedded 1.5 mm cores. Using the primary antibodies to XPO5 (Sigma Aldrich polyclonal Prestige Antibodies, diluted 1:1,000) slides were stained using citrate buffer antigen retrieval protocol. Appropriate positive controls (LAPC4 cells human prostate cancer cell line overexpressing a wild type XPO5 plasmid) were run concurrently. Similarly, cell lines were made by knocking down XPO5 with shRNA again the XPO5 as negative control, beside controls, mock sections were also treated in an identical fashion except for replacing the primary antibody with non-immune rabbit IgG. All these stains were manually scored separately in the nucleus/nucleolus and cytoplasm of (cancer, non-neoplastic) cells by one pathologist who was blinded to patient identity. Immunoreactive Scores were calculated by the percentage of immunoreactive cells (0% = 0; 1-10% = 1; 11-50% = 2; 51-80% = 3; 81-100% = 4) by staining intensity (negative = 0; weak = 1; moderate = 2; strong = 3). Cores with the immunoreactive score of 0 or 1 were considered negative (0), and those with the immunoreactive scores of 2-4, 6-8, and 9-12 were considered weakly positive (1+), moderately positive (2+), and strongly positive (3+), respectively.

Q-RT PCR analysis

mRNA (1 µg) from cancer cells were reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen). Sybr green–based real-time qRT-PCR was performed using SYBR GreenER qPCR SuperMix (Invitrogen) according to the manufacturer's instructions. All reactions were done in triplicate. Standard curves were generated by serial dilution of each sample, and the relative amount of target gene mRNA was normalized to Actin mRNA. Sequences of the primers for XPO5 and Actin was published previously (Hoti et al., 2017).

MTS assay

The metabolic viability of the cells was monitored using 3-(4,5)-Dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS assay kit CellTiter 96) from Promega (Madison, WI) as described by (Hoti et al). Briefly cells were seeded onto 96-well plates and cultured in the presence of test agents for indicated time intervals. MTS solution was added, and cells were incubated for 2h at 37 °C Formazan formed from the reduction of MTS was quantified by measurement of absorbance of the medium at 490 nm using a microplate reader (All data have been normalized to the background signals).

Fluorescence microscopy

The immunofluorescence method was performed to evaluate the XPO5 localization in Kidney and breast cancer cells. Briefly, cells grown on poly-d-lysine coated glass coverslips in the presence of complete or cFBS containing media were fixed with 2% paraformaldehyde-PBS for 10 min. After fixation, cells were washed three times with PBS, followed by permeabilization in 0.1% Triton X-100 for 5 min. The fixed and permeabilized cells were blocked in 1% bovine serum albumin in PBS for 15 min, followed by a 1h incubation with the primary antibodies against XPO5. After washing three times with TPBS cells were then probed with secondary antibodies (FITC-conjugated anti-rabbit IgG), and the DNA was stained with DAPI for 10 min. Images were taken on Zeiss Axio Observer 40X fluorescence microscope (Carl Zeiss, Jena, Germany). Figures were constructed using ZEN imaging software and Adobe Photoshop (Adobe Systems, CA).

Statistical analysis

The MTS assay and the qRT-PCR experiments were done in triplicate or quadruplicate. All statistical analyses were performed using Microsoft Excel running on an IBM-PC compatible computer on the Windows 7 operating system. Statistical significance was defined as a P-value < 0.05.

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