Background: cancer, tissue microarray, tramp, polysome background: about

Background: cap-dependent translation is necessary due to high protein requirement in cancer. An interaction between EIF4E and EIF4G is crucial for EIF4F complex formation and initiation of cap-dependent translation. In the present study, we analyzed Human prostate cancer tissue microarray(TMA) and mRNA data for EIF4G1 in clinical datasets, and prostate tumor tissue from TRAMP(Transgenic Adenocarcinoma of Mouse Prostate) model. We also assessed the functional role of EIF4G1 in commonly used PCa cell lines.

Methods: TMA was used to analyze the EIF4G1 protein levels in patient samples and mRNA data for EIF4G1 was analyzed from TCGA and Trento/Cornell/Broad clinical data sets. PCa cells LNCaP, C4-2b, 22Rv1, DU145, PC3 and human prostate cells RWPE-1 were used. For an in-vivo model of PCa, we used TRAMP and wild-type mouse.

Loss of function studies was performed by using siRNA/shRNA. Real-time(RT) PCR and Western Blot analysis were used to quantitate relative mRNA and protein levels respectively. Analysis of polysome was performed by sucrose density gradient fractionation and Polysome-to-Monosome(P/M) ratios were determined. Cell cycle, cell proliferation/migration, and Clonogenic activity were measured by standard methods. Results: TMA analysis showed that protein levels of EIF4G1 are high in PCa as compared to normal prostate tissue, and there is a graded increase in EIF4G1 as the disease progresses.

TCGA dataset revealed that EIF4G1 positively correlated with higher tumor grade and stages and Trento/Cornell/Broad dataset showed that 43% of castration-resistant prostate cancer(CRPC) patients have EIF4G1 mRNA up-regulation. PCa cells express a significantly higher level of EIF4G1 as

compared to normal prostate cells. Similarly, prostate tumor tissue from TRAMP tissue showed higher EIF4G1 expression as compared to normal wild-type prostate tissue. There is a shift in P/M ratio with the siEIF4G1 knockdown. Silencing of EIF4G1 causes decreases in Cyclin D1 and p-Rb levels and G0/G1 cell cycle delay and impaired Clonogenic activity as well as cell proliferation. RT-PCR data suggest that EIF4G1 knockdown decreases the level of EMT markers and limits the cell migration. Conclusions: Taken all together, our data indicate that EIF4G1 may function as an oncoprotein and is a novel target for intervention in PCa and CRPC.

Keywords: EIF4G1, Prostate Cancer, Tissue Microarray, TRAMP, Polysome

Background: About 161, 360 new prostate cancer cases has been predicted for this year 1. Current treatment for localized tumor is surgery or chemotherapy and for metastases tumor, the primary target is Androgen receptor, which is the key molecular driver of the disease. Despite different treatment options disease is advanced and thus further giving rise a different phenotype of the disease i.

e. castration-resistant prostate cancer in due course of time, which is unmanageable by current therapy and leads to estimated 26, 730 deaths in 2017 1. Thus, new treatment options are highly warranted for better prognosis of the disease. Translational control is critical for any cancer cell growth and progression, which requires a high protein synthesis levels and translation of specific mRNAs that are responsible for different tumorigenic properties. Cap-dependent translation is essential to maintain high protein synthesis in rapidly dividing cancer cells. Translational control occurs predominately during a rate-limiting, initiation step which is subjected to

extensive regulation 2, 3 and is governed by cap-binding complex, eukaryotic initiation factor 4F (eIF4F) which is composed of cap-binding protein eIF4E, eIF4A (helicase) and eIF4G (scaffolding protein). In the normal condition of cell EIF4E is constrained 4E-BPs and thus inhibits its binding with eIF4G, thus inhibiting the eIF4F translation initiation complex formation 5.

An interaction between eIF4E and eIF4G is crucial for the formation of the eIF4F complex and initiation of cap-dependent translation 4. EIF4G family comprises of three isoforms eIF4G1, eIF4G2 and eIF4G3 6, 7 among which eIF4GI is the major isoform (> 85%) 8. Isoforms eIF4G11, eIF4G2 and eIF4G3 have 50% identity and their genes have been mapped to 3q27. 1, 11p15 and 1p36. 12 respectively 7. Isoforms eIF4G1 and eIF4G3 are involved in cap-dependent translation, while eIF4G2 is associated with IRES-dependent translation in cells 6, 9. Several studies have shown that eukaryotic translation initiation factor 4 gamma 1 (EIF4G1) is overexpressed in different cancers such as breast cancer, lung cancer, nasopharyngeal cancer, cervical cancer, multiple myeloma and ovarian cancer 10-17 and related to tumorigenesis and pathogenesis 13. It’s known that interaction of EIF4G1-EIF4E not only govern the protein synthesis but also its quality and thus contribute to the cell phenotype and function.

18. Chromosomal location of eIF4G1 (3q27. 1) is known to amplified in PCa patients 19.

Although recent studies have demonstrated that EIF4G1 plays important roles in different cancers, its functional role in Prostate Cancer (PCa) is unreported. We wanted to explore the functional role of EIF4G1 in PCa cells.

We focused on the role of EIF4G1 in the disease progression, translation control, cell cycle distribution and cell characteristics such as growth and proliferation and migration in PCa. Materials and Methods: Materials: All chemicals were purchased from Sigma-Aldrich. Antibodies for EIF4G1 (Cell signaling #2498); Cyclin D1 (Santa Cruz sc-20044); pRb (Cell signaling #9308); ?-tubulin (Developmental Studies Hybridoma Bank E7); ?-actin (Sigma A2228) were used to probe respective proteins of interest. Human Prostate tissue Microarray & Immunohistochemistry (IHC): Tissue Microarray for prostate (US Biomax, Inc.

#PR1921a) was used to see the EIF4G1 protein levels in the patient sample. This TMA contains 80 cases of adenocarcinoma, 8 adjacent normal prostate tissues and, 8 normal prostate tissues. Following lab protocol antigen-retrieval was done, followed by blocking in 3% BSA and incubated with EIF4G1 antibody overnight in the humidified chamber at 4°C.

Further tissue section was incubated with the biotinylated secondary antibody, followed by DAB staining and counterstained with hematoxylin (Sigma-Aldrich). Images for representative tissue sections were taken by Olympus BX51 microscope at the magnification of X20 and X40 using CellSens Entry. Above IHC protocol was also used for EIF4G1 staining with wild-type as well as 30-week old TRAMP prostate tissue. TCGA data mining and retrieval of data from the clinical data set: mRNA expression and clinical data from TCGA (The Cancer Genome Atlas) data set for the prostate cancer and normal samples were analyzed by UALCAN (http://ualcan. path. uab. edu/) 20 web server and TCGA database.

The analysis was done on 497 primary tumors of prostate cancer and 52 normal samples from TCGA. Using a gene name (or more), the web server will mine the data available for the gene expression with cancer stage, Gleason score and survival analysis and statistical significant p-value for each group/subgroup analysis. To study gene expression changes from different clinical dataset we used freely accessible cBioPortal (http://www. cbioportal. org) tool 21, 22. All prostate tumors with mRNA expression data (n= 114) from the Neuroendocrine Prostate Cancer dataset 23 using a mRNA Z-score threshold of ± 2 as compared with normal prostate samples was used for EIF4G1.

Genetic alterations in percent mRNA upregulation were taken consideration for the present study. Cell Line/Culture & Tissue samples: Prostate cancer cell lines LNCaP, C4-2b, 22Rv1, DU145 were cultured in RPMI 1640 complete medium (Hyclone: Cat No.: 30255.

01) and PC3 cells were cultured in DMEM/F12 with 10% v/v Fetal Bovine Serum, 1% v/v Antibiotics (Penicillin and Streptomycin). The normal Prostate cell line RWPE-1 was cultured in Keratinocyte-SFM (ThermoFisher Cat No.: 17005042) with EGF 1-53 (Epidermal Growth Factor 1-53) and BPE (Bovine Pituitary Extract).

The cells were incubated at 37°C in a 5% CO2 humidified atmosphere. Further Prostate tumor tissue of 30 Week old Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice and prostate tissue from wild-type mice were used in present study. Polysome profiling: Polysome-bound RNA fractionation was done by the method described somewhere else 24 with

modifications. 10×106 cells for LNCaP and C4-2b siControl and siEIF4G1 cells were used per sucrose gradient. Briefly, before harvesting, cells were pulsed with 100 µg/ml of Cycloheximide for 10 minutes. And lysed in PL Buffer (Polysome lysis) containing 20 mmol/L Tris-HCl (pH 7. 5), 250 mmol/L NaCl, 15 mmol/L MgCl2, 0.

5% NP-40, 100 µg/mL Cycloheximide, 2 mmol/L DTT, 50 µg/mL heparin, and 200 U/mL RNasin (Promega) and homogenized. Lysing 15 minutes on ice, cell lysates were centrifuged and the supernatant was loaded onto a 10% to 60% sucrose gradient tube. Tubes were centrifuged at 35, 000g for 3 hours at 4°C and fractions were collected using Density Gradient Fractionation System by ISCO with continuous monitoring based on an absorbance at 254nm. To calculate polysome to monosome ratio graph were scanned and pixels of polysome and monosome were measured with tpsUtil64 and tpsDIG2w64 software. (http://life. bio. sunysb. edu/morph/).

Immunoblotting: Immunoblotting for EIF4G1 Protein expression was done in above PCa cell lines as well as in normal prostate tissue and in TRAMP prostate tumor tissue. Further immunoblotting for EIF4G1/pRb/CyclinD1 was done on siControl and siEIF4G1 in LNCaP & C4-2b cells. Blot was scanned for the respective protein of interest and loading control protein by LI-COR Odyssey CLx (LI-COR, Lincoln, USA) system by using IRDye 680 goat anti-mouse and IRDye 800 goat anti-rabbit secondary antibodies or HRP conjugated secondary mouse and rabbit and developed by the film.

siRNA/shRNA Mediated EIF4G1 knockdown: For the loss of function studies, we knockdown EIF4G1 in cells, we used siRNA for EIF4G1 (human) from Santa Cruz (sc-35286) and non-targeting siRNA-A as a negative control from

Santa Cruz (sc-37007) And we also used shRNA vector control (SHC001) and shRNA for EIF4G1 (SHCLNG-NM_182917, TRCN0000061770) from Sigma-Aldrich. Transfection of this siRNA/shRNA was performed in six-well plates using the HiPerFect transfection reagent (Qiagen, CA)/Lipofectamine® 200 reagents (Life Technologies, Invitrogen) respectively as per manufacturer's protocol. Effect of knockdown was checked by immunoblotting. Flow cytometry: Cell Cycle Analysis was done for siControl and siEIF4G1 in LNCaP and C4-2b cells as mentioned earlier 25.

Cell cycle distributions were analyzed by using BD LSR II Flow Cytometer and ModFit LT Software was used for analysis. RT-PCR: Total RNA was extracted from siControl and siEIF4G1 in LNCaP and C4-2b cells using a commercially available RNA isolation kit (OMEGA), followed by cDNA synthesis from 1µg of RNA. Further RT-PCR was done for EIF4G1/CyclinD1/EMT related genes such as N-Cadherin/Vimentin/Snail and Zeb 1. Cell proliferation and viability: Cell proliferation and viability assays were done on LNCaP& C4-2b siControl and siEIF4G1 for 24/48/72/96 h by MTT assay and Crystal Violet stating respectively as described previously 25. Clonogenic Assay: LNCaP and C4-2b cell transfected with siEIF4G1 or siControl (500 cells/well) were seeded in 6-well plates. After 14 days, the cells were stained with 0.

4% Crystal violet solution for 1hr at room temperature, wash and dry the plates and visible colonies were counted by ImageJ software. Cell Migration Assay: Trans-well cell migration assay was done for vector control and shEIF4G1 in LNCaP and C4-2b cells as mentioned earlier with some modifications 26. Briefly, a single cell suspension of VC and shEIF4G1 for LNCaP and C4-2b cells were seeded on 8-? m-pore size insert in a Transwell.

A total of 2×105 cells in 300 µl of 1% serum medium were seeded and media containing 20% FBS (600 ? l) was added to the lower chamber.

After 24 hr of migration time, transwell was stained with 0. 4% crystal violet. The upper side of the transwell chamber was cleaned with cotton before drying and Images of migratory cells on the underside of the transwell were captured using above mentioned microscope at 10X magnification. The migratory cell was counted by counting four fields per stained membrane. Statistical analysis: Data are expressed as means ± standard deviations (SD). The two-tailed Student t-test and ANOVA test were used for statistical analysis of experiments and GraphPad Prism5 was used for statistical analysis. Significant differences in p-values are indicated as *<0.

05, **<0. 01, ***<0. 001. Results: EIF4G1 is overexpressed in clinical samples of PCa: We first analyzed the for EIF4G 1 protein levels in a TMA slide.

We take account high level of EIF4G1 if more than 60% field showed positive staining, expression was medium if it ranges from 40-60% positive EIF4G1 staining and expression were counted as low if less than 40% area showed positive EIF4G1 staining. EIF4G1 protein is present at very low/undetectable level in normal prostate while a high EIF4G1 level was detected with high-grade tumor. Moreover, EIF4G1 protein levels showed a graded increase as the disease progresses to an advanced stage. Analysis of TMA showed that 0000% tissue have high EIF4G1 in higher grade samples and 000% of the tissue have positive staining for EIF4G1 in medium grade tumor samples and low-grade tumor sample EIF4G1 expression was low (%), (Figure. 1B). For

low-grade tumor sample, 00% tissue scored positive for EIF4G1 while high-grade tumor showed an increase in 000% staining.

Summarizing all data from TMA indicates that there is a significantly positive correlation between expression of EIF4G1 and disease phenotype and aggressiveness (Figure. 1C). Further, we analyzed data from TCGA, which includes 497 primary PCa samples and 52 normal prostate tissues with help of ULCAN web server. We found overexpressed mRNA level of EIF4G1in primary tumor compared to normal prostate tissue with the significantly high p-value (p= 1.

62E-12) (Figure 2A) and we also analyzed EIF4G1 expression in a paired sample (n= 52) from TCGA database (Figure 2B). We observed a graded increase in EIF4G1 mRNA expression with increasing tumor grade (Gleason Score) with a significant p-value with comparison between normal to Gleason Score (GS) 6 (p= 1. 56E-05), GS 7 (p= 1. 62E-12), GS 8 (p= 6. 52E-13), GS 9 (p <1E-12) and GS 10 (p= 2.

83E-04) Figure 2C). Patients with high EIF4G1 expression has less survival (Approx. 9. 58 years) compared to the patient with low/Medium EIF4G1 expression (Approx. 13.

69 years) (p <0. 0036) (Figure 2D). Furthermore, we also looked the expression of EIF4G1 in publically available clinical data set for patients with CRPC and neuroendocrine phenotype through the cBioPortal web server. Using cBioPortal, we investigated the mRNA expression data of EIF4G1 from the Neuroendocrine Prostate Cancer (Trento/Cornell/Broad 2016) dataset (n= 114) using Z-score threshold of ± 2. We observed that 59% of patients have

genetic alteration such as amplification and mRNA upregulation in EIF4G1. Among which 28% patients have amplification in EIF4G1 gene and 43% patients' accounts for mRNA upregulation of EIF4G1 (Figure 2E).

Interestingly we found that there is no change in EIF4E in these patients. Overall, these data suggest that the alterations/changes in the EIF4G1 expression may be associated with changes in cellular functions that may affect patients’ outcome. EIF4G1 expression in PCa cell lines and in tissue sections from the in-vivo model of PCa: We checked the EIF4G1expression in different PCa cell lines viz. LNCaP, C4-2b, 22Rv1, DU145, PC3 and in TRAMP prostate tumor tissues. We found that EIF4G1 expression was high in all PCa cell lines used in the present study when compared to normal human prostate cell line, RWPE-1 (Figure 3A). Further, we also analyzed the EIF4G1 mRNA expression level in these cell line and found a significant increase with LNCaP, C4-2b, DU145, and PC3 (Figure 3B). Furthermore, we found that EIF4G1 expression was significantly up-regulated in 30-week old TRAMP prostate tumor tissues ($p < 0.0001$) when compared to the normal wild-type prostate tissue in western blot (Figure 3C).

Further, we also did the immunohistochemistry for EIF4G1 in 30-week old TRAMP prostate tumor tissues and wild-type tissue and found a significantly high EIF4G1 with TRAMP prostate tissue ($p < 0.0001$) (Figure 3D). Taken together these data suggest that EIF4G1 may have an important role in PCa progression. Knockdown of EIF4G1 affected the polysome profiling: Polysome profiles generated from LNCaP & C4-2b cells with siEIF4G1 or siControl suggests that EIF4G1 play important role in translation initiation step in PCa cells and decrease in polysome peak based on an absorbance at 254 nm for

siEIF4G1 in compare to siControl, and allowing ribosome run-off when EIF4G1 was knockdown and causes a shift from heavier fractions of polysomes to monosome fraction. For global translation activity, estimation of Polysome-to-Monosome (P/M) ratio was determined.

There is a change in polysome (P) to monosome (M) ratio from siControl (0. 95) to siEIF4G1 (0. 72) in LNCaP cells as well as in C4-2b cells P/M ratio of siControl 1.

14 to 0. 93 (Figure 4A & B). These results suggest that EIF4G1 play important role at initiation step in PCa cells. EIF4G1 regulates expression of cell cycle-related genes and is required for cell cycle progressionSilencing of EIF4G1 affected several cell cycle/checkpoint regulatory proteins, including such as reduced Cyclin D1 & p-Rb expression in both cell lines at protein as well mRNA levels (Figure 5A& B). To further investigate the mechanisms of EIF4G1 regulation in PCa cell growth, we assessed the effect of the knockdown on PCa cells, by cell cycle distribution. We found that silencing of EIF4G1 causes G0/G1 cell cycle delay in LNCaP (Top) and C4-2b (Bottom) cell lines (Figure 5C). Taken together, our data suggested EIF4G1 regulates the expression of the cell cycle for PCa growth. EIF4G1 is required for PCa cell growth and colony formation: To study the functional role of EIF4G1 in PCa cell growth, we knock-down the EIF4G1 by using siRNA specifically targeting EIF4G1 in LNCaP & C4-2b cells.

A non-targeting (control)-siRNA was used as a negative control for both cell line. Knockdown of EIF4G1 by siRNA decreases its (EIF4G1) expression when compared to the siControl in LNCaP as well as in C4-2b cells (Figure 6A).

Silencing of EIF4G1 significantly impairs growth/proliferation of LNCaP and C4-2b cells at the different time point (48/72/96h) assay done by crystal violet staining (Figure 6B). Cell viability was done by MTT assay showed a significant decrease in cell viability at the different time point (24/48/72/96h) (Figure 6C). Next, we did the colony formation assay on PCa cells and data indicated that silencing of EIF4G1 effectively impairs the efficiency of colony formation of LNCaP & C4-2b cells (Figure 6D). Concluding these results that EIF4G1 is required for PCa cell growth and colony formation. Knockdown of EIF4G1 Down regulated EMT markers in PCa Cell Lines and limited cell migration: Cell migration is a main feature of EMT, we hypothesized that EMT gene will be down-regulated in EIF4G1 silenced PCa cells. Thus, we set out to examine the expression of EMT markers by RT-PCR in EIF4G1 Knockdown PCa cell lines (LNCaP & C4-2b).

N-Cadherin is known to have a critical role in the EMT. We found a decrease in mRNA levels of N-Cadherin (a mesenchymal marker), Snail and ZEB1 that suppresses EMT (Figure 7A). Cell migration was done by transwell® migration assay on vector control (VC) and shEIF4G1 with LNCaP & C4-2b cells and found a significant ($p < 0.001$) inhibition in cell migration with knockdown of EIF4G1 cells (Figure 7B).

Altogether, our data indicate that EIF4G1 promote PCa cells to EMT and that inhibition of their function facilitates EMT properties of cells. Discussion: Prostate cancer is most common malignancy in men with poor survival rate as diseases progressed. It is now known that RNA-binding proteins (RBP) are an important component of effective protein machinery and regulates the translational efficiency of different mRNAs. EIF4G1, which is an also RBP, acts

as a scaffold protein that together with eIF4E and eIF4A helps to recognize the 5′ end of the mRNA to initiate the translation and help in mRNA recruitment to the ribosome assembly 27. Further EIF4G1 can put together the 5′ and 3′ ends of the mRNA to form a ‘ closed-loop structure by interacting with the other RBPs that play a role in termination and recycling of translation machinery with subsequent translation initiation on the same mRNA 28. Taken account the role of EIF4G1 in cap-dependent translation, it has been shown that overexpression of EIF4G1 has reported in different cancer and played an important role in disease pathogenesis and progression in different cancers 10-17. We analyzed the EIF4G1 protein expression in the human prostate tissue microarray.

We further corroborated our finding in clinical datasets patients with PCa, by data mining in TCGA database and clinical dataset on neuroendocrine PCa (NEPC). We found increases in EIF4G1 in TCGA database and its positive correlation with tumor grade. And in a clinical dataset with NEPC 43% patients have altered mRNA expression. Collectively, results from clinical dataset showed an inverse correlation between the expression of EIF4G1 and clinical outcome of patients with PCa. Further, we evaluated the expression levels of EIF4G1 in prostate cancer cell lines and TRAMP prostate tumor tissue in comparison with normal prostate cells and wild-type mice prostate tissue. Our data showed that EIF4G1 is significantly overexpressed in PCa cell lines as well as in TRAMP prostate tissue (which morphologically represents an advanced form of the disease).

IHC staining for EIF4G1 showed a significant difference between the expression of EIF4G1 with wild-type and 30 Week old TRAMP prostate tissue.

To the best of our knowledge, the role of EIF4G1 in PCa was not investigated yet. This is the first study to describe elevated expression levels and function of EIF4GI in PCa. Further, We also look for polysome distribution based on absorbance at 254nm for siControl and siEIF4G1 in LNCaP and C4-2b cells and found that there is an inhibition of overall protein synthesis as evident by fewer polysome peaks recorded at an absorbance at 254nm compared to siControl in both cell lines. Compared to siControl cells, the P/M ratio decreases ~25% in siEIF4G1 cells in case of LNCaP cells and decreases ~20% in case of C4-2b cells. To further investigate the mechanisms of EIF4G1 regulation in PCa, we assessed the effect of the knockdown on PCa cells, by cell cycle distribution and found that silencing EIF4G1 delays the cells at the G0/G1 phase and affects the cell cycle regulatory protein and mRNA such as Cyclin D1 and Rb. This result was in support from the previous study on lung cancer 10. For the loss of function studies, we used the knock-down approach by siRNA.

To achieve this we use androgen-sensitive LNCaP and androgen insensitive C4-2b cell lines in the present study. We observed that EIF4G1 is required for PCa cell survival/proliferation and colony formation in both cell lines. These results from functional assays in PCa cells were in line with other reported study on different cancer such as lung cancer 10. Furthermore knockdown of EIF4G1 also affected the EMT markers in PCa cells. There was downregulation of N-Cadherin, Snail, and Zeb1 in knockdown cells compared to the siControl cell in LNCaP & C4-2b.

In transwell cell migration assay, we found knockdown of EIF4G1 limits the cell migration in both cell lines. A study in lung cancer also showed a similar

pattern of EMT inhibition in EIF4G1 knockdown cells 12. The beneficial effect of targeting translation lies within its strength to affect the expression of multiple oncogenic pathways that are associated with disease and progression. The potential of targeting translational machinery will help the patients with failure in targeted therapies or can be combined with current therapy in PCa treatment for improving the disease outcome.

Overall, our data indicate that EIF4G1 may function as an oncoprotein and is a novel target for intervention in PCa and CRPC.