

# TR4 nuclear receptor promotes clear cell renal cell carcinoma (ccRCC) vasculogenic mimicry (VM) formation and metastasis via altering the miR490-3p/vimentin signals



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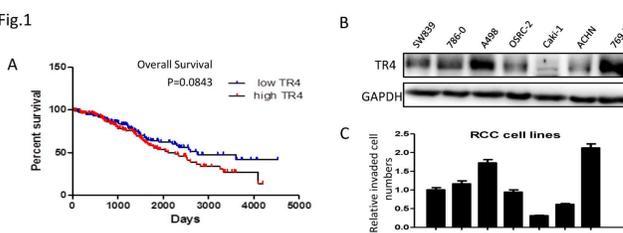
## Abstract

**INTRODUCTION:** While TR4 nuclear receptor plays key roles to promote prostate cancer progression, its roles to alter the progression of clear cell renal cell carcinoma (ccRCC), remains unclear. Here, we demonstrate that TR4 can promote the ccRCC cell vasculogenic mimicry (VM) formation and its associated metastasis via modulating the miR490-3p/vimentin(VIM) signals.

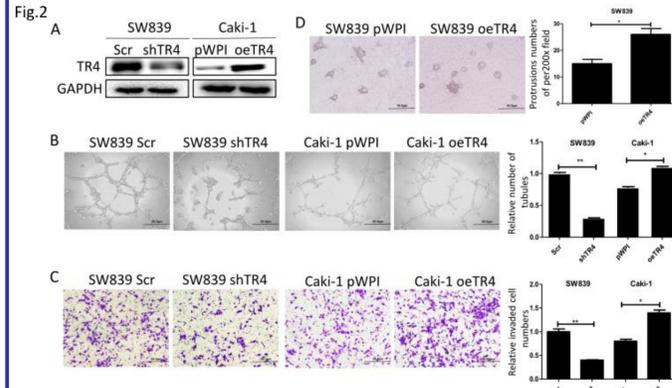
**METHODS:** We first studied a database in TCGA, including gene expression data from 606 primary ccRCC samples to examine the TR4 expression. To further confirm this clinical data in the various ccRCC cell lines (786-0, SW839 and Caki-1), we assayed TR4 expression and applied the Matrigel-coated Transwell invasion assay and 3D invasion assay to examine their invasion capacity. We then assayed its influences on the VM tube formation and cell invasion via altering the TR4 expression. To further dissect the molecular mechanism how TR4 could decrease the VIM expression, we first searched for potential TR4 response elements (TR4REs) on the VIM 5' promoter region using JASPAR database. We then applied the Chip-on in vivo binding assay to examine TR4 binding to these TR4REs. To verify how miR-490-3p can directly regulate VIM expression through targeting its 3'UTR, we identified some potential binding sites located on the 3'UTR of VIM-mRNA and applied the reporter assay with the pscheck2 vector carrying the wild type and mutant miRNA-target sites. To prove the above in vitro cell line data in the in vivo mouse model, we applied the orthotopic ccRCC xenograft mouse model. We generated stable clones of Caki-1 cells with luciferase expression with overexpressed TR4 and/or overexpressed miR-490-3p as well as a control, with 8 mice/group (1: Scr+luc; 2: oeTR4+luc; 3: oemir490-3p+luc; and 4: oeTR4+oemir490-3p+luc). Tumor progressions were evaluated via the non-invasive in vivo imaging system (IVIS). IHC staining from these ccRCC xenograft tumors were also evaluated.

**RESULTS:** Here we provide the first preclinical study data showing that TR4 may play positive roles in RCC VM formation and metastasis. Mechanism dissection revealed that TR4 might increase the oncogene VIM expression via decreasing the miR-490-3p expression through direct binding to the TR4-response elements (TR4REs) on the promoter region of miR-490-3p, which might then directly target the 3'UTR of VIM-mRNA to increase its protein expression. Preclinical studies using the in vivo mouse model with xenografted RCC Caki-1 cells also found that TR4 could promote the ccRCC VM and its associated metastasis via modulating the miR490-3p/VIM signals.

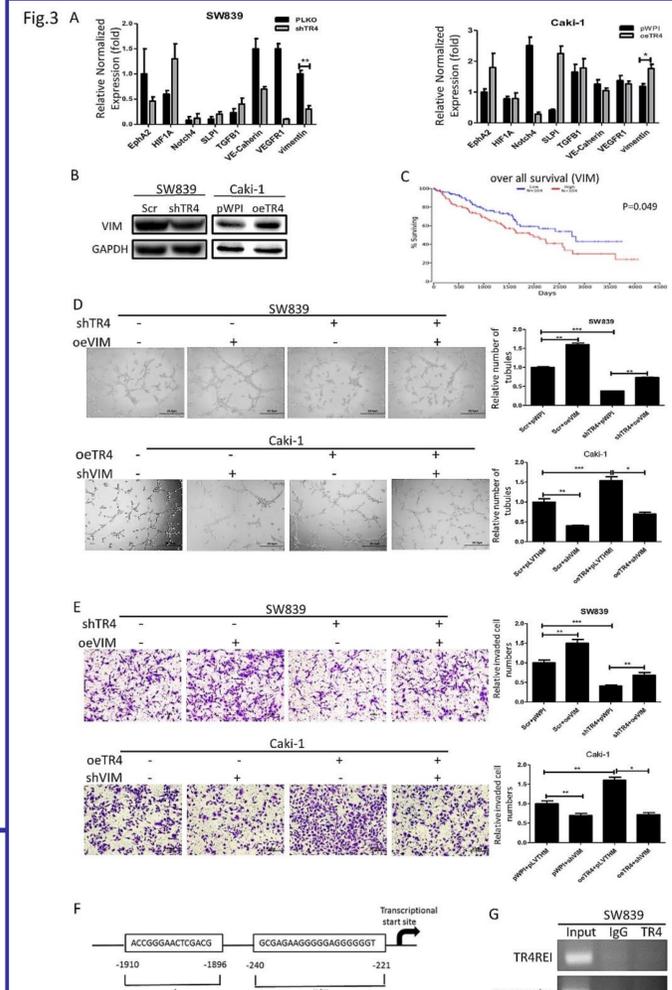
**CONCLUSIONS:** Our results from preclinical studies using multiple RCC cell lines and the in vivo mouse model all conclude that TR4 may play a key role to promote ccRCC VM formation and metastasis and targeting the newly identified TR4/miR-490-3p/VIM signals with small molecules may help us to develop a new therapeutic approach to better suppress the ccRCC metastasis.



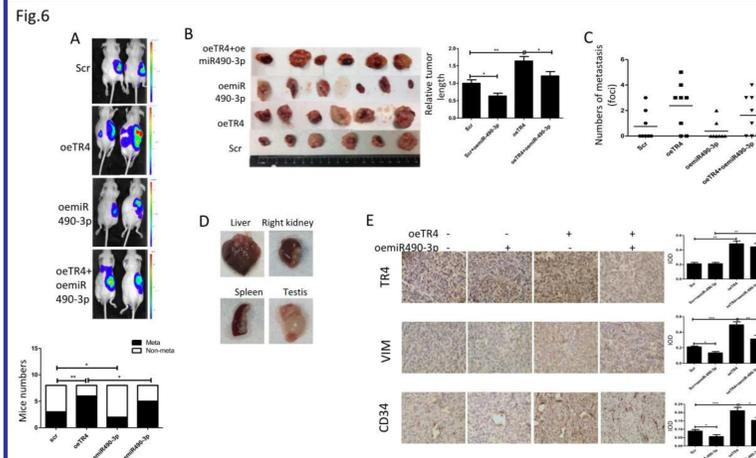
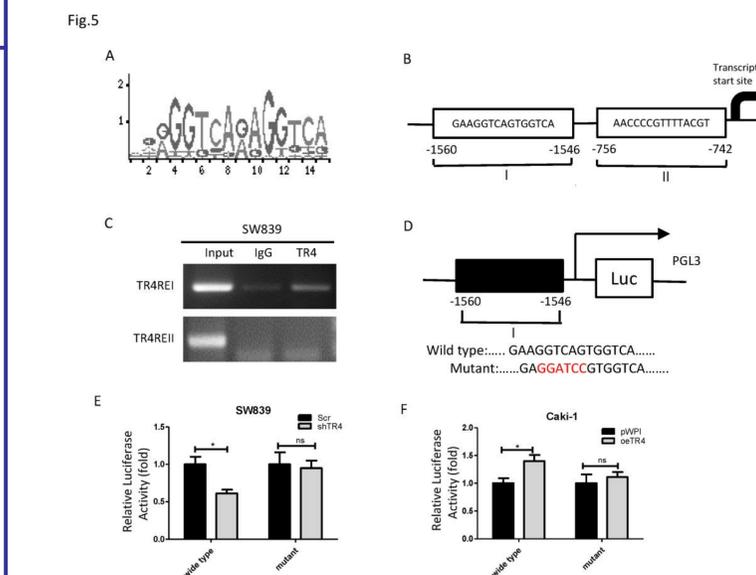
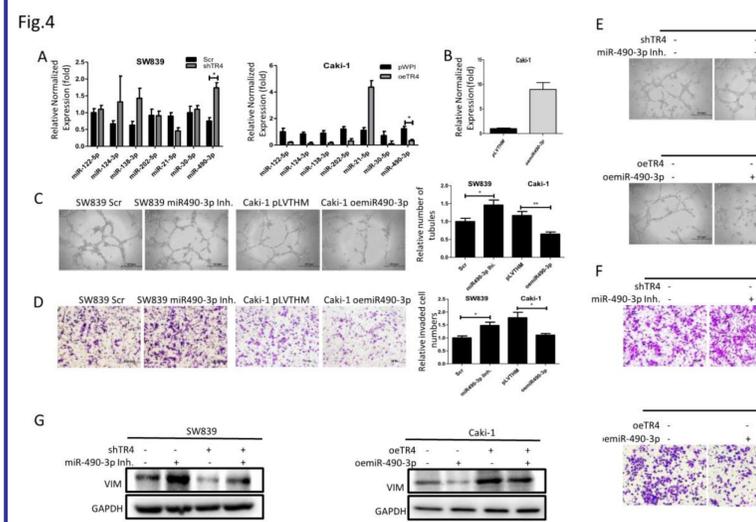
**Fig 1.** Higher expression of TR4 was correlated with RCC patients' survival and cancer cell invasiveness. (A) Overall survival curves of ccRCC patients was associated to the TR4 expression based on TCGA database analysis. (B-C) The correlation between TR4 expression using western blot (B) and ccRCC cell invasive capacity (C) based on results from Matrigel-coated Transwell assay in several ccRCC cell lines.



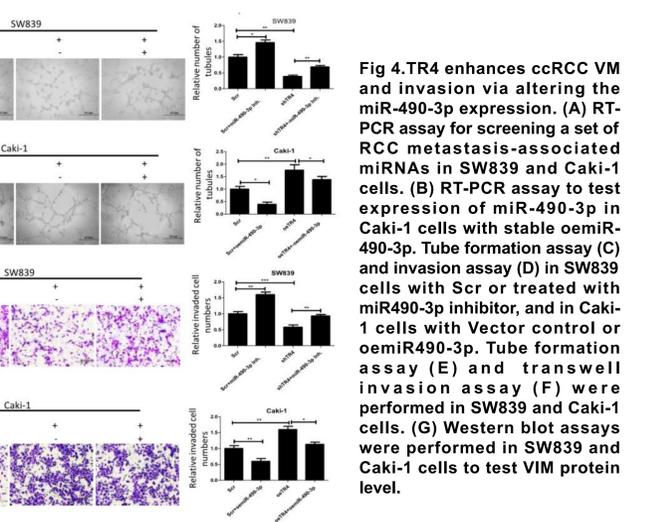
**Fig. 2.** TR4 promotes ccRCC cell VM formation and invasion in vitro. (A) TR4 expression in ccRCC cells with TR4 knockdown or overexpression. Western blot assay for vector control (Scr) and shTR4 in SW839 cells, control (pWPI) or oeTR4 in Caki-1 cells. (B) VM tube formation assays in SW839 cells with Scr or shTR4 and in Caki-1 cells with pWPI or oeTR4. (C) Chamber-transwell invasion assay in SW839 cells with Scr or shTR4 and in Caki-1 cells with pWPI or oeTR4. (D) Invasion ability of overexpressed TR4 in SW839 cells by 3D invasion assay. Data are expressed as the mean  $\pm$  SD and significant differences are indicated by \* $P < 0.05$ , \*\* $P < 0.01$  compared to the controls.



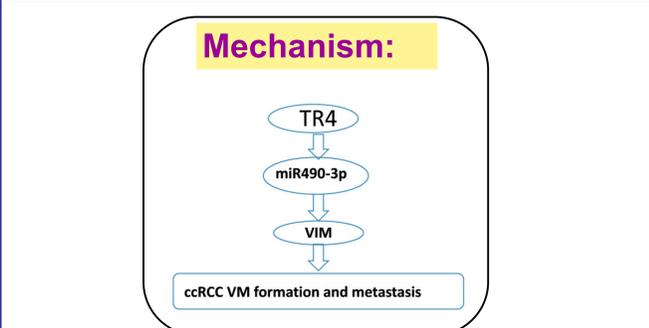
**Fig 3.** TR4 promoted ccRCC cell VM formation and invasion via altering VIM expression. (A) RT-PCR of 8 genes related to VM formation or metastasis in SW839 cells with vector control or shTR4 and in Caki-1 cells with pWPI control or oeTR4. (B) Western blot assay for VIM expression in SW839 and Caki-1 cells. (C) Overall survival curves of ccRCC patients was associated with the VIM expression based on TCGA database. (D) Tube formation assays were performed in SW839 and Caki-1 cells. (E) Chamber-transwell invasion assays were performed in SW839 and Caki-1 cells. (F) Three putative TR4REs were predicted by JASPAR on the VIM promoter. (G) ChIP assay results of the three TR4REs of the VIM promoter in SW839 cells.



**Fig 6.** In vivo mouse RCC model confirmed the role of TR4 and miR-490-3p in ccRCC metastasis. The RCC cells were orthotopically implanted in the left kidney of each mouse. (A) IVIS imaging was used to determine the metastasis in four groups (1: Scr-luc; 2: oeTR4-luc; 3: oemir490-3p-luc; 4: oeTR4+oemir490-3p-luc), metastasis (Meta) vs non-metastases (Non-meta) in the 4 groups of mice. (B) The mice were sacrificed and the primary tumor sizes in each group were observed and weighed. (C) Numbers of the total metastatic foci in each group of mice. (D) Representative images of metastatic foci in liver, right kidney, spleen, and testis. (E) Representative images of IHC staining for TR4, vimentin and CD34 in the 4 groups of mice. For (A, B, and E), each sample was run in triplicate and in multiple experiments.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Fig 4.** TR4 enhances ccRCC VM and invasion via altering the miR-490-3p expression. (A) RT-PCR assay for screening a set of RCC metastasis-associated miRNAs in SW839 and Caki-1 cells. (B) RT-PCR assay to test expression of miR-490-3p in Caki-1 cells with stable oemir-490-3p. Tube formation assay (C) and invasion assay (D) in SW839 cells with Scr or treated with miR490-3p inhibitor, and in Caki-1 cells with Vector control or oemir490-3p. Tube formation assay (E) and transwell invasion assay (F) were performed in SW839 and Caki-1 cells. (G) Western blot assays were performed in SW839 and Caki-1 cells to test VIM protein level.



**Summary**

- ccRCC patients with higher TR4 expression have lower survival rate.
- TR4 promotes ccRCC cell VM and invasion in vitro and in vivo via altering miR-490-3p / VIM expression.
- Targeting the newly identified TR4/miR-490-3p/VIM signals with small molecules may help us to develop a new therapeutic approach to better suppress the ccRCC metastasis.