A miR-7/GAS6/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma

Kabir TD¹, Ganda C¹, Brown RM¹, Beveridge DJ¹, Richardson KL¹, Chaturvedi V¹, Candy P¹, Epis M¹, Wintle L¹, Kalinowski F¹, Kopp C^{1,2}, Stuart LM¹, Yeoh GC³, George J⁴, Leedman PJ^{1,5}

¹Laboratory for Cancer Medicine, Harry Perkins Institute of Medical Research and the University of Western Australia Centre for Medical Research, 6 Verdun St, Nedlands, WA, 6009, Australia

²Institute of Molecular Medicine, Goethe University Frankfurt Am Main, Germany

³Liver Disease and Carcinogenesis Laboratory, Harry Perkins Institute of Medical Research and the University of Western Australia Centre for Medical Research, 6 Verdun St, Nedlands, WA, 6009, Australia

⁴Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital and University of Sydney, NSW, Australia

⁵School of Medicine and Pharmacology, the University of Western Australia, 35 Stirling Highway, Nedlands, WA, 6009, Australia.

Key words: microRNA, sorafenib resistance, PI3-kinase/AKT pathway, TAM, RelA.

Correspondence: Peter J Leedman MD, PhD

Laboratory for Cancer Medicine, Harry Perkins Institute of Medical Research and the University of Western Australia Centre for Medical Research, 6 Verdun St, Nedlands, WA, 6009, Australia. Ph +61 8 6151 0704; Fax +61 8 9463 1469; email: peter.leedman@perkins.uwa.edu.au.

Abbreviations: AKT, protein kinase B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinases; GAS6, growth arrest specific 6; miR-7, microRNA-7/hsa-miR-7-5p; HCC, hepatocellular carcinoma; RelA, Transcription factor p65 or nuclear factor NFκB p65 subunit; RTKs, receptor tyrosine kinases; SR, sorafenib resistance; TAM, TYRO3/Axl/MER tyrosine kinase receptor; AFP, α-feto protein.

Supported by grants from The Medical Research Commercialisation Fund (MRCF), the National Health and Medical Research Council (NHMRC), and the Cancer Council of Western Australia (CCWA).

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version record](#). Please cite this article as [doi:10.1002/hep.29478](https://doi.org/10.1002/hep.29478).

Abstract:

Sorafenib remains the only approved drug for treating patients with advanced hepatocellular carcinoma (HCC). However, the therapeutic effect of sorafenib is transient and patients invariably develop sorafenib resistance (SR). Recently, TYRO3, a member of the TAM family of receptor tyrosine kinases, was identified as being aberrantly expressed in a significant proportion of HCC, however its role in SR is unknown. In this study, we generated two functionally distinct sorafenib resistant human Huh-7 HCC cell lines, in order to identify new mechanisms to abrogate acquired SR, as well as new potential therapeutic targets in HCC. Initially, we investigated the effects of a microRNA, miR-7, in both *in vitro* and *in vivo* preclinical models of human HCC and identified miR-7 as a potent tumour suppressor of human HCC. We identified TYRO3 as a new functional target of miR-7, which regulates proliferation, migration and invasion of Huh-7 cells via the PI3-Kinase/AKT pathway and is markedly elevated upon acquisition of SR. Furthermore, miR-7 effectively silenced TYRO3 expression in both sorafenib-sensitive and sorafenib-resistant Huh-7 cells inhibiting TYRO3/GAS6 mediated cancer cell migration and invasion. In conclusion, we identified a novel mechanism for acquiring SR in HCC which is via the aberrant expression of the TYRO3/PI3-Kinase/AKT signal transduction pathway, a mechanism that can be overcome by miR-7 over-expression. Taken together, these data suggest a potential role for miR-7 as an RNA-based therapeutic to treat refractory and drug resistant HCC.

Liver cancer ranks sixth amongst all malignancies in the world and is the 2nd leading cause of cancer-related mortality (1). The rising incidence of hepatocellular carcinoma (HCC), the most common primary malignant neoplasm of the liver, has been linked to hepatitis B or C infection and increasing world-wide obesity associated with fatty liver disease (2). In 2017 it is estimated that 40,710 patients will be diagnosed with HCC in the US and more than 70% of them will eventually die (2). Less than 30% of patients with HCC can be managed by curative therapy and sorafenib, a multi-kinase inhibitor, is the only available drug for managing patients with advanced tumours (3). The clinical benefit of sorafenib is modest, adverse effects are common and almost all patients become refractory within a few months, associated with a very poor prognosis (5). Recently, regorafenib, another multi-kinase inhibitor was approved as a second line therapy in HCC patients resistant to sorafenib by the U.S. Food and Drug Administration (4). Although regorafenib can improve overall survival modestly (an increase in median overall survival from 7.8 months to 10.6 months), it is associated with a range of side-effects and some severe drug-associated toxicity has been reported (5). Based on this evidence, there is an urgent need to better understand the molecular mechanisms underlying the development of SR and to identify novel therapeutic targets to improve clinical outcome.

Recently, TYRO3, a member of the TAM family of transmembrane tyrosine kinase receptors was found to be aberrantly expressed in HCC (6). Of 55 HCCs 42% had elevated expression of TYRO3 (>2-fold) compared to adjacent normal tissue, and higher levels of TYRO3 were associated with higher levels of serum alpha-fetoprotein (AFP) and an increased tumour diameter. These data suggested that inhibition of TYRO3 and its signaling pathways in tumors with high expression could be of therapeutic benefit in HCC. The role of TYRO3 in SR is unknown.

Oligonucleotide therapeutics is an emerging field being driven by advances in technology resulting in new RNA-based drug therapies as an alternative to conventional chemical inhibitors or antibodies (eg. RNAi to PCSK9 to treat hypercholesterolemia) (7). This technology includes: the short interfering RNAs (siRNAs), anti-sense oligonucleotides and microRNAs (miRNAs) (8). Our interest in the RNA-based drug development field has been with miRNAs, which are a family of endogenously transcribed ~22 nucleotide noncoding RNAs (ncRNAs) that post-transcriptionally regulate gene expression by inducing either translational repression or message decay (9). Abnormal expression of several miRNAs has been observed in HCC (10-13). Here we focused on a tumor suppressor miRNA, miR-7-5p (miR-7), which is down-regulated in several human cancers, including HCC, and has been shown to regulate a number of oncogenic signal transduction pathways such as the EGFR signalling pathway, as well as PI3-Kinase/AKT and RAF-MEK-ERK pathways (13-17). We aimed to explore the role of miR-7 in HCC and in sorafenib resistant HCC, and to identify new targets that could be useful clinically. Although the EGFR is frequently overexpressed in HCC, and therefore an excellent target, to date anti-EGFR treatments have not been found to benefit patients. Given that miRNAs target multiple mRNAs in multiple pathways, we hypothesized that miR-7 would simultaneously target the EGFR and several other pathways in HCC, resulting in enhanced inhibition of tumor growth.

We demonstrate that miR-7 is a potent inhibitor of HCC cell growth *in vitro* and *in vivo*, and that TYRO3 is a new direct miR-7 gene target. Furthermore, TYRO3 was found to be an important mediator of SR in HCC cells. miR-7 significantly reduced TYRO3 expression in parental and sorafenib resistant HCC cells. Thus, miR-7 could potentially be exploited as a novel RNA-based therapeutic for HCC, with specific emphasis on patients with sorafenib resistant tumors.

Materials and Methods:

Cell Culture: The Huh-7 and Hep3B cell lines used in this study were kindly provided by A/Prof. Nicholas Shackel, Centenary Institute, Sydney, Australia. Two sorafenib resistant sublines were established, namely Huh-7/SR1 and Huh-7/SR2, by culturing the cells in increasing doses of sorafenib. The origin and culture conditions of these cells are detailed in the Supplementary Materials and Methods.

Reagents: A full list of reagents is included in the Supplementary Materials and Methods.

Cell viability, proliferation and colony formation assay: To determine the sensitivity of HCC cell lines to sorafenib, miR-7 and TYRO3, cells were treated with increasing concentration of drug, miRNA and siRNA, or after transfection with a single concentration of miRNA/siRNA, the effect on cell viability was measured over time, as previously described (18). 2D and 3D colony formation assays were performed as described previously (15). Further details are outlined in the Supplementary Materials and Methods.

Migration and invasion assays: Migration and invasion was assessed using transwell assays with polycarbonate membrane inserts with 8.0 μ M pore size (Costar) coated with or without Matrigel (Corning) as described in Supplementary Materials and Methods.

RNA extraction, qRT-PCR and miRNA target validation (luciferase reporter assays): Full methods are described in Supplementary Materials and Methods.

Western blot analysis: Immunoblotting was performed using antibodies detailed in the Supplementary Materials and Methods. Densitometry was performed using ImageJ 1.51h v software (<http://rsb.info.nih.gov/ij/>).

Phospho-receptor tyrosine kinase (Phospho-RTK) array: Phospho-RTK array (R&D Systems) was performed according to manufacturer's instructions. Lysates were prepared from Huh-7 cells transfected with 30 nM miRNA precursor molecules for 48 h and 500 μ g of protein was used for each array. Densitometry was performed using ImageJ 1.48 v software (<http://rsb.info.nih.gov/ij/>).

Orthotopic liver cancer model: For details on methodology, please see Supplementary Materials and Methods.

Public array data: To investigate the clinical significance of TYRO3 expression in human HCC a Kaplan-Meier survival curve was plotted using datasets from The Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>; accessed October 2015) in R survival package. For information about patient samples and expression analysis refer to Supplementary Materials and Methods.

Statistical Analysis: All results are represented as mean $\pm$ standard error means (SEM) except for qRT-PCR which is presented as mean $\pm$ SEM of $\Delta\Delta Ct$. Graph Pad Prism (Version 7.0, GraphPad Software) was used to calculate statistical significance between test (e.g. miR-7-5p/siTYRO3) and control (e.g. miR-NC/Negative control siRNA) treated groups using Student's *t* test (one-tailed, unpaired), one-way ANOVA, two-way ANOVA, ANOVA with repeated measures and Mann-Whitney U-test as appropriate. P-value <0.05 was considered statistically significant wherein * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ and **** $p<0.0001$.

Results:

miR-7 is a potent suppressor of HCC growth *in vitro* and *in vivo*

The constitutive activation of EGFR in HCC leads to persistent downstream signaling through the PI3-Kinase and AKT, p21-activated kinases (PAK) and the Raf/MEK/ERK pathways, transducing growth signals to tumour cells and promoting tumour progression and dissemination (19). As miR-7 is down-regulated in HCC (13, 20), its replacement should reduce EGFR signaling, together with other pathways containing miR-7 targets. We initially evaluated the effects of miR-7 overexpression *in vitro* using a human HCC derived cell line Huh-7, and found that miR-7 produced a significant anti-proliferative ($p=0.0435$), anti-migratory ($p<0.0001$) and anti-invasive ($p=0.0024$) phenotype in Huh-7 cells and reduced

their clonogenic capacity ($P < 0.01$) (Fig. 1Ai-iv, S1A). miR-7 was cytotoxic at very low concentrations to Huh-7 ($EC_{50} = 1.514$ nM), HEP3B ($EC_{50} = 2.115$ nM) and PLC/PRF/5 ($EC_{50} = 0.4812$ nM) cells (Fig 1B, S1Bi, S1Bii). The growth inhibitory effect of miR-7 on HCC cell viability was unrelated to the level of endogenous miR-7 which varies across the three cell lines (Fig S1Biii). miR-7 induced a G0/G1 cell cycle arrest associated with apoptosis, as evidenced by an increase in caspase3/7 activity and cleaved PARP protein in western blot (Fig S1C).

We next explored the effects of miR-7 in an orthotopic model of human HCC, using a NOV340-miR-7 mimic, as described in methods. The NOV340-miR-7 mimic had a similar effect on Huh-7 cell viability as the Ambion miR-7 mimic used for the *in vitro* work (Fig. S1D).

Initial *in vivo* investigation found that miR-7 expression was enriched in both murine liver and human HCC cell line derived-tumor tissues following tail vein injection of the NOV340-miR-7 formulation (Fig S1E). We then conducted a short miR-7 replacement therapy trial in which NSG immunodeficient mice received Huh-7 cells orthotopically and once the cells reached a critical mass (equivalent to AFP secretion of 1000 ng/ml), they commenced a two-week alternate day intravenous treatment regimen of miR-7 or control (Fig 1C). Analysis of the mice at 14 days showed that the tumour burden in the mice receiving miR-7 was dramatically reduced, accompanied by markedly lower AFP levels (almost one hundred fold lower) (Fig 1D). This was associated with a major reduction in tumor size and weight (Fig 1F-G, Fig S1Fi). Remarkably, in the miR-7 recipient group ($n=8$) no visible tumours were observed macroscopically in 6 of the mice, explaining why there are only two tumour weights plotted for miR-7 in Fig 1F (Fig S1Eii, S1Fii). Taken together, these data indicate that miR-7 is a potent inhibitor of human HCC cell proliferation, migration and invasion *in vitro* and tumour growth *in vivo*.

The TAM family member TYRO3 is elevated in HCC and is a novel miR-7 target gene

Given the magnitude of effect induced by miR-7 in HCC, we set out to identify new targets of the microRNA within the receptor tyrosine kinase (RTK) family using an unbiased phospho-RTK antibody array. Densitometry analysis of the 42 member RTK array showed that miR-7 reduced the amount of phosphorylated EGFR and IGF1R, consistent with each being a validated target of miR-7 (Fig 2A, #2 and #5, respectively). In addition, miR-7 reduced the levels of phosphorylated FGFR4 and TYRO3 (Fig 2A, #3 and #6, respectively) and Target Scan analysis revealed both FGFR4 and TYRO3 contain putative miR-7 seed sequences in their 3'UTR (Fig S2A), which are well conserved across primates. FGFR4 has been implicated in HCC and is a potential therapeutic target (21). Small molecule inhibitors have been developed against FGFR4 (eg. BLU9931) and much is known about its expression in HCC (22). In contrast, interest in TYRO3 in HCC is just emerging and given its association with resistance to both chemotherapy and targeted-therapies in chronic myeloid leukaemia and ovarian cancer (23, 24), we speculated that aberrant TYRO3 expression might be an important determinant of SR in HCC. To date, there are no specific small molecule TYRO3 inhibitors that have been developed, as they either target all of the TAM family members or Axl alone. Thus, miR-7, by directly targeting TYRO3 could represent an alternative approach to specifically inhibit TYRO3 expression in HCC.

When miR-7 was overexpressed in Huh-7 or HEP3B cells we observed TYRO3 mRNA and protein expression were significantly reduced (Fig 2B, Fig S2B). Furthermore, we identified TYRO3 as a direct functional gene target of miR-7 using a luciferase reporter assay with constructs containing either the wild type (WT) or mutant (Mut) TYRO3 3'UTR (Fig 2C). Transient overexpression of miR-7 significantly reduced luciferase activity of the WT TYRO3, but not the mutant, compared to the miR-NC (Fig 2C ii). Additional clinical validation of the importance of TYRO3 expression in HCC was achieved within a cohort of 368 patients from

the TCGA, which showed that TYRO3 expression is significantly increased in tumor compared to surrounding normal tissue (Fig 2D). Further, HCC patients with higher expression of TYRO3 had a significantly worse prognosis and reduced survival (Fig 2E).

TYRO3 regulates proliferation, migration and invasion of HCC cells via the PI3-Kinase/AKT pathway

To evaluate the effects of regulating TYRO3 expression in HCC (Huh-7 and HEP3B cells), we used two commercially available TYRO3 siRNAs (named #5 and #6), each targeting different regions at the 3'-end of TYRO3 mRNA. This region is distinctive amongst all three transcript variants ensuing from alternative 5'-end splicing which can regulate subsequent biological function (25). We found a functional difference between the two siRNAs in migration assays: whereby TYRO3 #6 siRNA did not alter migration in HEP3B cells (Fig S3). However, using both the xCELLigence and transwell migration assay TYRO3 siRNA #6 was found to be more potent than TYRO3 siRNA#5 in Huh-7 cells as it decreased both migration ($P<0.0001$) and invasion ($P=0.0043$) (Fig 3B & C, Fig S3). TYRO3 function has been previously examined in HEP3B, but not Huh-7, cells (6). Thus, we elected to investigate TYRO3 function in Huh-7 cells for all the functional assays in this study. Transient knock down of TYRO3 in Huh-7 cells significantly reduced their rate of proliferation ($P<0.05$) (Fig 3A). To identify TYRO3-induced signalling pathways involved in oncogenic processes in HCC, the siRNA mediated TYRO3 depleted Huh-7 cells were analysed for various downstream signalling molecules. For the PI3-Kinase/AKT pathway, the amount of phosphorylated AKT (serine 473) is considerably reduced in TYRO3 deficient Huh-7 cells whereas PTEN (a negative regulator of the pathway (19)) remained intact (Fig 3D). In contrast, for the NF κ B and RAF/MEK/ERK pathways, phosphorylated Rel A (serine 536) and phosphorylated ERK1/2 (threonine 202/204) are increased upon TYRO3 depletion (Fig 3D, ERK1/2 increased with siTYRO3 #6 only). These results suggest that the canonical PI3-

Kinase/AKT pathway transduces the pro-tumorigenic signaling of TYRO3 in HCC. Furthermore, in cells in which TYRO3 expression is reduced, alternative pathways may become active, such as RelA/NF κ B signalling. Of note, we have previously reported that RelA is a direct target of miR-7 (15).

● Endogenous miR-7 regulates the TYRO3-mediated metastatic phenotype in HCC

To comprehend the biological significance underlying the miR-7/TYRO3 axis in Huh-7 cells “rescue of function” experiments were conducted, whereby the endogenous miR-7 levels were depleted using anti-miR-7 for 48 h, followed by sequential depletion of TYRO3 for a further 24 h (Fig S4A). We hypothesized that if TYRO3 is a physiological direct target of miR-7, elimination of endogenous miR-7 would induce a phenotype characteristic of TYRO3 over-expression. Subsequent introduction of TYRO3 siRNAs should antagonize this gain of function. We found using anti-miR-7 that depletion of endogenous miR-7 did not affect Huh-7 cell proliferation (Fig 4A, Fig S4B) but significantly stimulated their migration and invasion (Fig 4C-D). When the cells lacking endogenous miR-7 were exposed to TYRO3 siRNA we observed anti-miR-7 rescued the level of TYRO3 protein, consistent with the anti-miR-7 nullifying the effect of any endogenous miR-7 (Fig S4C). Subsequent depletion of TYRO3 in these cells did not alter Huh-7 cell viability (Fig 4B) but significantly antagonised the extent of migration and invasion provoked by anti-miR-7 (Fig 4C-D). We further observed that the TYRO3-dependent pro-metastatic effect in HCC is partly mediated by altered expression of E-cadherin (Fig S5, Fig 3D). Taken together, these data provide additional evidence for an important inverse regulation relationship between miR-7 and TYRO3 in HCC.

miR-7 reverses the rebound surge of pro-inflammatory chemokines upon TYRO3 depletion in Huh-7 cells

Loss of TAM receptors in HCC has been associated with altered expression of some key pro-inflammatory cytokines and chemokines (26), which could contribute to increased growth. Thus, we investigated the regulation of expression of CXCL10 and IL-8/CXCL8, two well-characterised HCC-associated chemokines with putative miR-7 binding sites. We found with qRT-PCR that TYRO3 depletion significantly increased expression of CXCL10 ($\rho=0.943$, $P=0.017$) and IL-8 ($\rho=0.886$, $P=0.033$), suggestive of a rebound surge of pro-inflammatory chemokines (Fig 4E). Furthermore, overexpression of miR-7 significantly decreased the expression of IL-8 ($P=0.025$) and CXCL10 ($P=0.0034$) in Huh-7 cells (Fig 4F). Based on these findings, we concluded that miR-7 not only fine-tunes TYRO3 expression in Huh-7 cells but it also down regulates key pro-inflammatory chemokines. This effect of miR-7, combined with the other data above, serves to illustrate how broad its effects are and also exemplifies its potency as an inhibitor of HCC cell viability.

Sorafenib resistance in HCC is overcome via miR-7 suppression of TYRO3 function

To understand the mechanisms driving acquired resistance to sorafenib in HCC, two sorafenib resistant cell lines Huh-7/SR1 ($EC_{50} = 42.92 \mu M$) and Huh-7/SR2 ($EC_{50} = 75.94 \mu M$) were established (Fig 5A). miR-7 expression was significantly reduced in both sorafenib resistant cell lines (Fig 5Bi) whereas TYRO3 expression was markedly increased in the more resistant Huh-7/SR2 cell line (Fig 5Bii). We found that the EGFR pathway becomes less active during acquiring SR (Fig 5C). The expression of TYRO3 protein is elevated in the Huh-7/SR2 cell line consistent with its mRNA profile, and is accompanied by higher levels of pRelA (Fig 5C). pAKT and pERK1/2 were differentially expressed between the two sorafenib resistant lines (Fig 5C). In addition, we found increased TYRO3 expression in HEP3B cells, and these cells were less sensitive to sorafenib than the parental Huh-7 cells, with an EC_{50} of $\sim 14 \mu M$ (Fig S6). Further characterization showed the two resistant cell lines were functionally very distinct from their precursors. Compared to the parental cells, the Huh-

Huh-7/SR1 cells were more proliferative whereas the Huh-7/SR2 cell lines were less proliferative in nature (Fig 5Di). Both sorafenib resistant lines were more migratory and invasive than the parental cell line (Fig 5Dii-iii). Aberrant activation of TYRO3 is reported to stimulate epithelial-mesenchymal transition (EMT) in colorectal cancer and regulate tumour dissemination (27). We observed that Huh-7/SR2 cells lost their epithelial polarity by expressing more mesenchymal cell markers: α -SMA and vimentin, and less epithelial E-cadherin compared to their parental counterparts (Fig 5Ei-ii). The sorafenib resistant cells also transcribed more TWIST1 (Fig 5Eiii). Furthermore, depleting TYRO3 levels in both the parental and Huh-7/SR2 cells reduced their viability in a dose-dependent manner and the EC_{50} of siTYRO3 in the Huh-7/SR2 cells was much less than that of the parental cells (0.009 nM versus 2.516 nM) (Fig 5Fi-ii). Finally, by using concentrations near the EC_{50} of siTYRO3 we found that combination therapy of siTYRO3 with sorafenib (5.5 μ M ~highest achievable dose in plasma) significantly increased the cytotoxic effect of sorafenib in the parental Huh-7 cell line (Fig 5Fiii), although this effect was not synergistic (using the Bliss Additivity model (28)).

We next investigated the effects of miR-7 on the sorafenib resistant cells. We observed that similar to siTYRO3, miR-7 significantly reduced viability of both Huh-7/SR1 and Huh-7/SR2 cells in dose-dependent fashion and markedly attenuated their migration and invasion (Fig 6A-B). miR-7 reinforced E-cadherin expression in the Huh-7/SR2 cell line and induced a global reduction of key survival molecules (TYRO3, pAKT, panAKT, pERK and total RelA), previously shown to be upregulated in these cells (Fig 6C, and compare to Fig 5C). We next stimulated the Huh-7/SR2 cells with the ligand to TYRO3, namely Growth Arrest Specific 6 (GAS6), in the presence and absence of miR-7. We observed that in the absence of miR-7 GAS6 significantly stimulated the migration and invasion of the Huh-7/SR2 cell lines, and this effect was dramatically reduced in the presence of miR-7 (Fig 6D). Further, in the presence of miR-7, the GAS6-induced increase in migration and invasion was abolished, consistent with miR-7 effectively inhibiting the GAS6-TYRO3 pathway (29). Finally, when we treated

sorafenib resistant Huh-7 cells with a constant dose of miR-7 and increasing doses of sorafenib the EC₅₀ curve shifted significantly downwards (Fig 6E-F). The Bliss Additivity model (28) demonstrated synergy between miR-7 (at 5 nM) and sorafenib in both sorafenib resistant cell lines (Table S1 and S2; 0.1-75 μ M and 25 μ M sorafenib in SR2 and SR1, respectively).

Discussion:

HCC targeted therapies, such as sorafenib, which are directed against multiple growth factor receptors or downstream signalling kinases do offer some therapeutic benefit, although the acquisition of therapeutic resistance is a major clinical problem. Here we show that miR-7 is a potent inhibitor of HCC growth *in vitro* and in an orthotopic liver model of HCC *in vivo*. A significant component of that action is via direct inhibition of TYRO3 expression and its downstream signalling network, driven predominantly via the canonical PI3-kinase/AKT pathway. Unexpectedly, TYRO3 expression is also amplified in sorafenib resistant HCC cells and was associated with their mesenchymal phenotype. We, therefore, propose combining miR-7 with TYRO3 inhibition will afford greater inhibition of HCC cell growth, and represent a novel strategy to overcome SR (see Fig 7).

The interaction of miR-7 with members of the EGF/EGFR pathway in a number of solid tumours such as glioblastoma (16), melanoma (15) and HCC (13) and its ability to sensitize cancer cells to radiotherapy (30) and targeted-therapies (Erlotinib) (18) demonstrate its potential as a therapeutic strategy. In this study we report another novel target of miR-7 belonging to the TAM family of RTKs; TYRO3 and identified it as a key driver of the oncogenic PI3-Kinase/AKT pathway in HCC. There is increasing emphasis on developing TAM receptor inhibitors for the treatment of cancer, and Axl inhibitors have recently entered Phase I clinical trial for treatment of several different solid tumours (31). The early data

suggest that these small molecule Axl inhibitors have shown some therapeutic efficacy towards HCC, especially as Axl is reported elevated in pre-clinical models of human HCC and in patient samples (32, 33).

Our TCGA HCC data demonstrated that TYRO3 is aberrantly overexpressed in HCC and associated with a poor prognosis. Our results also suggest that TYRO3 may play an important pro-proliferative and pro-invasive role in parental as well as sorafenib resistant cells. Significantly, inhibition of TYRO3 not only reduced proliferation, migration and invasion of sorafenib sensitive HCC cell lines via massive inhibition of the pAKT-pathway but inhibition of TYRO3 significantly inhibited growth of sorafenib resistant cells in a dose-dependent fashion. Hitherto we did not observe any synergism between simultaneous inhibition of TYRO3 and sorafenib therapy in the resistant cell lines, potentially because of a subtle paradoxical increase in pERK levels following loss of TYRO3 which has been implicated in conferring SR (34).

TYRO3 is reported to modulate several oncogenic pathways including survival, altered cellular morphology, cell-cycle transition, proliferation, adhesion, motility and release of a number of inflammatory cytokines and is observed elevated in 42% cases of HCC (6, 35, 36). It is also known to induce neoplastic transformation in non-malignant cells and becomes activated in dormant tumours about to proliferate (37, 38). In the presence of limited therapeutic options for HCC patients, TYRO3 represents a promising option for developing new HCC-targeted drug therapies. In that context, TYRO3-specific inhibitors are being identified and tested as new-targeted therapies for HCC (39). In the absence of any small molecule inhibitors of TYRO3, therapeutic targeting of TYRO3 and its downstream signaling pathways by miR-7 is an exciting prospect for treating sorafenib-sensitive and resistant HCC.

In HCC, SR may be inherent wherein the cancer cells typically express less pERK and PTEN but more pAKT (40, 41). More commonly, however, resistance is acquired where the cells augment critical cell-autonomous survival mechanisms by, for example, increasing expression of EGFR (42) or exploiting non-cancerous cells of the tumour microenvironment (43), to sustain a drug-resistant tumour permissive niche. Surprisingly, in the resistant cell lines the EGF/EGFR pathway was minimally active compared to GAS6/TYRO3 driven PI3-Kinase/AKT signalling, the latter accompanied by elevated pRelA, indicative of additional TYRO3-independent complementary pathways activated in these cells. miR-7 retained its cytotoxicity in the sorafenib resistant cells at a low concentration, significantly reduced their migratory and invasiveness *in vitro* and was synergistic with sorafenib. Furthermore, a reciprocal association exists between expression of miR-7 and its target genes in the sorafenib resistant cells underpinning the potential clinical importance of miR-7 in refractory tumours.

The negative association between TYRO3 and CXCL10, and TYRO3 and IL-8 is concerning since CXCL10 is a homing factor of immune cells to the tumour microenvironment and IL-8 is a well-characterized proangiogenic factor in HCC (44, 45). A higher level of CXCL10 is reported in recurrent HCC while elevated IL-8 is observed in patients having poor overall survival (44, 46). Thus, although TYRO3 siRNA inhibited HCC cell growth and P-Akt, there was an accompanying increase in each of CXCL10 and IL-8, in part negating the effects of siRNA to TYRO3. In contrast, miR-7 significantly inhibited P-Akt and expression of both CXCL10 and IL-8 in HCC, the latter each containing a miR-7 target site in their 3'-UTR. These data emphasize a major advantage of using a miRNA as therapeutic in HCC because it simultaneously targets multiple members of multiple signaling pathways.

With the availability of very few therapeutic interventions for patients with HCC and an inescapable poor patient outcome, there is an urgent need to develop newer treatment options. RNA-based therapeutics is a rapidly emerging field, with particular reference to hepatic disorders because of the recent phase 2 clinical trial success of specifically delivering siRNAs to hepatocytes (7, 47). These exciting developments in RNA-based therapeutics result from synthesizing oligonucleotides with 2nd generation chemistry, which are more stable than previously and using conjugates that target the hepatic asialoglycoprotein receptors which retain their expression even during development of HCC (7, 47). Thus, generation of 2nd generation stable miR-7 mimics which would be delivered efficiently to human hepatocytes, could become an option as a microRNA replacement therapy for HCC.

In conclusion, we identified a novel miR-7/GAS6/TYRO3 axis that regulates the growth, migratory and invasive phenotype of parental and sorafenib resistant HCC cells, predominantly via inhibition of the PI3-Kinase/AKT pathway. Given the recent advances in RNA-based therapies and improved delivery methods, these data lay a foundation for using miR-7 in the treatment of advanced and sorafenib resistant HCC, potentially in combination with a small molecule inhibitor of TYRO3.

Conflict of Interests: No potential conflict of interest.

Author's contribution: TDK and CG are co-first authors. TDK, VC, LW, ME, FK, RB, KR, LS, MDP and CK helped with the experiments. PC performed TCGA analysis. TDK, CG and PJL planned the experiments, interpreted the data and wrote the manuscript. DJB, GCY, JG and RMB critically reviewed, edited and significantly contributed to the manuscript.

Acknowledgement: We thank Dr Shane Colley and Dr Andrew Woo for proof reading this manuscript. We also thank A/Prof Nicholas Shackel of Centenary Institute Sydney for gifting

the Huh-7 and HEP3B cell lines to do our research. We thank David Brown, ex-Mirna Therapeutics, for advice and technical help for some of the studies.

References:

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359-E386.
2. American Cancer Society. American Cancer Society Cancer Statistics Center. www.cancer.org/cancer/liver-cancer/; accessed on March 2017.
3. Gauthier A, Ho M. Role of sorafenib in the treatment of advanced hepatocellular carcinoma: An update. *Hepatol Res* 2013;43:147-154.
4. FDA. FDA expands approved use of Stivarga to treat liver cancer. In: U.S. Food and Drug Administration; <https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm555608.htm> 2017. accessed on May 2017.
5. Bruix J, Qin SK, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017;389:56-66.
6. Duan Y, Wong W, Chua SC, Wee HL, Lim SG, Chua BT, Ho HK. Overexpression of Tyro3 and its implications on hepatocellular carcinoma progression. *Int J Oncol* 2016;48:358-366.
7. Fitzgerald K, White S, Borodovsky A, Bettencourt BR, Strahs A, Clausen V, Wijngaard P, et al. A Highly Durable RNAi Therapeutic Inhibitor of PCSK9. *N Engl J Med* 2017;376:41-51.
8. Levin AA. Targeting Therapeutic Oligonucleotides. *N Engl J Med* 2017;376:86-88.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
10. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 2009;137:1005-1017.
11. Li N, Fu H, Tie Y, Hu Z, Kong W, Wu Y, Zheng X. miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. *Cancer Lett* 2009;275:44-53.
12. Wong CC, Wong CM, Tung EK, Au SL, Lee JM, Poon RT, Man K, et al. The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. *Gastroenterology* 2011;140:322-331.
13. Fang Y, Xue JL, Shen Q, Chen J, Tian L. MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. *Hepatology* 2012;55:1852-1862.
14. Zhang X, Hu S, Zhang X, Wang L, Zhang X, Yan B, Zhao J, et al. MicroRNA-7 arrests cell cycle in G1 phase by directly targeting CCNE1 in human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2014;443:1078-1084.
15. Giles KM, Brown RA, Ganda C, Podgorny MJ, Candy PA, Wintle LC, Richardson KL, et al. microRNA-7-5p inhibits melanoma cell proliferation and metastasis by suppressing RelA/NF-kappaB. *Oncotarget* 2016;7:31663-31680.

16. Kefas B, Godlewski J, Comeau L, Li YQ, Abounader R, Hawkinson M, Lee JW, et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. *Cancer Res* 2008;68:3566-3572.
17. Shi Y, Luo X, Li P, Tan J, Wang X, Xiang T, Ren G. miR-7-5p suppresses cell proliferation and induces apoptosis of breast cancer cells mainly by targeting REGgamma. *Cancer Lett* 2015;358:27-36.
18. Kalinowski FC, Giles KM, Candy PA, Ali A, Ganda C, Epis MR, Webster RJ, et al. Regulation of epidermal growth factor receptor signaling and erlotinib sensitivity in head and neck cancer cells by miR-7. *PLoS One* 2012;7:e47067.
19. Psyrris A, Arkadopoulos N, Vassilakopoulou M, Smyrniotis V, Dimitriadis G. Pathways and targets in hepatocellular carcinoma. *Exp Rev Anticancer Ther* 2012;12:1347-1357.
20. Kalinowski FC, Brown RAM, Ganda C, Giles KM, Epis MR, Horsham J, Leedman PJ. microRNA-7: A tumor suppressor miRNA with therapeutic potential. *Int J Biochem Cell Biol* 2014;54:312-317.
21. Zheng N, Wei W, Wang Z. Emerging roles of FGF signaling in hepatocellular carcinoma. *Transl Cancer Res* 2016;5:1-6.
22. Hagel M, Miduturu C, Sheets M, Rubin N, Weng W, Stransky N, Bifulco N, et al. First selective small molecule inhibitor of FGFR4 for the treatment of hepatocellular carcinomas with an activated FGFR4 signaling pathway. *Cancer Discov* 2015;5:424-437.
23. Gioia R, Leroy C, Drullion C, Lagarde V, Etienne G, Dulucq S, Lippert E, et al. Quantitative phosphoproteomics revealed interplay between Syk and Lyn in the resistance to nilotinib in chronic myeloid leukemia cells. *Blood* 2011;118:2211-2221.
24. Lee C. Overexpression of Tyro3 receptor tyrosine kinase leads to the acquisition of taxol resistance in ovarian cancer cells. *Mol Med Rep* 2015;12:1485-1492.
25. Biesecker LG, Giannola DM, Emerson SG. Identification of alternative exons, including a novel exon, in the tyrosine kinase receptor gene Etk2/tyro3 that explain differences in 5' cDNA sequences. *Oncogene* 1995;10:2239-2242.
26. van der Meer JHM, van der Poll T, van 't Veer C. TAM receptors, Gas6, and protein S: roles in inflammation and hemostasis. *Blood* 2014;123:2460-2469.
27. Chien C, Hou P, Wu H, Chang Y, Lin S, Lin B, Lee J, et al. Targeting Tyro3 inhibits epithelial-mesenchymal transition and increases drug sensitivity in colon cancer. *Oncogene* 2016 ; 35: 5872-5881.
28. Bliss C. The toxicity of poisons applied jointly. *Ann. appl. Biol.* 1939; 26:585-615.
29. Taylor IC, Roy S, Varmus HE. Overexpression of the Sky receptor tyrosine kinase at the cell surface or in the cytoplasm results in ligand-independent activation. *Oncogene* 1995;11:2619-2626.
30. Lee KM, Choi EJ, Kim IA. microRNA-7 increases radiosensitivity of human cancer cells with activated EGFR-associated signaling. *Radiother Oncol* 2011;101:171-176.
31. Sheridan C. First Axl inhibitor enters clinical trials. *Nat Biotech* 2013;31:775-776.
32. Liu J, Wang K, Yan Z, Xia Y, Li J, Shi L, Zou Q, et al. Axl expression stratifies patients with poor prognosis after hepatectomy for hepatocellular carcinoma. *PLoS One* 2016;11:e0154767.
33. Pinato DJ, Trousil S, Caley M, Mauri FA, Aboagye E, Sharma R. Pharmacological inhibition of Axl tyrosine kinase as a novel therapeutic strategy in hepatocellular carcinoma. *J Clin Oncol* 2015;33: e15152.
34. Ezzoukhry Z, Louandre C, Trecherel E, Godin C, Chauffert B, Dupont S, Diouf M, et al. EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *Int J Cancer* 2012;131:2961-2969.
35. Tsou WI, Nguyen KQN, Calarese DA, Garforth SJ, Antes AL, Smirnov SV, Almo SC, et al. Receptor Tyrosine Kinases, TYRO3, AXL, and MER demonstrate distinct patterns and complex regulation of ligand-induced activation. *J Biol Chem* 2014;289:25750-25763.
36. Demarest SJ, Gardner J, Vendel MC, Ailor E, Szak S, Huang F, Doern A, et al. Evaluation of Tyro3 expression, Gas6-mediated Akt phosphorylation, and the impact of anti-Tyro3 antibodies in melanoma cell lines. *Biochem* 2013;52:3102-3118.

37. Lan ZD, Wu HY, Li WQ, Wu SC, Lu L, Xu M, Dai W. Transforming activity of receptor tyrosine kinase Tyro3 is mediated, at least in part, by the PI3 kinase-signaling pathway. *Blood* 2000;95:633-638.
38. Taichman RS, Patel LR, Bedenis R, Wang JC, Weidner S, Schumann T, Yumoto K, et al. GAS6 receptor status is associated with dormancy and bone metastatic tumor formation. *Plos One* 2013;8: e61873.
39. Ho HK, Chua BT, Wong W, Lim KS, Teo V, Ong HT, Chen X, et al. Benzylidene-indolinones are effective as multi-targeted kinase inhibitor therapeutics against hepatocellular carcinoma. *Mol Oncol* 2014;8:1266-1277.
40. Chen J, Ji T, Zhao J, Li G, Zhang J, Jin R, Liu J, et al. Sorafenib-resistant hepatocellular carcinoma stratified by phosphorylated ERK activates PD-1 immune checkpoint. *Oncotarget* 2016;7:41274-41284.
41. Zhai B, Zhang X, Sun B, Cao L, Zhao L, Li J, Ge N, et al. MK2206 overcomes the resistance of human liver cancer stem cells to sorafenib by inhibition of pAkt and upregulation of pERK. *Tumour Biol* 2016;37:8047-8055.
42. Blivet-Van Eggelpoel MJ, Chettouh H, Fartoux L, Aoudjehane L, Barbu V, Rey C, Priam S, et al. Epidermal growth factor receptor and HER-3 restrict cell response to sorafenib in hepatocellular carcinoma cells. *J Hepatol* 2012;57:108-115.
43. Crawford Y, Kasman I, Yu LL, Zhong CL, Wu XM, Modrusan Z, Kaminker J, et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* 2009;15:21-34.
44. Ouyang Y, Liu K, Hao M, Zheng R, Zhang C, Wu Y, Zhang X, et al. Radiofrequency ablation-increased CXCL10 is associated with earlier recurrence of hepatocellular carcinoma by promoting stemness. *Tumour Biol* 2016;37:3697-3704.
45. Zhu B, Lin N, Zhang M, Zhu Y, Cheng H, Chen S, Ling Y, et al. Activated hepatic stellate cells promote angiogenesis via interleukin-8 in hepatocellular carcinoma. *J Transl Med* 2015;13:365.
46. Ren Y, Poon RT, Tsui HT, Chen WH, Li Z, Lau C, Yu WC, et al. Interleukin-8 serum levels in patients with hepatocellular carcinoma: correlations with clinicopathological features and prognosis. *Clin Cancer Res* 2003;9:5996-6001.
47. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, Hall T, et al. Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol. *NEJM* 2017;376:1430-1440.
48. Shi B, Abrams M, Sepp-Lorenzino L. Expression of asialoglycoprotein receptor 1 in human hepatocellular carcinoma. *J Histochem Cytochem* 2013;61:901-909.
49. Daige CL, Wiggins JF, Priddy L, Nelligan-Davis T, Zhao J, Brown D. Systemic delivery of a miR34a mimic as a potential therapeutic for liver cancer. *Mol Cancer Ther* 2014;13:2352-2360.
50. GRAMBSCH PM, THERNEAU TM. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika* 1994;81:515-526.

Figure Legends:

Figure 1: miR-7 is a potent tumour suppressor of HCC viability *in vitro* and *in vivo*. A.

The anti-tumorigenic effect of miR-7 on human HCC derived cell line Huh-7 was initially investigated in *in vitro* system using MTS cell titre assay for proliferation; i, transwell assays without/with matrigel coating to assess cell migration; ii, and invasion; iii, respectively and

clonogenic assay; **iv**, to assess their tumorigenic potentials. **B**. Evaluation of the effective concentration (EC₅₀) of miR-7 by MTS assay. **C**. The study design demonstrating the time frame at which either miR-7 or the negative control was administered IV to an orthotopic model of HCC (n=8/ group). **D**. Measurement of serum α -fetoprotein (AFP) at day 4, 7, 10 and 14 post treatment. **E**, Representative livers of treated mice showing tumor burden; and **F**, the weights of the liver tumors. All *in vitro* assays were carried out on 3 independent days with technical repeats. *p<0.05, **p<0.01 and ****p<0.0001, by one-way repeated measure ANOVA (A) and Mann-Whitney U-test (B-C) . ****p<0.0001 for all *in vivo* experiments by one-way repeated measure ANOVA.

Figure 2. TYRO3 is elevated in HCC and is a novel miR-7 target gene. **A**. Phospho-RTK array of miR-7 versus miR-NC treated Huh-7 cells. The top panel shows the differential phosphorylation of a number of receptor tyrosine kinases and the bottom panel corresponds to the densitometry calculations: 1, Control; 2, P-EGFR; 3, P-FGFR4; 4, P-Insulin R; 5, P-IGF1R; 6, P-TYRO3 (p-Dtk). **B**. Effect of miR-7 over-expression on TYRO3 mRNA (**i**), and protein (**ii**), (respectively) in Huh7 cells; **C**. Luciferase reporter assay using the 3'-UTR of TYRO3 as a target of miR-7. Upper panel (**i**) shows the sequence of the seed site mutated within the TYRO3 3'UTR, bottom panel (**ii**) shows luciferase assay. **D and E**. TYRO3 expression in HCC versus normal surrounding tissue and associated Kaplan Meier survival curves in HCC patients from the TCGA cohort (n=368). ***p<0.001 by paired student's *t*-test, from three independent experiments with technical repeats.

Figure 3: TYRO3 regulates proliferation, migration and invasion of HCC cells via the PI3-Kinase/AKT pathway. Huh-7 cells were treated with siRNA to TYRO3 and the effects of subsequent TYRO3 depletion evaluated: **A**. Cell proliferation assay: **B**. Migration assay in matrigel; **C**. 2D invasion assay. **D**. Immunoblot analysis of PI3-Kinase/AKT, NF κ B and MAPK/ERK signaling pathways. Western blots quantified by relative band densitometry using Image J (version 1.51h) by normalizing the bands to β -actin and then to negative control. All experiments were performed on three independent days with technical repeats. Error bars

represent mean $\pm$ SEM and * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$ by one-way repeated measure ANOVA (A) and Mann-Whitney U-test (B-C).

Figure 4: Knock-down of endogenous miR-7 in Huh-7 cells induced a pro-migratory and pro-invasive phenotype that was rescued by sequential depletion of TYRO3. Huh-7 cells were treated with anti-miR-7 or anti-miR controls for 48 h and then with TYRO3 siRNA for 24 h and effects of these treatments assessed *in vitro* A-B. Cell proliferation assay: C. Migration assay: and D. Invasion assay. E and F. qRT-PCR for chemokines: CXCL10 (i) and IL-8 (ii) expression. All experiments were performed on three independent days with technical repeats. The error bars represent mean $\pm$ SEM of Δ CT. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ by paired student's t-test. All experiments were performed on three independent days with technical repeats. Error bars represent mean $\pm$ SEM. and * $p < 0.05$ and **** $p < 0.001$, by two-way ANOVA with post-hoc correction by Tukey method.

Figure 5: Sorafenib resistance in HCC is overcome via suppression of TYRO3 function. A. EC_{50} calculations of sorafenib treatment assessed by cell titre assay in Huh-7 parental and sorafenib resistant cell lines. B. qRT-PCR for miR-7 (i) and TYRO3 (ii) mRNA in parental and sorafenib resistant cell lines. C. Immunoblot analysis of phospho-EGFR (pEGFR), EGFR, TYRO3 and their downstream signalling pathways pAKT, pERK and pRelA in Huh-7 parental and sorafenib resistant cells. Western blots quantified by relative band densitometry by normalizing the bands to β -actin and then to the parental control. Using Image J (version 1.51h). D. Phenotypic evaluation of two sorafenib resistant cell lines by (i) cell proliferation; ii transwell migration; and iii invasion assays. E. Evaluation of the EMT marker alpha-smooth muscle actin (α -SMA) by immunofluorescence immunocytochemistry (i). Immunoblot for markers of EMT (E-cadherin and vimentin) in the parental and sorafenib resistant cell lines (ii). qRT-PCR for Twist mRNA from each of the same lines (iii). F. Cell proliferation assay following titration of TYRO3 siRNA in parental cells (i) and Huh-7/SR2 cells (ii). Cell proliferation assay of combining 5 nM siTYRO3 and 5.5 μ M sorafenib in Huh-7 parental cells (iii). All experiments were performed on three independent days with technical

repeats. Error bars represent mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ by one-way repeated measure ANOVA (D), and one-way ANOVA with post-hoc corrections by Dunnett's method (B, Dii-iii) and two-way ANOVA with post-hoc correction by Tukey method (E-F).

Figure 6: miR-7 abolished the TYRO3 dependent increase in invasiveness in sorafenib resistant Huh-7 cells. The tumour suppressive function of miR-7 on the two sorafenib resistant Huh-7 cell lines SR1 (A) and SR2 (B) was investigated by MTS cell titre assay for cytotoxicity (i) and by transwell assays to determine migration (ii) and invasive capacity (iii). C. Western blot analysis of the effect of miR-7 transfected into Huh-7/SR2 cells. D. Migration (i) and invasion assay (ii), of Huh-7/SR2 cells following stimulation of TYRO3 by its ligand GAS6. E and F. Cell viability assays of the combination of miR-7 with sorafenib in Huh-7/SR1 and Huh-7/SR2 cells. After transfecting miR-7 into the cells (5 nM), they were treated with increasing doses of sorafenib (0.01 to 100 μ M). EC₅₀ counts were determined by MTS cell viability assay after 48 h of treatment with sorafenib. All experiments were performed on three independent days with technical repeats. Error bars represent $\pm$ STDEV and * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, by Mann-Whitney U-test (Aii-iii, Bii-iii), and two-way ANOVA (Ai, Bi, D) with post-hoc correction by Tukey test.

Figure 7: A proposed model for how miR-7 rescues sorafenib resistance (SR) in HCC. Neoplastic transformation reduces endogenous levels of miR-7 in hepatocytes with subsequent elevated levels of its target genes. EGFR is the most well characterized gene target of miR-7 that is frequently elevated in HCC and upon acquisition of SR. Aberrant activation of TYRO3 signaling is a novel mechanism of acquiring SR in Huh-7 cells and is a new target of miR-7. Administration of miR-7 inhibits cancer cell proliferation in an orthotopic model of human HCC and overcomes SR *in vitro* via targeting both the EGFR and TYRO3 signaling pathways.

Accepted Article

Fig 1

345x298mm (300 x 300 DPI)

Acce

Fig 2

83x51mm (300 x 300 DPI)

Accepte

Fig 3

44x72mm (300 x 300 DPI)

Fig 4

53x79mm (300 x 300 DPI)

Fig 5

83x128mm (300 x 300 DPI)

Fig 6

61x119mm (300 x 300 DPI)

Fig 7

209x136mm (300 x 300 DPI)

Accepted

A miR-7/GAS6/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma

Supplementary Materials and Methods

Cell Culture: The Huh-7 cell line was derived from a primary well differentiated HCC from a 57 year old Japanese male. The Hep3B cell line was derived from a primary epithelial HCC of a black 8 year old male and is positive for the presence of Hepatitis B viral DNA sequences via PCR. To establish sorafenib-resistant cell lines Huh-7 cells were cultured with incrementing doses of sorafenib starting from 1.5 μ M for 4 weeks, 3 μ M for 6 weeks, 6 μ M for 8 weeks and 9.9 μ M for 8 weeks. Human liver cancer cell lines were maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS at 37°C with 5% CO₂. The identities of all cell lines (including sorafenib-resistant sublines) were confirmed via short tandem repeat (STR) profiling by Cell Bank Australia (Westmead, Australia).

Reagents: The synthetic miRNA precursor hsa-miR-7-5p (#AM17100, #PM10047), the negative control #1 miRNA (miR-NC; AM17100), the anti-miR-7 miRNA inhibitor (#AM17000) and the anti-miR miRNA inhibitor Negative control #1 (#AM17010) were sourced from Ambion (Thermo Fisher Scientific) and resuspended to a 50 μ M with RNase-free water. Flexitube siRNAs targeting TYRO3 (si-TYRO3-#5, SI00288344; si-TYRO3-#6, SI00288351) and negative control siRNA (#1027281) were sourced from Qiagen and resuspended to 20 μ M stock in RNase free water. The pmiR-REPORT-TYRO3 3'UTR WT and the pmiR-REPORT-TYRO3 3'UTR Mutant (thenceforth Mut) were synthesized by GenScript Inc. (Piscataway, NJ) to include the full length of wild-type 3'UTR sequences for TYRO3 (nt 2909-4001 of GenBank accession number NM_006293.3) and the predicted miR-7 target site modified at nt 3566-3572 to 'AGAAGGU' with the sequences confirmed prior to use. Sorafenib (LC Labs, #S-8502) was prepared in DMSO at a 100 mM stock and stored at -20°C. Transfections were performed in OptiMEM I reduced serum media (Thermo Fisher

Scientific) using Lipofectamine 2000 or 3000 transfection reagent (for HuH-7/SR lines) (Thermo Fisher Scientific), following manufacturer's instructions.

Cell viability and proliferation: To determine the sensitivity of HCC cell lines, the cells were either transfected with miR-7 or miR-NC (0.001 - 100 nM) or treated with an incrementing concentration of sorafenib (0 - 100 μ M). Cell viability was measured at 96 h post-transfection with miRNAs or 72 h post-treatment with sorafenib, using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) according to manufacturer's instructions. Absorbance was recorded at 490 nm using a spectrophotometer (Clariostar, BMG LABTECH). The EC₅₀ was calculated using GraphPad Prism Software (Version 5.04). To assess proliferation the cells were transfected with 30 nM of either miRNA precursors or siRNAs in 96-well plates and cell viability measured over 24-96 h, using the CellTiter 96 AQueous Solution.

2D and 3D colony formation assays: Huh-7 cells were transfected with 30 nM of miRNA for 48 h. Colony assays were performed as described previously (Giles et al. 2016), with a density of 3×10^3 into a 100 mm dish for 2D and 1×10^4 cells per well of 12 well plates for 3D assays, respectively. Colonies were stained with crystal violet for visualisation (~19 d post-transfection) and counted manually using ImageJ software (Version 1.51h).

Migration and invasion assays: Initially migration of HCC derived cancer cell lines were optimized by monitoring their motility in real-time using xCELLigence and electronic cell invasion and migration plates (CIM-plate-16). All the remaining experiments were carried out using 2D transwell assays described below. Huh-7, Huh-7/SR1 and Huh-7/SR2 cell lines were transfected and serum starved overnight prior to experimentation. Cells were trypsinized and resuspended at 1×10^6 cells/ml in serum free DMEM containing mitomycin C (1 μ g/ml) (to prevent cell proliferation). Cell suspension (200 μ L) was pipetted into the top of the transwell chamber with/without matrigel coating. FBS (5%) or GAS6 (200 ng/ml) with FBS (1%) in DMEM were used as chemoattractants in the lower chamber. The plates were then

incubated for 40 h and 72 h for migration and invasion, respectively. Cells adhering to the underside of the chamber were fixed with 100% (v/v) methanol and stained with 0.1% (w/v) crystal violet in PBS. The cells were counted by light microscopy at 40x magnification. Three fields of view from each insert were counted.

Luciferase reporter assay: Huh-7 cells were co-transfected with either the pmiR-REPORT-TYRO3 WT or Mut (0.5 µg) and pRL-CMV Renilla luciferase reporter (0.001 µg) in combination with either miR-7 or miR-NC (30 nM) using Fugene HD (Promega), according to the manufacturer's instructions. After 24 h incubation, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Luminescence was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech). Relative luciferase activity was determined by first normalising firefly luciferase values to *Renilla* luciferase values, followed by normalisation to miR-NC only values.

RNA extraction, reverse transcription and quantitative real time polymerase chain reaction (qRT-PCR): RNA was isolated by the miRNeasy Mini Kit (Qiagen) according to manufacturer's protocol. For cDNA synthesis, 200 ng RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) or 10 ng RNA for miRNA using the MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). To detect low basal expression of miR-7 in the Huh-7 cells 125 ng of RNA template was used for miRNA reverse transcription. For qRT-PCR for miRNA expression, TaqMan probes (Thermo Fisher Scientific) for miR-7 or RNU44 were used in TaqMan Universal Gene expression assay, with no UNG master mix (Applied Biosystem). For gene expression analysis, primers listed in table S3 were used with SensiMix SYBR Hi-ROX kit (Bioline). Quantitative PCR was performed using the ViiA7 Real-Time PCR System (Life Technologies). The ΔC_t values were calculated by normalizing to RNU44 (for miRNA) and GAPDH (mRNA) endogenous controls. The $\Delta\Delta C_t$ values of target genes were calculated

from normalizing their levels to miR-NC or the negative control siRNA. The mRNA expression is represented graphically as the mean fold change ($2^{-\Delta\Delta C_t}$).

Western blot analysis: Cell extraction buffer or CEB buffer was used to prepare protein lysates from Huh-7 and Hep3B cells supplemented with 1X cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and 1x PhosSTOP Phosphatase inhibitor (Roche). Protein was quantified by Bradford protein assay (BioRad). 15 µg of proteins per well were resolved on NuPAGE Novex 4-12% Bis-Tris protein gels (ThermoFisher) and transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich) before being probed with anti-EGFR (Abcam, ab52894), anti-pEGFR (Tyr1173, Santa Cruz, sc12351) anti-Akt (Cell Signaling Technology, #9272), anti-phospho-Akt (Ser473; Cell Signaling Technology, #4060), anti-TYRO3 (Cell Signaling Technology, #5585), anti-pERK (phosphor-p44/42 MAPK: thr202/204, Cell Signaling, #9101), anti-pRelA (Phospho-NF-κB p65: Ser536, Cell Signaling Technology, #3033), anti-RelA (Abcam, ab16502), anti-PTEN (Cell Signaling Technology, #9559) anti-PARP (214/215) cleavage site (EMD Millipore, AB3565) and anti-β-actin (Abcam, ab6276). Secondary horseradish peroxidase linked anti-mouse-IgG (GE Healthcare, NA931V), anti-rabbit-IgG (GE Healthcare, NA934V) and anti-goat-IgG (Santa Cruz Biotechnology, sc-2020) were used followed by detection using Luminata Crescendo Western HRP Substrate (Merck Millipore, Billerica, MA) and Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK).

Immunofluorescence immunocytochemistry: The Huh-7 and Huh-7/SR2 cells were seeded at the same density onto glass coverslips in 6-well plates for 48 h. The cells were methanol fixed, permeabilized with 0.1% Triton X-100, blocked with 1% BSA/10% normal goat serum and incubated with anti-alpha smooth muscle actin [1A4] antibody conjugated with Alexa Fluor 488 (ab184675 at 1/100) overnight at 4° C. The coverslips were rinsed thoroughly in ice cold PBS and mounted using ProLong gold anti-fade reagent with DAPI (Thermo Fisher Scientific, P36941) on superfrost plus glass slides (Thermo Scientific). Fluorescence was detected using Olympus IX71 inverted fluorescence microscope under 20X magnification.

Orthotopic liver cancer model to determine the effect of systemic delivery of NOV340-Formulated miR-7-5p mimic on tumour growth: All animal experiments were conducted by Mirna Therapeutics (Austin, TX) and carried out in accordance with the institutional guidelines for proper animal care and maintenance. Female NOD/SCID mice between 7 and 8 weeks of age were used for all experiments. The method for generation of the orthotopic liver cancer model has been previously described (48). Briefly, 1×10^6 Huh-7 cells were implanted in PBS (25 μ L) into the lateral lobe of the liver. Tumour growth was monitored by measuring serum α -fetoprotein (AFP) levels using sera prepared from mouse blood samples and a human AFP ELISA kit (DRG, EIA-4331). Once serum AFP levels reached an average of ~ 1000 mg/ml (at 17 d), mice were randomised into three groups of 8 mice per group and received tail-vein injections (200 μ L) of 1 mg/kg NOV340-formulated miR-7-5p (by Polymun Scientific, thenceforth: miR-7), empty liposome (equivalent to 1 mg/kg lipid) (by Polymun Scientific, thenceforth: miR-NC) or dilution buffer. Extensive prior studies by Mirna Therapeutics established that the effect of miR-NC and empty liposome were indistinguishable. Mice were dosed three times per week (M/W/F) for two weeks. During the dosing period, mouse blood samples were collected every 3 to 5 d and serum AFP levels measured to monitor tumour development. Twenty-four hours after the final dose, mice were sacrificed and tumours (where available) excised. Tumour weights were collected and serum AFP levels measured and compared between groups.

Correlation between TYRO3 expression and survival in human HCC patients: To investigate the clinical significance of TYRO3 expression profile in human HCC a Kaplan-Meier survival curve was plotted using datasets from The Cancer Genome Atlas (TCGA, <https://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>; accessed October 2015) in R survival package. The cohort consisted of 368 patients (Male=250, Female=118) with HCC wherein 170 patients were at stage I, 86 at stage II, 84 at stage III and 6 at stage IV. Specific recorded risk factors were chronic excess alcohol consumption (117 cases), haemachromatosis (5 cases), Hepatitis B (80 cases), Hepatitis C (34 cases), non-alcoholic fatty liver disease (NAFLD, 11

cases) and unknown (101 cases). At 20 months follow up a total of 280 patients survived whereas 88 had died. All deaths were used as an endpoint when plotting Kaplan-Meier survival curves. As indicated, analyses were adjusted for age and gender. The validity of proportional hazards assumptions were assessed by scaled Schoenfeld residuals (49). Correlation coefficients were calculated using the R “cor.test” function. The boxplot was produced using the R “graphics” package.

Supplementary figure legends:

Fig S1: A. Quantification of the 3D matrigel colony formation assay by counting the mean number of colonies from 3 wells /treatment with three fields of view/ well. The bars represent mean $\pm$ SEM. **B.** EC₅₀ of miR-7 was determined in HEP3B (i) and PLC/PRF/5 (ii) cell lines at 96 h post-transfection with incrementing doses of miR-7 (range: 0.001 to 100 nM) using the MTS cell titre assay. Examination of levels of miR-7 across HEP3B, Huh-7 and PLC/PRF/5 cells by qRT-PCR assay (iii). **C.** miR-7 induced G0/G1 arrest (i) and apoptosis (ii-iii) of Huh-7 cell as assessed by cell cycle analysis (i), Caspase Glo 3/7 assay (ii) and western blot of PARP-cleavage product (iii). **D.** The cytotoxic effect of miR-7 was compared to chemotherapeutic agent Kif11 inhibitor in Huh-7 cells by MTS assay. **E.** qRT PCR analysis using a standard curve method showed miR-7 was enriched in both the normal liver tissues of the mice (i) and human derived tumour tissues (ii) and were increased to 10 million copies per ng of total RNA in mice treated with the NOV340-miR-7 formulation. **F.** Measurements of liver tumour weights of mice treated with miR-NC and miR-7 (i) and H&E staining of liver samples treated with NOV340-miR-7 (ii). The bars represent mean $\pm$ SEM of Δ CT (A, B iii) or SEM (D, ii) of three independent experiments. * $p < 0.05$ and **** $p < 0.001$ by Mann Whitney U test (A), by Kruskal-Wallis test (B iii) and paired student's test (C ii).

Fig S2: A. Prediction of miR-7 complementary seed sequence by TargetScan Human (Release 7.1) in human FGFR4 and TYRO3 3'UTR. The position of the respective

complementary base pairs to miR-7-5p seed sequence in FGFR4 and TYRO3 are highlighted in bold. The context score and P_{CT} for FGFR4 and TYRO3 is -0.12 and -0.03, and 0.23 and <0.1, respectively. **B.** Western blot depicts miR-7 targets both EGFR and TYRO3 in Huh-7 and HEP3B cell lines and their downstream PI3-K/AKT pathway.

Fig S3: Optimization of TYRO3 siRNA for experimentation in Huh-7 cell lines. A. Western blot showing TYRO3 knock-down in HEP3B cell lines. **B.** Xcelligence was used to monitor migration in real time in HEP3B and Huh-7 cells depleted of TYRO3. Error bars represent mean $\pm$ SEM.

Fig S4: Schematic representation of rescue of function experiment: A. The HUH7 cells were transfected with anti-miR-7 for 48 h to a final concentration of 30 nM (left panel). It is assumed if TYRO3 is an endogenous target of miR-7, anti-miR-7 will bind to free and miRISC incorporated miR-7 in the cytoplasm and counteract its function and increase abundance of TYRO3 mRNA stimulating a gain of TYRO3 function phenotype. After 48 h when the TYRO3 mRNA increases, these are then antagonized with the siTYRO3 mRNA for 24 h (right panel) which produces a loss of function phenotype. **B.** qRT-PCR analysis of miR-7 transcript levels in anti-miR-7 and anti-miR-control transfected Huh-7 cells. **C.** Western blot illustrates anti-miR-7 rescues expression of TYRO3 in Huh-7 cell lines transfected with siRNAs targeting TYRO3 compared to the anti-miR-control. The bars represent mean $\pm$ SEM of ΔCT (**B**) of three independent experiments. * $p < 0.05$ by paired student's test (**B**).

Fig S5: Regulation of E-cadherin expression. qRT-PCR showing E-cadherin expression is induced by miR-7 over-expression (**i**) and loss of TYRO3 function (**ii**) in Huh-7 cells. Error bars represent mean $\pm$ SEM of ΔCT . * $p < 0.05$ and **** $p < 0.001$ by paired student's t-test.

Fig S6: Elevated TYRO3 expression in HEP3B cell lines is associated with higher EC_{50} of sorafenib compared to Huh-7 cell lines.

Accepted Article

Supplementary tables:

Sorafenib (µM)	Huh-7/SR2	$E_{miR-7} = 1 -$ Observation of miR-7 Rx	$E_{sorafenib} = 1 -$ Observation of sorafenib Rx	$E_{Bliss} = E_{miR-7} +$ $E_{sorafenib} - (E_{miR-7} \times$ $E_{sorafenib})$	$E_{obs} = 1 -$ observed (miR- 7+Sorafenib)
75	$E_{obs} > E_{Bliss}$	0.081355	0.605297	0.637408	0.705560
50	$E_{obs} > E_{Bliss}$	0.050847	0.556481	0.579032	0.70986
25	$E_{obs} > E_{Bliss}$	0.010169	0.388069	0.394292	0.698033
10	$E_{obs} > E_{Bliss}$	0.033898	0.229096	0.255228	0.60986
1	$E_{obs} > E_{Bliss}$	0.025423	0.194778	0.215250	0.44642
0.1	$E_{obs} > E_{Bliss}$	0.089830	0.203872	0.275389	0.531366

Table S1: Calculation of synergy between miR-7 and sorafenib using the Bliss Additivity model in Huh-7/SR2 cells.

Sorafenib (µM)	Huh-7/SR1	$E_{miR-7} = 1 -$ Observation of miR-7 Rx	$E_{sorafenib} = 1 -$ Observation of sorafenib Rx	$E_{Bliss} = E_{miR-7} +$ $E_{sorafenib} - (E_{miR-7} \times$ $E_{sorafenib})$	$E_{obs} = 1 -$ observed (miR- 7+Sorafenib)
25	$E_{obs} > E_{Bliss}$	0.081761	0.184035	0.250749	0.306390

Table S2: Calculation of synergy between miR-7 and sorafenib using the Bliss Additivity model in Huh-7/SR1 cells.

Name	Supplier	Sequence
Hsa-miR-7-5p Taqman miRNA assay	Thermo Fisher Scientific	Refer to Catalogue no: 4427975, 000268
Hsa-RNU44 Taqman miRNA assay	Thermo Fisher Scientific	Refer to Catalogue no: 4427975, 001094
GAPDH-F	Geneworks	5'-ATG GGG AAG GTG AAG GTC G-3'
GAPDH-R	Geneworks	5'-GGG GTC ATT GAT GGC AAC AAT A-3'
CXCL10-F	Geneworks	5'-GTG GCA TTC AAG GAG TAC CTC-3'
CXCL10-R	Geneworks	5'-TGA TGG CCT TCG ATT CTG GAT T-3'
TWIST1-F	Geneworks	5'-GTC CGC AGT CTT ACG AGG AG -3'
TWIST1 -R	Geneworks	5' -GCT TGA GGG TCT GAA TCT TGCT -3'
E-cadherin/ CDH-1 Quantitect Primer Assay	Qiagen	Hs_CDH1_1_SG Refer to Catalogue no: QT00080143
TYRO3 Quantitect Primer Assay	Qiagen	Hs_TYRO3_SG Refer to Catalogue no: QT00055482
IL-8 Quantitect Primer Assay	Qiagen	Hs_IL-8_SG Refer to Catalogue no: QT00000322

Table S3: List of Primer Sequences

Accepted Article

A**Matrigel colony formation** *miR-7 vs miR-NC, $p < 0.01$ **B i** **ii** **iii****miR-7 transcript levels** **C i** **ii** **iii** **Hepatology**

D
 + Negative control, $EC_{50} > 150$ nM
 + miR-7, $EC_{50} = 0.594$ nM
 + Kit11 inhibitor, $EC_{50} = 0.027$ nM

F i

Hepatology

ii

B

A

Page 45 of 48

HEP3B cell lines

B

E-cadherin mRNA

Normalized transcript levels of E-cadherin

E-cadherin mRNA

Normalized transcript levels of E-cadherin

■ Huh-7, $EC_{50} = 6.199 \mu M$

■ HEP3B, $EC_{50} = 14.11 \mu M$

Page 48 of 48

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Kabir, TD;Ganda, C;Brown, RM;Beveridge, DJ;Richardson, KL;Chaturvedi, V;Candy, P;Epis, M;Wintle, L;Kalinowski, F;Kopp, C;Stuart, LM;Yeoh, GC;George, J;Leedman, PJ

Title:

A microRNA-7/growth arrest specific 6/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma.

Date:

2018-01

Citation:

Kabir, T. D., Ganda, C., Brown, R. M., Beveridge, D. J., Richardson, K. L., Chaturvedi, V., Candy, P., Epis, M., Wintle, L., Kalinowski, F., Kopp, C., Stuart, L. M., Yeoh, G. C., George, J. & Leedman, P. J. (2018). A microRNA-7/growth arrest specific 6/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma.. *Hepatology*, 67 (1), pp.216-231. <https://doi.org/10.1002/hep.29478>.

Persistent Link:

<http://hdl.handle.net/11343/293939>